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Chemical Investigations of the Rhubarb Plant

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FOREWORD

The experiments with rhubarb leaves described in this Bulletin are presented in three parts. The first two parts give the details of separate experiments carried out with leaves collected in two successive years from the same farm. The two lots differed somewhat widely in initial composition and the behavior during culture in water in darkness was correspondingly different. In the experiments of the second year, the conditions of culture were extended to include culture in darkness in glucose solution and culture in light in water, the set of samples cultured in darkness in water serving as a control.

The description of these experiments is given in considerable detail since work that has occupied a laboratory staff for more than two years cannot be unduly compressed without risk of omitting many points concerning which information is essential to an appreciation of the significance of the results.

An interpretation of the data in terms of modern views of plant metabolism has been attempted in Part III. In developing this interpretation, full advantage has been taken of the clear and stimulating presentation of present-day knowledge of protein metabolism in plants given by Professor Chibnall in his Silliman Lectures at Yale in October, 1938. It is a pleasure to acknowledge the help obtained from many personal discussions of the present data with him and also from the opportunity to study the text of his lectures in advance of publication.

H. B. V.

Chemical Investigations of the Rhubarb Plant

I. Chemical Changes that Occur in Leaves During Culture in Darkness

Hubert Bradford Vickery, George W. Pucher, Alfred J. Wakeman and Charles S. Leavenworth

INTRODUCTION

Most of the published chemical investigations of the rhubarb plant have dealt chiefly with the organic acids present in high proportion in the fleshy petiole, or with the substances in the root responsible for the medicinal properties which have been held in high esteem from early antiquity. Culpepper and Caldwell (21) in their general review of the subject have pointed out that the widespread use of the rhubarb petiole for human food is comparatively recent, but there is a considerable literature that deals with the food value of this part of the plant (22) and also with the alleged toxicity of the leaf blade (9).

In their own investigations, Culpepper and Caldwell studied the changes in the general chemical composition of the rhubarb plant that take place during an entire growing season. Allsopp (5) in England, on the other hand, in somewhat similar experiments, has restricted his attention to the behavior of the organic acids. By the use of modern methods of analysis, he was able to demonstrate transport of the acids from the rhizome to the leaf during the early stages of growth, and also observed the rapid increase in the quantity of acids that occurs in the shoot during the season of active photosynthesis. This increase was accompanied by a continuous transport of acids back to the rhizome, and the variations in the actual quantities of the individual acids in a single plant clearly indicated that these substances play an active part in the general metabolism.

Our own previous work with the rhubarb plant has also had to do with the nature and distribution of the chief organic acids in the leaf (65, 66). This plant has been grouped by Ruhland and Wetzel (69, 70) among the so-called "acid plants" which are characterized by highly acid saps and are held to behave differently from more nearly neutral plants with respect to the metabolism of ammonia. According to the views of Prianischnikow (64), ammonia, whether produced by oxidative deamination of amino acids within the cells, or absorbed from the solution that bathes the roots, is promptly converted into asparagine or glutamine, the ammonium ion concentration being usually maintained at a very low level. The reaction probably involves carbohydrates, or metabolites of carbohydrates, as the

Note: The chemical investigations of rhubarb leaves herein described were carried out as part of a general project under the title "Cell Chemistry", by the Department of Biochemistry of the Connecticut Agricultural Experiment Station. A portion of the expense was borne by the Carnegie Institution of Washington.

source of the carbon compound with which the ammonia combines, although this is a matter of debate at the present time. This view of the function of the amides has been shown in this laboratory to agree in general with observations on the tobacco (85) and beet (81) plants. The German investigators, however, believe that in acid plants, particularly in *Begonia, Oxalis*, and *Rheum* species, ammonia is chiefly dealt with by direct neutralization by an organic acid. They quote experimental results that show concentrations of ammonia nitrogen in the rhubarb petiole far higher than are usually encountered in other species, and call attention to the parallel production of organic acids and ammonia during growth as an evidence of neutralization as the mechanism for dealing with these large amounts of ammonia. Whether or not rhubarb possesses an amide synthesizing mechanism of the usual type is left uncertain by their experiments, but Kultzscher (44) maintains that amide synthesis plays a very small part in acid plants and distinguishes between ammonium ions and free ammonia. He makes use of an idea that probably originated with Prianischnikow (64) according to which only free ammonia is thought to be toxic and, thus, in the acid plant where, at high hydrogen ion activities, the ammonia tension is reduced to infinitesimal proportions, there is no necessity for conversion into a neutral amide.

These observations induced us to examine the organic acids of the rhubarb plant in some detail, and it was found that, in a series of samples of leaves collected at different times in the season, there was no evidence of correlation between the changes in the quantities of ammonia and of any of the three chief organic acids, *l*-malic, oxalic, and citric acids (66).

The method of culture in water that has been applied in this laboratory to the study of the tobacco plant is one that is particularly effective for the investigation of nitrogen metabolism. In the tobacco leaf cultured in water in the dark, ammonia is produced by the oxidative deamination of amino acids that result from the hydrolysis of the proteins under the influence of intracellular enzymes. Synthesis of asparagine begins promptly, and for many hours the ammonia nitrogen is maintained at a very low concentration. Ultimately, however, at about the time that extensive chlorophyll degeneration becomes obvious from the yellow color of the leaves, ammonia begins to accumulate rapidly, although asparagine synthesis continues at an undiminished rate. Consideration of the quantities of nitrogen involved in these reactions enabled us to present an interpretation of the results in terms of the hypotheses of nitrogen metabolism in plants proposed many years ago by Schulze and more recently extended by Prianischnikow. Careful study of the organic acids in the same series of samples furnished no evidence that these substances shared directly in the nitrogen metabolism of the tobacco plant and no definite conclusions could be drawn with respect to the origin of the carbon compounds from which the amides were formed.

In the rhubarb leaf, however, if the views of Ruhland and Wetzel are correct, one might expect to find an ammonia metabolism of a different type. Under conditions of culture that would be expected to lead to an accumulation of ammonia in the tissues, the organic acids should increase in proportion and the production of amide nitrogen should be a minor phenomenon. If, however, the production of ammonia during culture is accompanied by a prompt increase in amide nitrogen, there would be no

reason to assume that the ammonia metabolism of rhubarb differs in any essential feature from that of the tobacco or beet plant.

Although the question whether or not there is an amide metabolism in rhubarb leaves analogous to that in the leaves of a neutral plant such as tobacco may be readily answered by relatively simple experimentation, the answer would have only trivial significance unless evidence were secured of the origin of the ammonia, and of the changes in the other components of the tissue which might be expected to be involved in the reactions. Accordingly a study of the chemical changes that occur during culture of rhubarb leaves was planned so as to give information not only with respect to the transformations of the nitrogenous components but also of the carbohydrates and organic acids.

The interpretation of the data, in terms of the somewhat meager theoretical background that was all that was available at the time our study of tobacco leaves was completed, is enormously simplified today by the complete summarization and review of the field of protein metabolism in plants by Chibnall in his Silliman Lectures at Yale (20). His discussion of the relationships between the behavior of the protein on the one hand and of the carbohydrates and organic acids on the other has shown, by a marshaling of evidence secured in many laboratories, that chemical changes that affect the one also have their effect upon the other; these components of the cells are therefore linked in equilibrium relationships with each other and the equilibria are controlled by enzyme reactions, some of which are well recognized phenomena of widespread occurrence in nature, others being merely postulations at the present time. However, by a careful consideration of all the data, Chibnall has shown that it is possible to set up a general scheme of reactions arranged as a cycle, many of the intermediate compounds being related to substances extraneous to the cycle which can therefore in turn influence the behavior of substances to which they have no obvious chemical relationship whatever.

The existence of a complete set of analytical data for a single series of samples of tissue is essential to an interpretation in terms of this cycle. The present investigation of rhubarb leaves therefore furnishes an opportunity not only to attempt such an interpretation but also, conversely, to test the validity of the assumptions involved in the series of reactions to see to what extent predictions founded upon the cycle are fulfilled.

PREPARATION OF MATERIAL

The leaves studied in 1936 were obtained from a crop grown from rhizomes some 16 years old, in a field that had been heavily dressed in the spring with commercial fertilizer that contained nitrogen, phosphoric oxide and potassium oxide in the ratio 5:8:7. The plants were vigorous and grew rapidly despite a somewhat dry season. The leaves were collected at 9 A.M., June 6, following a clear day and night. Two lots of samples were rapidly selected, with care to exclude leaves of unusual size or that had been damaged. One lot consisted of samples of 20 leaves each and received the key letter D, to signify that the tissue was to be dried for analysis, followed by a numeral to designate the individual sample. Two of these samples were reserved for immediate analysis (FD1 and FD2), and the remainder were placed in the dark room and were supported in pails that contained a meas-

ured volume of water. The second lot of samples consisted of 10 leaves each and received the key letter E, to signify that the tissue was to be extracted with water. The management of this series of samples was the same as the D series. The material assembled for analysis at the end of the experiment consisted of the following:

Fresh leaf control:	Blade tissue and petiole tissue in duplicate from 20 leaves (FD1, FD2); dried.
	Blade tissue and petiole tissue extracts in duplicate from 10 leaves (FE1, FE2).
	Residues from extraction of blade tissue and petiole tissue, dried.
Cultured leaf:	Blade tissue and petiole tissue from 20 leaves cultured from 24 to 260 hours; eight successive samples of each, dried.
	Blade tissue and petiole tissue extracts from 10 leaves cultured from 24 to 260 hours; eight successive samples of each, dried.

Each sample of the D series, on being removed from the culture solution, was weighed and rapidly dissected into blade and petiole fractions, the three main veins of the leaf being left attached to the petiole and forming a part of this fraction. The blade tissue and the petiole tissue, the latter cut into thin slices, were then spread on trays and dried in a ventilated oven with a rapid circulation of air heated to 80°. When thoroughly dry, the material was again weighed ("crude dry weight") and was ground in a Wiley mill and preserved in closed containers.

The samples of the E series were likewise dissected and the blade tissue and petiole tissue, the latter cut into thin slices, were separately immersed in ether for 30 minutes. The tissues were then strained off on cheesecloth, enveloped in stout canvas, and pressed between steel plates in a hydraulic press. The pressed residue was ground in a plate mill, treated with a little ether and disintegrated with the addition of sufficient water to make a soft mass. After being allowed to stand 10 minutes, this was again pressed, and the process was repeated twice more, the pressed residue being finally dried. The ether employed was collected and washed three times with water in a separatory funnel, and, to inactivate enzymes and precipitate proteins, all aqueous extracts and washings were combined and rapidly heated to 80° on a steam bath with careful stirring. After being held at 80° for 6 minutes, the extracts were rapidly cooled and made to a definite volume—usually 1050 ml. for the blade extracts and 1400 ml. for the petiole extracts. These were centrifuged, filtered through a plug of glass wool in a dry funnel, and a sample was preserved with toluene in a closed container in a refrigerator for subsequent analysis. Extensive tests have shown that such extracts can be kept for several months unchanged with respect to the components in which we are particularly interested.

The analytical methods employed have been fully described in recent publications from this laboratory. References may be found in Bulletin 399 (85). The only important modification of the technic therein described is that the moisture determinations were made by drying the tissue in an electric oven at 105° for exactly 4 hours. The samples were cooled in covered weighing bottles in a desiccator over sulfuric acid and were weighed rapidly. Ash was determined after ignition of samples in open capsules in an electric muffle furnace at 600° for 16 hours. By careful attention to the routine of cooling and weighing, reproducible results were secured in this

way. We fully recognize that the determination both of "moisture" and of "ash" in plant tissue is a highly empirical procedure. From the analytical point of view, however, reproducibility of the results is more important than the exact interpretation of the figures obtained. Considerable experience with attempts to dry tissues over various dehydrating reagents *in vacuo* at room temperatures has convinced us that this possibly more desirable method can be used only at the expense of much time and with no compensating increase in precision.

GENERAL BEHAVIOR OF LEAVES

The complete set of samples was assembled in the dark room by 11:00 A.M. of the day of collection and this was taken as the zero of time; samples were removed at the points noted in Table 1. At two-day intervals, the bases of the petioles were scraped with a knife and the water was changed; at no time was there evidence of serious bacterial infection.

Table 1. General Behavior of Rhubarb Leaves Cultured in Water in the Dark

Sample (D and E)	Hours	Appearance
1	24	Turgid; few petioles split; all petioles curved longitudinally, the anterior surface concave.
2	48	Turgid; one petiole split for 8 cm., curved as above.
3	72	Less turgid; yellow on margins but otherwise green.
4	96	Blades becoming flaccid; yellow regions in a few leaves extending to center.
5	117	Main veins still turgid, blades flaccid; nearly all yellowish green, margins becoming brown and curling; petioles still turgid.
6	165	Main veins still turgid; blades yellowish green, margins brown; petioles turgid.
7	213	Blade tissue shriveled and mainly brown, main veins still somewhat turgid but showing signs of collapse, difficult to separate quantitatively from blade tissue; petioles turgid.
8	261	Main veins partly collapsed although still turgid on the back of the leaf; small veins shrunken; blades brown and margins curled; some infection with molds; petioles softened and becoming flaccid.

The general appearance of the samples is shown in Table 1. The leaves remained in apparent full health for 72 hours and, even after 96 hours, the degeneration of chlorophyll had not become extensive. Chlorophyll breakdown, as evidenced by yellowing of the leaves, became noticeable first at the margins of the leaves. This is in contrast to the behavior of tobacco leaves under similar circumstances. In these, yellowing becomes evident first along the main veins. On the other hand, tobacco leaves cultured in light become yellow first at the margins. Michael (52) has observed that nasturtium leaves (*Tropaeolum majus*), cultured in darkness in water, first become yellow in the regions adjacent to the main veins and petiole, and that the products of protein decomposition are in part transported to the petiole; the rates of chlorophyll destruction and protein digestion in his experiments were parallel. It will become evident from the data to be presented below that protein digestion in rhubarb leaves proceeded most rapidly during the period when the rate of extension of the yellow areas was most noticeable.

Towards the end of the period of culture, the changes in the rhubarb blade tissue were clearly those of autolysis, but the fleshy petioles retained their turgidity and showed evidence of serious breakdown only at the end of 261 hours. The main veins resembled the petioles in their capacity to withstand a protracted period of culture, and this behavior is sufficient justification for including most of the main vein tissue in the petiole fraction.

In general, it may be assumed that the changes noted in the blade tissue, during the first 72 hours, were those of metabolism rather than of autolysis; subsequently, however, the changes became definitely and increasingly autolytic although at what point death of the cells occurred is not certain. In the petiole, however, there was little evidence of autolytic change for 200 hours. The behavior of the rhubarb leaf blade and petiole in many ways resembles that respectively of the tobacco leaf and tobacco stalk (85, 86),and instances of this will be pointed out in detail as they are encountered.

THE VARIATION IN WEIGHT OF THE SAMPLES

The degree with which random samples of rhubarb leaves may be expected to duplicate each other in composition may perhaps be best appreciated from the variation in the original fresh weights of the several lots employed in this experiment. The material at collection was selected to a certain extent; that is to say, leaves of roughly the same size and stage of development only were taken, but these were secured from a large number of plants and may be regarded as representative. The fresh weights of the samples are shown in Table 2; with the exception of sample D5, which is manifestly out of line with the others, the variation from a mean weight of 1948 gm. for 20 leaves and of 962 gm. for 10 leaves is not excessive. Even including this somewhat heavy sample, the standard error is only ± 3.5 percent for the 20-leaf samples and was as little as ± 1.6 percent for the 10-leaf samples. If this one sample is excluded, the standard error of the weight of the 20-leaf samples is reduced to ± 2.6 percent. It will be noted, in the discussion of the analytical determinations below, that this sample, which was cultured for 117 hours, occasionally gave results somewhat out of line with the other samples, but there are few cases where this leads to any doubt as to the general behavior. Accordingly, there seems no justifiable reason to exclude the data from this sample. On the other hand, it furnishes an example of the magnitude of the effect of a somewhat unusual sampling error. The E samples were definitely more uniform, although somewhat lighter than the D samples.

The ratio of weight of blade tissue to total weight of sample for the four fresh leaf samples and for the D and E samples, after these had been subjected to culture, shows little change for 72 hours. The mean of the ten observations secured before the ratio began to change is 0.323 ± 0.003 and may therefore be used to calculate the fresh weight of the blade tissue of each sample before culture with an uncertainty of only 1 percent. The changes in this ratio, as culture continued beyond 72 hours, illustrate the conclusions already drawn from the general appearance of the leaves; the decreasing turgidity of the blade is at once reflected in its relative loss of fresh weight.

TABLE 2. WEIGHTS AND BLADE WEIGHT RATIOS OF THE SAMPLES OF RHUBARB LEAVES COLLECTED IN 1936

20-leaf samples	Initial weight	Ratio: blade / whole leaf	10-leaf samples	Initial weight	Ratio: blade / whole leaf
	gm.			gm.	
FD1	1864	0.313	FE1	969	0.318
FD2	1957	0.330	FE2	942	0.314
D1	1994	0.314	E1	962	0.335
D2	1994	0.329	E2	992	0.320
D3	1900	0.322	E3	956	0.335
D4	1929	0.292	E4	960	0.302
D5	2099	0.271	E5	980	0.296
D6	1889	0.180	E6	964	0.195
D7	1895	0.091	E7	951	0.098
D8	1958	0.085	E8	946	0.072
Mean	1948			962	
Standard error	±68(±3.5%)			±15(±1.6%)	

EXPRESSION OF DATA

The present study introduces a complication into the expression of the data that was not encountered when dealing with the sessile leaves of tobacco (85). The samples consisted of blade and petiole pulled from the crown of the plant, any adhering basal sheath tissue being removed. The chemical changes that occur in the two parts of the leaf may be expected to be quite different in view of the difference in function and, accordingly, it was necessary to analyze the two parts of the tissue separately. It seems best to express the results in terms of a biological unit, the entire leaf, but to discriminate in the tables and graphs between what happened in the blade and in the petiole with its attached main veins. The data are therefore calculated in terms of the grams per 1000 gm. of original fresh weight of the whole leaf, but show in detail the behavior of the two main tissues. The justification for this procedure is the relatively constant ratio of the weight of the blade to the weight of the whole leaf at the start of the culture.

The analytical results were obtained as percentages of the "crude dry weight" (CDW) of the D samples and as grams in the total volume of extract respectively from the blade or petiole tissue of the E samples. In order to simplify the calculations, factors for the conversion of an analytical result into grams per 1000 gm. of whole leaf were established for each sample. These factors, expressed as logarithms, were tabulated and the calculation was effected in tables, one for each constituent, by entering the logarithm of the analytical result under the proper factor; the result was then computed, the antilogarithm was ascertained, and the result was checked by another calculator. An example will make the method clear. The blade tissue of sample D1 weighed 84.8 gm. after being dried. It was then ground and was found to contain 95.85 percent of dry solids, i.e., it still retained 4.15 percent of moisture. This 20-leaf sample weighed 1994 gm. (CL) at the start of the culture and 2118 gm. (RL) when removed 24 hours later, and of this, 670.3 gm. was blade tissue. The separated blade tissue, which weighed 84.8 gm. after being dried (CDW), is therefore equivalent to 1994

gm. of original whole leaf. If the initial weight of the sample had been 1000 gm., the crude dry weight of the blade tissue would have been 1000 ÷ 1994 times the actual crude dry weight. Hence 3 — log CL is the logarithm of the factor to convert crude dry weight to a basis of 1000 gm. of original fresh weight. Its value for sample D1 is $\bar{1}.7002$. Thus the crude dry weight on a basis of 1000 gm. of original fresh weight is the antilog of log 84.8 + $\bar{1}.7002$, or 42.52 gm. All analytical results were obtained as a percentage of the dried tissue. The quantity wanted is the amount in grams per 1000 gm. of original fresh weight. Hence, in the sample under discussion, the amount of the analytical constituent in 42.52 gm. of the dry preparation is desired.

This number 42.52 appears in all calculations of the composition of sample D1 and an analogous number is found for each of the other samples. These numbers are denoted in our tables as the A factor. As an illustration of its use, to obtain the total solids of sample D1, the calculation is simply log A + log 95.85—2 = 1.6286 + 1.9816 − 2 = 1.6102, the antilog of which is 40.76. Thus the total solids of sample D1 blade were 40.76 gm. per kilo of original whole leaf.

The results of all the analytical determinations were calculated in this way and the final data are collected in Table 13, p. 54, to which frequent reference will be made in the following.

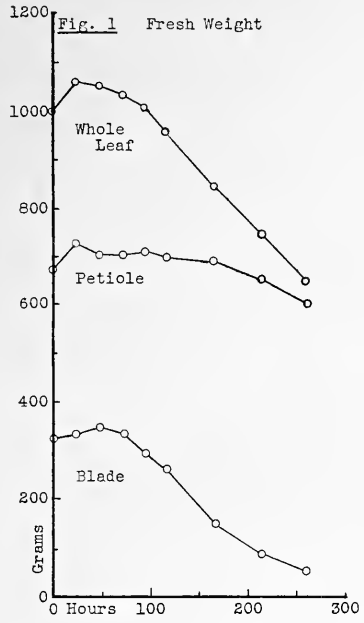
WATER AND ORGANIC SOLIDS

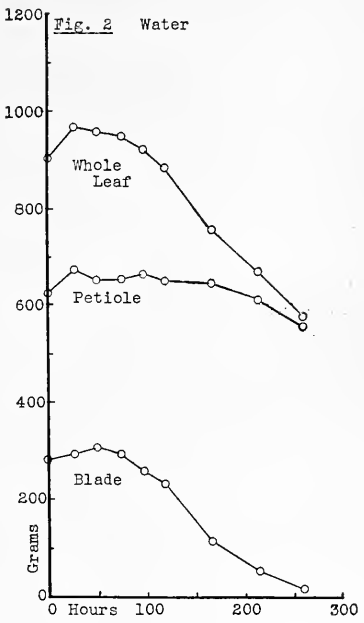
The weight of the successive samples of leaves, when removed from the culture solutions, is plotted in Figure 1, data from the D samples only being presented. Comparison of the data for the D and E samples in Table 13 shows that both groups behaved in essentially the same manner. The course of the curves corresponds to what might be anticipated from the general appearance of the leaves (Table 1); the early samples were markedly turgid and remained in good condition for 72 to 96 hours. Loss of weight to compensate for the initial uptake of water did not occur until after more than 100 hours. The blade tissue imbibed less water than the petiole, and retained the excess for a shorter time; the blades had returned to their initial fresh weight in less than 96 hours, while the petioles remained above their initial weight for more than 165 hours. The rate of final collapse of the blade tissue was rapid while the rate of loss of weight of the petiole was slow.

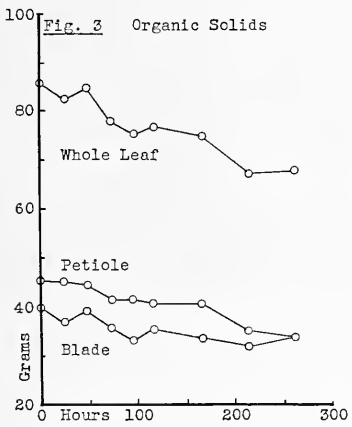
The water relationships are more definitely shown in Figure 2. The leaves as a whole imbibed and retained an excess of water for more than 100 hours, but then lost water at a rate that can be quite accurately expressed by a straight line. The changes in water content were greater in the blade tissue, although the actual amount of excess water in the petiole in the early stages was larger than that in the blades. As might be expected, the curves are very nearly superposable upon the curves for fresh weight.

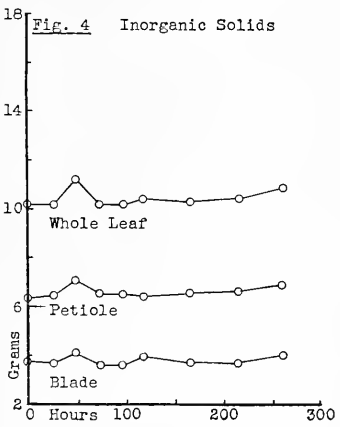
Figure 3 shows the continuous loss of organic solids from the leaves. The petioles lost somewhat more than the blades in spite of the more obvious changes in the blade tissue in the later phases of the culture. Owing to the minor irregularities in these curves, an analysis of the data by the method of least squares was made. The curve for the whole leaves indicates that 85.0 gm. of organic solids were present at the start and that the loss

in 200 hours was 14.4 gm. Of the 38.5 gm. of organic solids of the blade tissue, 5.0 gm. had disappeared in this interval, and of the 46.4 gm. of organic solids in the petioles, 9.2 gm. vanished. Thus the petioles lost organic solids, presumably in part through respiration, to a considerably









greater extent than the blade tissue. This is a somewhat extraordinary fact; although it is possible that a part of the loss in the early phases of the experiment may have been due to transport to the blade, it is difficult to believe, in view of the condition of the blade tissue, that this was a signifi-

cant factor after the expiration of 100 hours or so. Furthermore such evidence of transport as we have been able to secure has shown that nitrogen, and therefore also organic solids, moved from the blade to the petiole. As will become clear when the data for carbon losses are presented in Part III, the actual respiration, i.e., loss of carbon dioxide, was not greatly different over the whole period in the two tissues, but was much more rapid from the blade in the early phases of the experiment and became really important in the petiole only towards the end of the period of culture. The expression of the changes of solids by means of calculated straight lines is thus undoubtedly misleading.

The export of soluble substances from the petiole to the blade, if it occurred at all during the early stages of culture while the blades were still more or less turgid and in a healthy condition, must have been something other than a mere translocation of soluble substances *en masse*. The relative amounts of inorganic solids of blade and petiole showed no significant change during the entire period of culture. This is clearly shown by Figure 4 in which the inorganic solids of the leaves are plotted. The sample at 48 hours is a little out of line but, in spite of this, an analysis of the data for the whole leaves shows that they can be represented adequately by a straight line that departs very slightly indeed from the horizontal. This is true also for the separate data for the inorganic solids of the petiole and blade tissues, the apparent increase in inorganic solids of the blade tissue being of the order of less than 0.1 gm. in 200 hours, a quantity materially less than the error of the determinations.

There is little doubt that the losses of organic solids, shown by the curves in Figure 3, represent oxidation of organic substances to volatile end-products, that is chiefly the combined effects of the oxidation of carbon and of hydrogen to carbon dioxide and water. The magnitude of the loss may be compared with similar data for the tobacco plant. Tobacco leaf tissue may lose from 20 to 25 percent of its organic solids during 200 hours of culture in the dark. The rhubarb blade tissue lost 5 gm. from a total of 38.5 gm., or about 13 percent. Tobacco stalk tissue may lose nearly 5 percent of its organic solids in the same period; the rhubarb petioles lost about 9 gm. from a total of 46.4 gm., or nearly 19 percent. Thus the loss from the blades of rhubarb leaves appears to be relatively less extensive than that from tobacco leaves, but that from the petioles, in terms of the proportion of organic solids that disappeared, is markedly greater than that from the stalk of the tobacco plant. It should be remembered, however, that the tobacco stalk is a distinctly woody tissue and a considerable part of the organic solids is present in a form which probably does not share in the oxidation reactions. The comparison of the two tissues in terms of the relative proportion of the losses of organic solids is made chiefly to emphasize the great reactivity of rhubarb petioles. To what extent this reactivity should be regarded as an expression of transport to the blade is unfortunately left uncertain by the present data.

NITROGENOUS CONSTITUENTS

Total Nitrogen

The quantities of nitrogen in the successive samples of dried blade and petiole tissue are plotted in Figure 5. The mean value of the total nitrogen

of the whole leaf was 2.79 ± 0.06 gm., a variation of ± 2 percent, and indicates that the initial composition of the samples was remarkably uniform.

A careful consideration of the curves for the nitrogen of the blades and petioles suggests that transport from the blade to the petiole occurred during the early stages of the culture, while the blades were still in good condition. The increase in petiole nitrogen, at the end of 117 hours, was 0.18 gm., the loss of blade nitrogen was 0.11 gm. These quantities are only a little greater than the error of the determinations, but the consistency of the results, as indicated by the slopes of the curves, strongly suggests that a small quantity of nitrogen was in fact withdrawn from the blade tissue to the petiole. This conclusion is supported by the data for the E samples (see Table 13) which show a similar and even greater increase in petiole nitrogen and decrease in blade nitrogen, and, as will be shown in Part II of this Bulletin, the leaves examined in 1937 gave evidence of a similar but even more striking transport of nitrogen. A similar phenomenon has been reported by Michael (52) as well as by others.

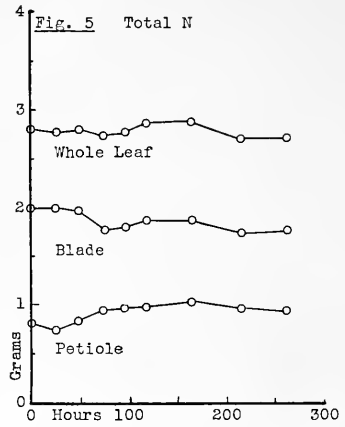
Protein Nitrogen

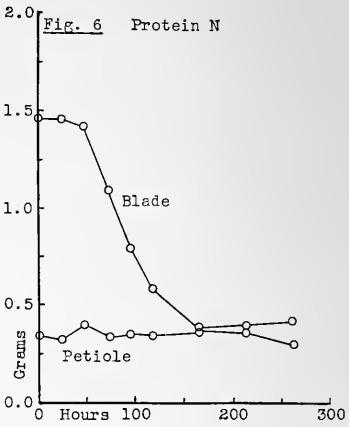
An estimate of the protein nitrogen of leaf tissue can be arrived at in a number of ways, and data for three such estimates upon the present material are given in Table 13. What are probably the most trustworthy values are secured by determination of the nitrogen that remains insoluble after the dried tissue has been exhaustively extracted with hot alcohol and then with hot water. Evidence in support of this has been given in detail in Bulletin 399, and the data obtained in this way are plotted in Figure 6.

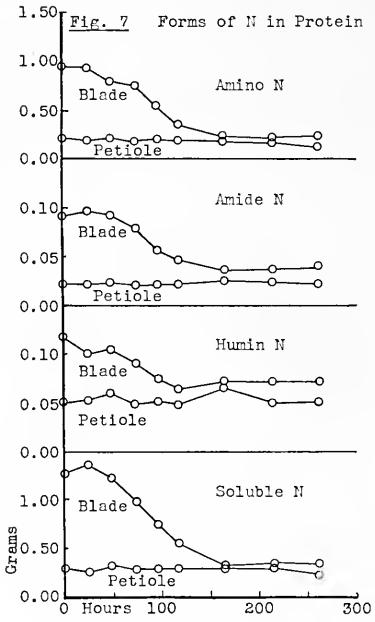
Another method consists in calculating the insoluble nitrogen from determinations of the total nitrogen and the nitrogen dissolved by treatment of the dried tissue with water at 80°. Such values are invariably somewhat higher, and obvious sources of error are the chlorophyll and the nitrogenous lipoids which are removed in the first method by the treatment with hot alcohol. A third method is the determination of the nitrogen of the residues after the fresh tissue is cytolyzed with ether, pressed, ground, and thoroughly washed with cold water. It is necessary to emphasize that very little leaf protein passed into solution during the preparation of this material, although the technic is not unlike that employed by Chibnall for the preparation of solutions of leaf cell proteins. The values secured for protein nitrogen in the residues were frequently somewhat higher than those given by either of the other methods and are less accurate in any case because no account is taken of the traces of protein that did pass into solution and that were subsequently coagulated by heat and discarded. Comparison of the three methods shows a general agreement in the form of the curves obtained, but there is little doubt that the first method yields the most reliable results.

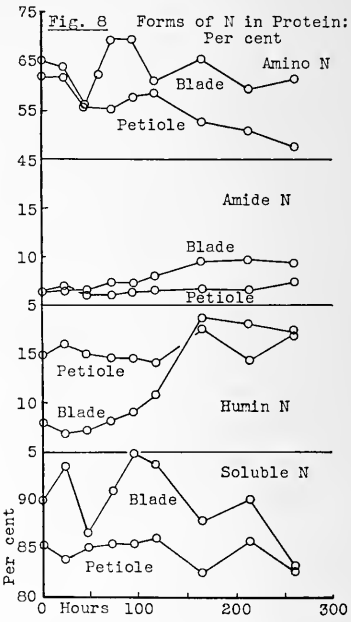
Figure 6 shows that the protein of the present samples of rhubarb leaf did not immediately begin to diminish in quantity. The value at 48 hours is undoubtedly high, the determination in the petiole being distinctly out of line with the others, but it will be recalled that this was also true of the organic solids of this sample.

The failure of the protein of the blade to show any detectable loss in the earliest stage of the culture period is particularly interesting and merits further consideration. In the first place, the observation was not confirmed by the data to be presented in Part II for another experiment conducted









under essentially identical conditions. In this later experiment there was a detectable loss at the end of 25 hours, although it was only a little greater than the experimental error of the determination. The significant point in the present connection is that the digestion of protein in rhubarb leaves dur-

ing culture in darkness may be extremely sluggish at the start of the experimental period. This is in contrast to the behavior of the protein of tobacco leaves in similar circumstances. When cultured in darkness in water, the digestion of protein begins at once and follows a nearly straight line course for more than 100 hours (85).

The significance of this type of behavior is emphasized by Yemm (92), who demonstrated beyond question that the protein of barley leaves underwent prompt digestion during culture in darkness, and that the nitrogen of that part of the protein that disappeared during the first 24 hours could be accounted for as the nitrogen of soluble amino acids and of glutamine. Thus, in this plant, the general scheme of nitrogen metabolism, protein nitrogen $\longrightarrow$ amino acid nitrogen $\longrightarrow$ ammonia $\longrightarrow$ nitrogen of amides, which has been found to hold for tobacco leaves and which is shown below also to hold in the case of rhubarb leaves, is initiated at once. That is to say, this sequence of reactions is probably an expression of the behavior of the nitrogen in the intact leaf under normal conditions, at least in darkness.

After the lapse of 48 hours, digestion of protein in the present series of rhubarb leaves became rapid, and one-third of the protein of the whole leaf had become soluble in water by the ninety-sixth hour. The curves for the protein of the blade and petiole tissue show that about four-fifths of the protein of the whole leaf is located in the blade, and that this protein alone underwent extensive digestion, the protein of the petiole changing little, if at all, until more than 200 hours had elapsed. Consideration of the condition of the blade tissue (Table 1) suggests a correlation between the onset of rapid protein digestion and the beginning of obvious chlorophyll degeneration, together with the first evidences of loss of turgidity.

It is necessary to discriminate between the rates of protein hydrolysis in rhubarb leaves in the early and in the later phases of culture in darkness. Under the admittedly artificial conditions of water culture, it is impossible to assert that the behavior in the early phase is a continuation of that to be expected in the normal leaf, yet this seems probable. Thus one gains the impression that protein digestion is not rapid in the intact leaf. On the other hand, such judgments are purely relative. The rate of digestion of protein in rhubarb blades in culture during the interval between 48 and 165 hours is indeed rapid, but this is a measure of digestion of protein in a tissue that is becoming moribund, and which, toward the end, is clearly undergoing autolysis. Rapidity of protein hydrolysis is to be expected and the actual rate observed bears no necessary relation to the rate of hydrolysis in the healthy tissue at the start.

The apparent sluggishness with which hydrolysis of the protein begins in no way indicates that protein shares to only a small extent in the metabolism. What is observed is merely the net loss of protein; there is no reason to suppose that protein synthesis suddenly ceases when the leaf is excised from the plant. The acceleration of apparent digestion rate after 48 hours may be in part an indication of a slowing up of the regenerative reactions.

A further indication that the rapid hydrolysis in the later stages is correlated with the onset of entirely abnormal conditions is its obvious relationship to the beginning of and the increasingly rapid extension of yellowing, that is in the initiation and later progress of the decomposition of the

green pigment. Michael (52) has pointed out the correlation between chlorophyll degeneration and protein hydrolysis in leaves of *Tropaeolum majus* subjected to culture in water in darkness, and the present experiments are in close analogy.

The ultimate extent of the digestion of the blade protein is remarkable; about two-thirds had become soluble at the end of 165 hours, but the protein that remained at this time resisted further digestion. This is of interest in connection with the condition of the tissue at this point. The leaves were flaccid and mostly yellow with some brown color at the margins. In the later samples, the brown areas were much larger, the mesophyll tissue had become shriveled and collapsed and there is little doubt that most of the cells were dead. As will be shown in Part III, loss of carbon from the blade tissue suddenly ceased at the 165-hour point and the failure of the supply of energy evidently had an immediate effect upon the protein metabolism.

In marked contrast to the behavior of the blade protein, the protein of the petioles changed little if at all until nearly the end of the culture period. This may be an expression of the fact that the petiole remained turgid and in an apparently healthy condition for nearly 200 hours, but, as will appear later, it also reflects the fact that the chief metabolic changes detected in the petiole were among the carbohydrate and organic acid components. The demands upon the protein appear therefore to have been postponed. In any case, however, protein is a minor constituent of the petiole tissue and its behavior has many analogies with that of the protein of tobacco stalk tissue under similar circumstances.

Although a less trustworthy method to determine protein was employed in the analysis of the 10-leaf E samples, the behavior in this parallel but independent culture experiment was very nearly the same. Digestion of the blade protein had become appreciable at the expiration of 48 hours, and continued rapidly until 165 hours had elapsed when two-thirds of the protein had been converted into soluble products. As in the D samples, protein digestion then abruptly ceased and, in fact, the analysis at 213 and 261 hours indicated a slight increase. The protein of the petiole remained constant, within the limits of error, for more than 165 hours but slowly diminished thereafter, the total loss at the end being definitely greater than the standard error of the determinations.

In order to obtain a better understanding of what happened to the protein of these samples of leaves during culture, the residues of tissue from the D samples, after exhaustive extraction with hot alcohol and hot water, were subjected to careful examination. Attention was drawn in Bulletin 399 to the difficulties inherent in the operations of protein analysis when the conventional methods are applied to plant tissues which may contain from two to three times as much of other substances as they do of protein. The extraction of the tissues with alcohol and hot water removes nearly all nonprotein nitrogen of types with which we are familiar, and evidence has been obtained to show that very little of the nitrogen in such residues belongs to substances other than protein. But the residues contain large proportions of complex carbohydrates some of which, especially those derived from pentose sugars, may be expected to yield aldehydes during the hydrolysis operation. Such products condense with amino acids and give rise to an insoluble material designated as humin, and, accordingly, may contribute

to a distortion of the apparent relative proportion of the various forms of nitrogen present after hydrolysis.

The literature of humin formation during the hydrolysis of proteins is scanty; perhaps the most nearly adequate discussion is that of Lugg (45) in the appendix to Chibnall's Silliman Lectures (20). Lugg shows that the amino acid residues in the humin may be combined with two furfural residues and that the sulfur-containing amino acids cystine and methionine, together with tryptophane and tyrosine contribute largely to humin formation during acid hydrolysis; in addition, the basic amino acids and proline are also probably involved to some extent. Chibnall (20) has also pointed out from the work of Tristram that, of the three basic amino acids, lysine alone seems to survive acid hydrolysis in a constant proportion in the presence of carbohydrate impurities. The dicarboxylic amino acids likewise seem not to be affected by carbohydrates in the hydrolysis mixture, and tryptophane and tyrosine can be accurately estimated in impure preparations of protein after alkali hydrolysis. Thus a somewhat restricted group of amino acids can be selected which may be employed for the characterization of leaf proteins with some assurance that gross errors of interpretation can be avoided. But determinations of total amino nitrogen and even of the ammonia nitrogen, after hydrolysis with strong acid solutions, are now known to give misleading results in the case of leaf proteins even when these have been isolated from the bulk of the carbohydrate materials in the tissues. The difficulties cannot be entirely surmounted until a technic is devised to separate the protein from these carbohydrate and other contaminants. Formic acid has been recommended as a solvent for this purpose by Clarke (49) and is employed by him for the removal of plant tissue proteins from residues not unlike those we have studied. Large proportions of humin nitrogen were still encountered, however, after hydrolysis of the formic acid extracts, and furthermore, the extraction of the protein, even after the tissue had been boiled with 90 percent formic acid for many hours, was incomplete. A careful consideration of this and other technics has convinced us that little advantage is gained by any method hitherto suggested for extraction of the protein from the tissue previous to hydrolysis. The use of proteolytic enzyme preparations for the purpose, while reasonably effective, contributes so much unknown nitrogen to the solution that the analytical results are difficult if not impossible to control and interpret.

Notwithstanding the fact that the procedure violates many of the fundamental principles of protein analysis, we have subjected the extracted residues directly to hydrolysis with sulfuric acid. The justification for this is that we are interested in the differences between the effects on successive samples rather than in the absolute values. The method employed is as follows: The tissue remaining after extraction with alcohol for 16 hours for the removal of soluble carbohydrates, etc., is dried and weighed in order to compute a factor to relate the extracted residue to the original dried tissue. A sample of 0.500 gm. of this residue is extracted twice with 20 ml. of water, being heated 5 minutes on the steam bath each time and centrifuged, the water extract being discarded. The residue is then suspended in 20 ml. of 10 N sulfuric acid and is heated in a boiling water bath with an air reflux condenser for 24 hours, being occasionally thoroughly agitated. The hydrolysate is cooled, diluted to 50 ml., centrifuged, and the clear fluid is decanted through asbestos on a Gooch crucible with gentle suction. The humin is

thoroughly washed with 1 N sulfuric acid, transferred to a Kjeldahl flask and nitrogen is determined.

Aliquots of 5 ml. are taken for total nitrogen, the result yielding the value given in Tables 3 and 13 for soluble nitrogen after hydrolysis, and 5 ml. aliquots are also used for the determination of ammonia. This is distilled off *in vacuo* at 40° in the presence of a slight excess of sodium hydroxide and the residues from two determinations are combined, acidified with acetic acid and made to 50 ml., suitable portions of this solution being in turn used for determinations of amino nitrogen in the manometric Van Slyke apparatus.

For the determination of the protein nitrogen, a separate series of samples of the dry alcohol-extracted tissue are extracted with hot water as before, transferred directly to Kjeldahl flasks, and subjected to the customary digestion. The sum of the humin nitrogen and the soluble nitrogen should agree with these determinations of the protein nitrogen; the values given in Table 13 show that this is true within ± 5 percent.

The data obtained by these methods are plotted in Figure 7 and the smoothness of the curves as a whole suggests that the sampling errors are insignificant and that the methods yield consistent results. Curves for the whole leaf tissue have been omitted since the uniform values for the petiole throughout indicate that the whole leaf curve would be nearly identical with that of the blade tissue in detail, being merely placed somewhat higher on the scale of ordinates.

There is a close resemblance between the curve for the amino nitrogen of the blade protein after hydrolysis and the curve for protein nitrogen of the blade shown in Figure 6, suggesting that the amino nitrogen is a fixed fraction of the protein nitrogen throughout, and providing further evidence for the validity of the protein nitrogen determinations. The curve clearly indicates the order of magnitude of the quantity of amino nitrogen which must have been converted into a soluble form during the period of culture. Inasmuch as the ultimate fate of this nitrogen in the tissues is a matter of great concern in the development of the theory of protein metabolism, the quantities liberated respectively in the blade and in the petiole tissue are shown in Table 3. The values for protein nitrogen and of its various prod-

Table 3. Changes in Protein and in Products of Hydrolysis of Protein During Culture of Rhubarb Leaves for 213 Hours in the Dark

Figures are grams per kilo of fresh weight of whole leaves.

	Blade			Petiole		
	Initial	Final	Change	Initial	Final	Change
Protein N	1.46	0.398	1.06	0.349	0.344	0.005
Amino N	0.953	0.248	0.705	0.214	0.175	0.039
Amide N	0.091	0.024	0.067	0.022	0.024	0.002
Humin N	0.118	0.071	0.047	0.051	0.056	0.005
Acid soluble N	1.29	0.347	0.943	0.297	0.288	0.009
Soluble non-amino N	0.357	0.099	0.258	0.083	0.113	0.030

ucts of hydrolysis for the initial sample at zero time are the means of the duplicate initial samples. Proteolysis practically ceased at 165 hours, and it is therefore permissible to average the results at 165, 213, and 261 hours to obtain a value somewhat more trustworthy than that secured from the last individual sample in order to calculate the total change that occurred. This value should be valid for a culture time of 213 hours, or, in round numbers, 200 hours. The data clearly show that the changes in the petiole protein are insignificant, being for the most part of the order of magnitude of the error of the determinations. The changes in the blade protein are, however, very extensive; more than 72 percent of the protein nitrogen was digested, and about 74 percent of the potential amino nitrogen of the protein and 73.6 percent of the amide nitrogen were rendered soluble. The acid soluble nitrogen (i.e. nitrogen rendered soluble by acid hydrolysis) likewise diminished by 73 percent.

The consistency of these data for the distribution of the protein nitrogen in the tissue residues warrants further analysis of the results. The percentage of the protein nitrogen in each of several forms is plotted in Figure 8, and the percentage of the nitrogen rendered soluble by hydrolysis is likewise shown. The ratio of amino nitrogen gives a series of points somewhat erratically distributed above and below the 63 percent level, and a calculation of the best straight line that expresses these ratios gives a line that originates at 64.8 percent and drops to 62.5 percent at 200 hours. Evidently, therefore, the apparent amino nitrogen ratio of the residual protein changed very little throughout the period of culture. That the mean value of about 63 percent of the protein nitrogen as amino nitrogen after hydrolysis is an accurate characterization of the leaf blade protein is, however, doubtful. The fact that the values can be expressed reasonably well by a line that does not depart much from the horizontal indicates that the general procedure of hydrolysis in the presence of the carbohydrate impurities is, to some extent at least, a justifiable one, but the actual magnitude of the mean value is undoubtedly depressed below that characteristic of the pure protein. The nitrogen in the humin actually diminished (see Figure 7), but the ratio of humin nitrogen to protein nitrogen increased materially. There is little doubt that proportionately more of the protein nitrogen, and therefore probably of the potential amino nitrogen was converted into humin during hydrolysis as the culture progressed. The slight drop in the amino nitrogen ratio, therefore, is not necessarily significant from the protein chemistry point of view. So high a proportion as 8 percent of humin nitrogen for the initial sample is clear indication that some of the protein nitrogen which, if hydrolysis could have been effected in the absence of carbohydrates, would have been liberated as amino nitrogen was diverted to the humin fraction. The increase in the relative proportion of humin nitrogen as culture progressed is probably largely an effect of the increasingly higher proportion of carbohydrates to protein in the samples subjected to acid hydrolysis.

The curve for amide nitrogen indicates what appears to have been a definite change in the composition of the residual protein as culture progressed. Starting from a value of 6 percent, the amide ratio increased regularly to about 9.5 percent of the protein nitrogen. The initial value corresponds to data of Miller in Chibnall's laboratory (53) on a series of leaf proteins isolated in pure form by them, and is also similar to the amide

ratio of tobacco leaf portion (85). The increase suggests that the part of the protein which was digested possessed on the average a lower dicarboxylic amino acid content than the part that remained.

Another entirely different interpretation of the behavior of the apparent amide nitrogen is, however, much more probably the correct one. The observation is, after all, merely an increase in the relative proportion of ammonia produced by the hydrolysis of the residual protein of the leaf blade. The hydrolysis takes place in successive samples in the presence of a rapidly increasing relative proportion of carbohydrate impurities, and these in turn give rise to an increase in the relative proportion of humin nitrogen. Chibnall in his Silliman Lectures has specifically withdrawn the data of analyses of leaf proteins made in his laboratory previous to 1936, and in an appendix has pointed out that reliable values for the amide nitrogen of leaf proteins can only be obtained by hydrolysis with dilute acid as was indeed shown in this laboratory for the protein gliadin in 1922 (78). The decompositions incident to humin formation on hydrolysis of impure preparations with strong acid lead to the production of a certain amount of ammonia, and these side reactions are obviously, from the present data, the more serious the greater the proportion of impurity to protein. Hence the increase in ammonia found probably has nothing to do with the true amide nitrogen of the protein. The dilemma is an excellent example of the difficulties that arise when one employs the methods of protein analysis under unsuitable, not to say improper, conditions.

The ratio of humin nitrogen of the blade protein increased during culture, although the absolute amount decreased sharply. This is also doubtless a result of the conditions under which hydrolysis occurred. In the fresh leaf samples, approximately 9 gm. of protein were present together with 17 to 18 gm. of non-protein solids. In the last samples of the series, there were only 2.5 gm. of protein, but about 18 gm. of non-protein solids still remained. Much of the non-protein solids consisted of complex carbohydrates, insoluble in water and alcohol, which became soluble during the process of hydrolysis and, accordingly, the hydrolysis of the protein in the later samples took place in the presence of a much higher relative concentration of carbohydrate decomposition products than in the earlier. The higher proportion of humin nitrogen under these conditions is thus to be anticipated.

The distribution of the nitrogen in the products of hydrolysis of the petiole protein calls for little comment. The total quantity of protein changed very little during the entire period of culture, and the curves in Figure 8 do not suggest that the composition was materially altered. The amino nitrogen ratio appears to be somewhat lower than that of the blade protein and there is a suggestion that this ratio fell slightly during culture, but both amide and humin nitrogen ratios were constant with the possible exception of the final value. The humin nitrogen was unusually high, but the tissue analyzed contained about 2 gm. of protein together with about 21 gm. of non-protein solids in each case and, as has already been pointed out, these are conditions that render the data of very dubious significance.

Soluble Nitrogen

The rate at which water soluble nitrogen appeared in the leaves during culture is shown in Figure 9. The general appearance of the curves indicates

that this nitrogen is derived from the digestion of the protein. If the curves for the whole leaves in Figures 6 and 9 are compared, it will be noted that the initial delay in the digestion of the protein is reflected by an initial delay in the production of soluble nitrogen; that the rapid digestion between 48 and 165 hours is contemporaneous with the rapid appearance of soluble nitrogen, while the check upon protein digestion that occurred after 165 hours is likewise shown in the decrease in rate of formation of soluble nitrogen. Comparison of the respective curves for the petiole, however, shows a conspicuous lack of agreement in detail. Whereas the protein curve indicates that the petiole contained about 0.35 gm. of protein nitrogen at the start and that this changed little if at all until the extreme end of the period studied, the curve for soluble nitrogen in the petiole reveals an increase of nearly 0.2 gm. in the first 96 hours of culture, and shows that this increased quantity was retained until the end of the experiment. This is clear evidence of transport of soluble nitrogen from the blade to the petiole during the early stage of culture while the blade tissue was still in good condition, and is in agreement with observations recorded by Michael (52) who noted a translocation of nitrogen from blade to petiole in leaves of *Tropaeolum majus* during culture in water in the dark.

The magnitudes can be better appreciated from the data collected in Table 4. As before, in Table 3, the initial value is an average of the determinations in the duplicate fresh leaf samples, and the final value is an average of the three observations after 165 hours. Comparison shows that, in the D samples, the actual increase in soluble nitrogen of the whole leaves is about 20 percent greater than the decrease in protein nitrogen, whereas, in the E samples, it is about 13 percent less. A closer agreement between the two experiments could scarcely be expected in view of the different methods used to determine both protein and soluble nitrogen in each case. The quantities of protein nitrogen digested agree well with the increase in soluble nitrogen in order of magnitude, and leave little doubt that most of the newly formed soluble nitrogen must have arisen from the protein. The evidence for transport of soluble nitrogen from the blade to the petiole is also very clear, and the order of magnitude of the quantity indicates that this was by no means an unimportant phenomenon.

There is an interesting and very definite difference between the soluble nitrogen of the D samples and that of the E samples. The one represents nitrogen extracted by hot water from previously *dried* tissue, the other

Table 4. Comparison of the Quantities of Protein Nitrogen Digested with the Quantities of Soluble Nitrogen Produced During 213 Hours of Culture

Figures are grams per kilo of fresh weight.

Samples		Blade			Petiole			Whole leaf		
		Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
Protein N	D	1.46	0.398	1.06	0.349	0.344	0.005	1.81	0.743	1.07
Soluble N	D	0.333	1.36	1.03	0.365	0.601	0.236	0.698	1.96	1.26
Protein N	E	1.79	0.576	1.21	0.390	0.308	0.082	2.18	0.884	1.29
Soluble N	E	0.199	0.991	0.792	0.346	0.702	0.356	0.545	1.70	1.16

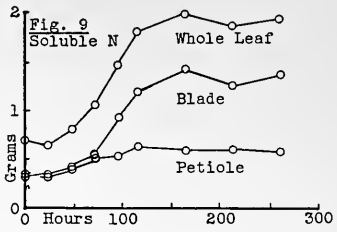


Fig. 9
Soluble N
Whole Leaf
Blade
Petiole
Grams
2
1
0
0 Hours 100 200 300

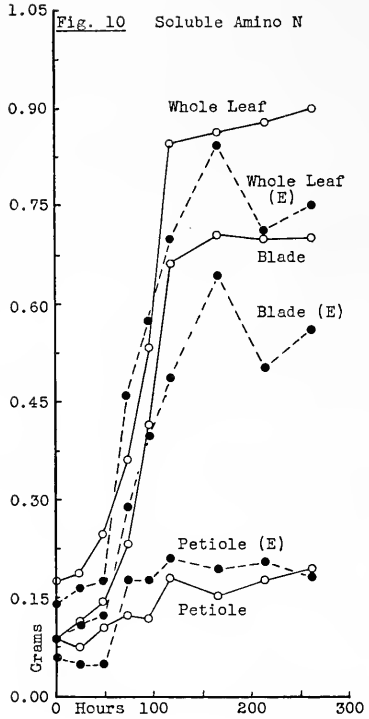


Fig. 10 Soluble Amino N
Whole Leaf
Whole Leaf (E)
Blade
Blade (E)
Petiole (E)
Petiole
Grams
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0.00
0 Hours 100 200 300

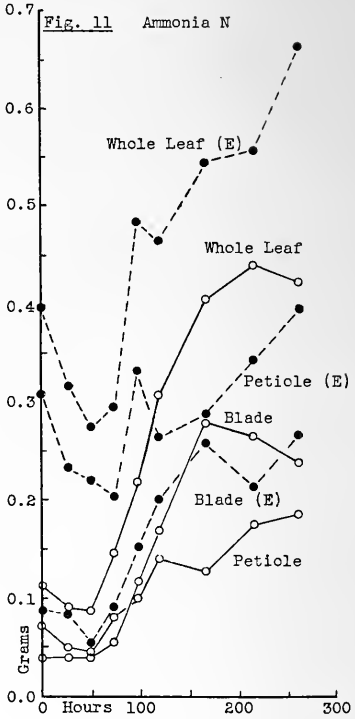


Fig. 11 Ammonia N
Whole Leaf (E)
Whole Leaf
Petiole (E)
Blade
Blade (E)
Petiole
Grams
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 Hours 100 200 300

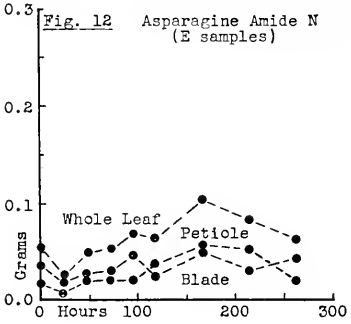


Fig. 12 Asparagine Amide N
(E samples)
Whole Leaf
Petiole
Blade
Grams
0.3
0.2
0.1
0.0
0 Hours 100 200 300

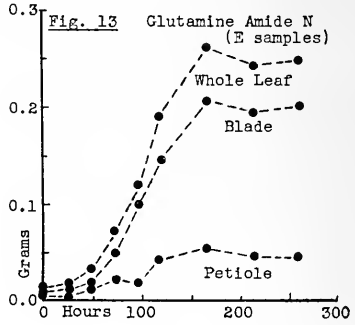


Fig. 13 Glutamine Amide N
(E samples)
Whole Leaf
Blade
Petiole
Grams
0.3
0.2
0.1
0.0
0 Hours 100 200 300

nitrogen extracted by cold water by means of grinding and pressing from tissue that had been cytolyzed with ether. The extract was heated to coagulate any soluble protein, of which only small amounts were present, and was centrifuged. A small deposit that slowly separated after some time in the refrigerator was centrifuged off before analysis. The sum of the soluble nitrogen and that of the insoluble residue is, accordingly, somewhat less than the total nitrogen of the leaves because of the removal of these small precipitates, but the difference is not great as can be seen by comparing the total nitrogen of the D samples with that of the E samples (Table 13).

But the quantity of nitrogen extracted by hot water from the dried *blade* tissue is in every case greater than that extracted by cold water from the cytolyzed tissue. This was not true of the petiole tissue: In the early stages of culture, the D samples showed a slightly higher soluble nitrogen; in the later stages the E samples were higher; in general there was no great difference, and the two methods yield substantially similar results. The difference in the solubility of the nitrogen of the blade tissue is, however, significant; it varies somewhat irregularly from 0.1 gm. of nitrogen in the early stages to more than 0.3 gm. at the end.

A similar observation has been made in connection with some experiments on the drying of tomato plant tissue (82). Tomato leaves and stems were dried at various temperatures in the same drying equipment as that employed for our rhubarb experiments, and samples from the same lots of tissue were extracted with cold water after cytolysis with ether. The results for both leaf and stem tissue are given in Table 5 together with data from a previously unpublished experiment in which the drying was carried out at 60°.

The samples dried at 60° were very young and required a longer period of drying than the other samples at this low temperature; some opportunity

Table 5. The Soluble Nitrogen of Tomato Leaf and Stem Tissue as Determined from Analyses of Samples Dried at Specified Temperatures and Similar Samples Extracted with Cold Water After Ether Cytolysis

Four batches of plants collected at weekly intervals were employed, one for each temperature given. Figures not otherwise designated are grams per kilo of fresh weight.

Dried tissue			Fresh tissue extract	
Temperature of drying	Time of drying	Soluble N	Soluble N	Soluble N corrected for N soluble on extraction after residue had been dried
°C	hrs.			
		LEAVES		
60	2.25	0.972	0.420	0.705
70	1	2.54	1.91	2.18
80	1	2.25	1.79	1.96
90	1	2.55	2.09	2.38
		STEMS		
60	5	0.622	0.420	0.668
70	2	2.94	2.68	2.80
80	1.5	2.66	2.47	2.79
90	1.25	3.28	3.20	3.33

for digestion of protein by the tissue enzymes before these were inactivated was provided, and it is not surprising that the soluble nitrogen of the dried tissue should be materially greater than that extracted by cold water after cytolysis with ether. This consideration applies to a very small extent to the samples dried at higher temperatures, but in these also the soluble nitrogen of the dried was greater than that of the cytolyzed samples. The residues, after expression of the cold water soluble constituents, were dried and subsequently examined by the methods employed for the dried fresh tissue. In all cases additional soluble nitrogen was found, but even when these quantities were applied as corrections on the soluble nitrogen after cytolysis, there was still a deficit from the quantities found after the fresh tissue was dried. On the other hand, the stem tissue extracts, when corrected in this manner, gave results that agree moderately well with the results of extraction after the tissue was dried. The behavior of tomato leaf tissue is accordingly very like that of the rhubarb leaf blade and a difficult question is raised as to the precise meaning of the term soluble nitrogen. Obviously the value secured differs, sometimes quite widely, with the method of extraction of the soluble constituents and with the treatment of the tissue before extraction.

The consideration of the quantity of protein digested and the quantity of soluble nitrogen produced during culture of rhubarb leaves indicates clearly that these two quantities are closely alike. There is, however, no reason to assume that they are identical. Nitrogenous compounds other than protein are present in the leaves and undergo decomposition during culture; chlorophyll is an obvious example and there are doubtless others. The quantities of these other substances are, however, of minor significance in comparison with that of the protein.

The problem may accordingly be restricted to a consideration of the protein decomposition products alone; 1.06 gm. of protein was decomposed in the blade tissue and afforded 0.705 gm. of amino nitrogen, 0.067 gm. of amide nitrogen and 0.258 gm. of non-amino nitrogen (soluble N − (amino N + amide N)). These quantities of nitrogen underwent metabolic changes in the tissue, a small part being transported to the petiole. It is necessary to consider what these changes may have been.

Nitrate Nitrogen

In the present series of samples, no nitrate nitrogen could be detected and the complication in the study of the nitrogen metabolism that nitrate introduces was therefore not encountered. The presence or absence of nitrate nitrogen in a leaf is a result of the conditions in the soil at or just previous to the time of collection of the samples. Although nitrate is usually to be found in rhubarb leaves, and, as Culpepper and Caldwell have shown, may reach an extraordinarily high concentration in this species, it happens that the samples collected in 1936 contained none and none was found after culture of the leaves for many hours. As will be shown in Part II, leaves collected from the same field in 1937 contained considerable nitrate and the quantity present changed significantly during culture. The difference was probably in part due to the use of a different fertilizer in the two seasons, and may also be an expression of the difference in weather conditions in the period immediately before sampling.

Soluble Amino Nitrogen

Digestion of the protein of the tissues into amino acids would be expected to give rise to soluble amino nitrogen in quantities equivalent to those lost by the protein. The data for soluble amino nitrogen are plotted in Figure 10, determinations made upon the dried leaf and also on the cytolyzed fresh leaf (Samples D and E) being included. Particularly in the early stages of culture, there is close agreement between the two sets of samples, but the significantly higher results for the soluble amino nitrogen of the blade tissue in the last four D samples is difficult to understand. It may be that the operation of drying these four samples gave rise to additional amounts of amino nitrogen, though this seems improbable in view of the agreement in the previous samples, and the results of earlier work on tobacco leaves do not support such a view. In the tobacco leaves, *cultured in darkness*, the amino nitrogen as determined in the dried samples checked closely with the amino nitrogen of the extracted samples, save in two or three cases.

On the other hand, it may be that the cold water extraction after cytolysis was less effective in removing amino nitrogen from the last four E samples. There is little justification for this view, however, although one of the E sample values is out of line with the other three.

The soluble amino nitrogen of the petiole tissue is of the same order of magnitude as that of the blade at the start, but it increased much less rapidly during culture. The agreement between the analyses of the D and E samples is for the most part satisfactorily close, and it would appear that the data are measurements of essentially the same thing in the two cases. This is of some importance as will become apparent in connection with the discussion of the ammonia nitrogen.

Table 6 shows the increase in soluble amino nitrogen in both sets of samples compared with the decreases in amino nitrogen derived from the protein. As in previous tables, the values given as initial are averages of the duplicate samples at the start; the final values are the average of the results after 165, 213, and 261 hours of culture. There is a general agreement in order of magnitude between the increase in soluble amino nitrogen in the D samples and the increase that should be expected from the quantity of protein that was digested, but there is very poor agreement when the E samples are so compared.

Table 6. The Change in Soluble Amino Nitrogen of Rhubarb Leaf Tissue During 213 Hours of Culture in Darkness

Figures are grams per kilo of fresh weight.

Samples		Blade			Petiole			Whole leaf		
		Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
Soluble amino N	D	0.088	0.705	0.677	0.089	0.177	0.088	0.177	0.882	0.705
" " "	E	0.082	0.572	0.490	0.058	0.196	0.138	0.140	0.768	0.628
Protein amino N	D	0.953	0.248	0.704	0.214	0.175	0.039	1.17	0.423	0.747
Amino N from protein, calc.	D	1.11	0.302	0.808	0.265	0.261	0.004	1.37	0.563	0.807

At this point it is necessary to anticipate a little with respect to the composition of the soluble nitrogen of these extracts. A large part of the soluble nitrogen of rhubarb leaves consists of ammonia and the other main known component is glutamine. The amino nitrogen determinations do not, therefore, give a true picture of the actual quantity of soluble amino nitrogen, since glutamine behaves abnormally with nitrous acid in the Van Slyke apparatus and yields gas equivalent to 180 percent of the true amino nitrogen. A correction of the amino nitrogen values has therefore been made, and the corrected values are given in Table 13. The results show the rate at which amino acids other than glutamine are produced but this rate is not a measure of the rate of hydrolysis of the protein. The amino acids are in part subjected to oxidative deamination, the ammonia produced being in part employed for the synthesis of glutamine; the amino acids that survive this oxidation accumulate as such. Accordingly, the corrected amino nitrogen values represent the difference in the rates of amino acid production by proteolytic enzymatic digestion of the protein and of amino acid oxidation. A plot of the figures shows that this difference in rate is substantially constant over a large part of the period of culture.

Soluble Peptide Nitrogen

Samples of water extracts prepared from the dried leaf samples were subjected to hydrolysis with 6 N sulfuric acid for 6 hours; ammonia was then removed and the amino nitrogen was determined. If easily hydrolyzable peptides were present in these extracts, the amino nitrogen after hydrolysis should be greater than that before, the difference furnishing a measure of the quantity of peptide nitrogen. A correction must be applied to the amino nitrogen values obtained before hydrolysis because of the abnormal behavior of glutamine.

Examination of the data in Table 13 shows that the blade tissue contained 0.085 gm. of nitrogen apparently in the form of hydrolyzable peptides and that no significant change occurred until nearly the end of the period of study. The individual determinations, during the interval between 48 and 165 hours in which protein digestion proceeded very rapidly, scarcely changed. Whatever this form of nitrogen may really have been, the fact that it was not increased during the period of most rapid protein digestion shows that the enzymes of the blade tissue brought about complete conversion to amino acids of the part of the protein that was digested.

In the petiole, there was an apparent increase in peptide nitrogen during the period of maximal protein digestion in the blade. The initial value of 0.060 gm. increased to 0.150 gm. at 96 hours and the amount subsequently diminished to approximately the initial value at 213 hours. During this period, very little protein digestion took place in the petiole and the results may represent the transport of some product from the blade that was later transformed. We hesitate to interpret the findings to refer definitely to peptide nitrogen, however. The quantity of nitrogen involved is small and has no particular bearing on the general course of the nitrogen metabolism. The most important inference is that peptide nitrogen was not produced in any significant quantity during the period of culture studied.

Ammonia Nitrogen

The rhubarb leaf contains a far larger part of its nitrogen in the form of ammonia than the tobacco leaf, and the ammonia content increased re-

markably during culture in darkness. The reactions which produce this substance are therefore, from the quantitative standpoint, among the most important that occur, and it is necessary at the outset to consider how the nitrogen present as ammonium ions in this tissue may be most accurately determined. Experience with tobacco leaves showed that cold water extraction of the cytolyzed tissue gave extracts in which the ammonia was invariably slightly higher than that found by analysis of tissue that had been dried. The difference was small and for most purposes negligible, particularly as the total quantity present was itself small. The method of extraction after cytolysis is effective; experiments designed to test this in which other soluble constituents such as nicotine or nitrate were determined in both extract and residue have shown that the quantities that remained unextracted are entirely negligible, and there is no reason to suppose that the method is less efficient as a means of extracting ammonium salts. Accordingly, data on the ammonia content of rhubarb tissue obtained by the cold water extraction method are probably reliable.

Results secured in this way are usually somewhat higher than results obtained by analysis of dried rhubarb blade tissue but are invariably very much higher for the petiole tissue. In fact there is no relation whatever between the quantities of ammonia found in petiole tissue by analysis of cold water extracts of the cytolyzed fresh material and the quantities found by direct analysis of the tissue after it has been dried, and it is obvious that grave losses of ammonia occur during the operation of drying the rhubarb petiole. This matter is of fundamental importance in the interpretation of the results of the present culture experiment.

The data are plotted in Figure 11. The two curves for the blade tissue are similar in general, but the curve for the extracted samples suggests a diminution of ammonia during the first 48 hours of culture which is not shown by the data for the D samples, but is clearly shown by the data for the petiole extract and, to a small extent, by the data for the dried petiole. In the interval between 48 and 165 hours, the quantity of ammonia in both blade and petiole increased strikingly, the results by the two methods being similar for the blade. After 165 hours of culture, the ammonia in the blade appeared to change very little; one result appears low and out of line and probably the value at 165 hours for the D samples is a little high.

The petiole, on the other hand, showed a continuous accumulation of ammonia and, save for the sample at 96 hours, the data are consistent and follow a smooth curve.

The curves for the ammonia of the whole leaf furnish an explanation of the irregularities of the respective curves for blade and petiole. The results for the E samples clearly indicate utilization of ammonia during the early stages of culture, and then show a rapid and continuous production of ammonia during the later stages. The apparently abnormal value at 96 hours for the ammonia in the petiole is nearly smoothed out when the value for the blade is added, and the inference is clear that transport of appreciable amounts of ammonia from blade to petiole occurred at about this time. The data for the D samples show a particularly satisfactory curve and suggest that a maximal ammonia content was reached after about 200 hours of culture.

The discrepancy between the two sets of results is difficult to explain. There is no reason to reject the data from the E samples and it therefore seems most likely that the data from the D samples are misleading. To account for the lower results, one may, in the first place, assume actual loss of ammonia from the tissue during the process of drying. There are several reasons for believing this to be improbable, the chief being the high acidity of the tissues, especially the petiole, which had a pH in the vicinity of 3.5. The blade tissue gradually became less acid as culture progressed, but the reaction never exceeded pH 5.5; at such reactions, loss of ammonia by volatilization during drying is incredible and, in fact, experiments designed to test this point showed that losses occur only when the reaction approaches pH 7. Furthermore, the total nitrogen of the D samples remained constant (see Figure 5). A loss of 0.2 gm. of ammonia nitrogen from the petiole would have been apparent; on the contrary there was a distinct gain.

One is forced to the assumption therefore that, during the operation of drying, ammonia entered into reaction with other constituents of the tissue with the production of substances which are not decomposed during the distillation at low temperature in weakly alkaline solution employed for the determination of ammonia. As will be shown below, these hypothetical substances are not entirely decomposed either by acid hydrolysis, nor by a short hydrolysis with normal alkali at boiling temperature (see p. 38-39).

The results of the analysis of the E samples are accordingly accepted to represent most accurately the sequence of events in the leaves, and the following discussion of the ammonia metabolism will be largely confined to these. The problem involves two main questions: What is the explanation of the diminution of ammonia during the first 72 hours of culture, and what is the origin of the ammonia that subsequently appeared in very considerable amount?

Amide Nitrogen

Before an attempt can be made to obtain answers to these questions, it will be necessary to present the data for the amide nitrogen. Two forms of amide nitrogen can be recognized in most plant tissues, one of which is very readily hydrolyzed in buffered solution at pH 7.0 at boiling temperature, the other being stable under these conditions. The second form is hydrolyzed only on being heated with normal acid at boiling temperature for several hours. Accordingly, if the free ammonia present is first determined, then the ammonia present after hydrolysis at pH 7.0 and finally after hydrolysis with normal sulfuric acid, the quantities of the two forms of amide nitrogen can be obtained by subtraction. Experience has shown that determination of the less stable form of amide nitrogen is a convenient and accurate method to estimate glutamine if this substance is present in appreciable quantities. The increase in ammonia produced by hydrolysis with normal acid is in most cases largely due to the hydrolysis of asparagine, but it must be remembered that other substances may be present that are not entirely stable under these conditions. Accordingly estimations of asparagine by this method may be somewhat high, and in cases where asparagine is absent, or is present only in very small amounts, the results may be entirely misleading.

Rhubarb blade tissue furnishes a case in point. A considerable quantity of leaves collected in June, 1937, from the same field as those described in

the present connection, was separately subjected to culture in the dark room for 114 hours. The blade tissue was then carefully examined for glutamine and asparagine. Indirect analysis indicated that 17.9 gm. of glutamine and 4.5 gm. of asparagine were present in the cold water extract from the blades of 104 leaves. A careful fractionation of the amides (80) gave 15.1 gm. or 84.5 percent of the glutamine in pure crystalline form, but no crystals of asparagine could be detected although every effort was made to bring asparagine to separate. Treatment of the mother liquors from the glutamine crystals with mercuric sulfate resulted in the precipitation of only 5 percent of the apparent asparagine amide nitrogen in them, and the conclusion was drawn that the tissue contained no significant quantity of asparagine whatever, the indirect analytical values for asparagine amide nitrogen being entirely due to the presence of other substances which yield ammonia on dilute acid hydrolysis (79).

Examination of the data for asparagine for the E samples given in Table 13, and plotted in Figure 12, shows that only a small quantity of apparent asparagine amide nitrogen was present either in blade or petiole in any case, and that this changed very little during culture. The data for the dried tissue indicated even smaller amounts of asparagine in the blade, and the calculation for the petiole gave many small negative quantities. It may be concluded, therefore, that the asparagine values obtained in this way are not determinations of asparagine at all, but are an indication of the presence in relatively small amounts of some substance or substances which behave like asparagine on hydrolysis. Rhubarb is evidently not an asparagine-forming plant.

The data for glutamine, on the other hand, are unequivocal. Glutamine has been isolated in substantial amounts from rhubarb blade tissue after culture in darkness, and evidence to be presented later leaves little doubt that it is present, though in much smaller relative proportion, in the petiole. The quantities found in the extracted E samples are plotted in Figure 13, and indicate a slow synthesis of glutamine during the first 48 hours, followed by a period of about 100 hours when glutamine was synthesized with great rapidity in the blade. After the lapse of 165 hours, glutamine synthesis apparently ceased.

The D samples (Table 13) likewise show a slow followed by a rapid synthesis of glutamine, and the values obtained are uniformly somewhat higher than those in the E samples, an illustration of the fact that plant tissues can be dried successfully without loss of significant amounts of glutamine. The difference between the results on the D and E blade samples is appreciable in only three cases, the samples at 117, 219, and 261 hours; and for the petiole samples only in two cases, those at 0 and at 96 hours. On the whole, the data may be regarded as in excellent agreement.

Amide Metabolism

In connection with our investigations of the culture of tobacco leaves, it was assumed that protein digestion occurred and that the amino acids produced were largely deaminized by an oxidative process. Much of the ammonia liberated was transformed, by reaction with suitable non-nitrogenous precursors, into glutamine and asparagine. A small part of the nitrate present in these leaves was reduced to ammonia, and it was found that the

quantity of nitrogen ultimately present in the amides, or as ammonium ions, was equal to the sum of the amino nitrogen of that part of the protein that was digested and subsequently oxidized and the small amount of nitrate that was reduced. The equivalence of these quantities was taken as evidence in support of the early hypotheses of Schulze in respect of protein metabolism in leaves.

It is of interest to see to what extent a similar accounting of the nitrogen may be made in the present case. In Table 7 are shown two sets of calculations, one for the changes during 165 hours of culture, the other for the changes that occurred in approximately 213 hours, the data for the final value being the calculated mean of the results at 165, 213, and 261 hours. During the interval from 165 to 261 hours the protein changed very little although the ammonia increased.

Table 7. The Relationships Between Protein, Amino, Amide, and Ammonia Nitrogen Changes in Rhubarb Leaves During Culture for 165 and for 213 Hours

Data are given in grams per kilo of original fresh weight of whole leaves.

		165 hrs.	213 hrs.
1	Protein N loss	1.056	1.067
2	α-Amino N liberated from protein	0.721	0.745
3	Ammonia N gain (E)	0.148	0.191
4	Glutamine amide N gain (E)	0.247	0.237
5	Soluble amino N gain (E)	0.703	0.628
6	Protein amino N corrected for glutamine (2−8% of 1)	0.637	0.660
7	Glutamine amide N corrected for glutamine from protein (4−8% of 1)	0.163	0.152
8	Soluble amino N corrected for glutamine (5−180% of 4)	0.258	0.202
9	Amino N transformed (6−8)	0.379	0.458
10	Total N of synthesized glutamine (twice 7)	0.326	0.304
11	Glutamine N + ammonia N (10 + 3)	0.474	0.495
12	Difference (9−11)	−0.095	−0.037

The details of the calculation may require some explanation. In line 2 is given the amount of α-amino nitrogen that would be liberated if the quantity of protein shown in line 1 is digested by proteolytic enzymes. The value was obtained as described on p. 22. Lines 3, 4, and 5 show the increases in ammonia, glutamine amide, and soluble amino nitrogen. Line 6 is an estimate of the part of the α-amino nitrogen liberated by digestion of the protein due to amino acids other than glutamine. If the glutamic acid that results from acid hydrolysis of protein is actually combined in the intact protein molecule as glutamine, then, when the protein is subjected to the action of proteolytic enzymes that are capable of breaking a peptide bond but which do not decompose an amide grouping, glutamine will be produced directly from the protein. Under the conditions in rhubarb leaf where glutamine is being synthesized, one might expect the digestion of the proteins to proceed in this way. Unfortunately we have no information regarding the glutamic acid content of rhubarb leaf proteins, but a series of leaf proteins analyzed in Chibnall's laboratory has yielded results that are remarkably constant, the mean value being approximately 8 percent of the protein nitrogen as nitrogen of glutamic acid (53). If this value can be ac-

cepted as a rough estimate of the glutamic acid content of the protein that was digested during the culture period, then 8 percent of the protein nitrogen must be deducted from the amino nitrogen liberated from the protein to give the α-amino nitrogen due to amino acids other than glutamine. Thus the figures in line 6 are calculated by subtracting 8 percent of those in line 1 from those in line 2.

In order to avoid confusion, it must be borne in mind that the data of line 2 were obtained by acid hydrolysis and accordingly the glutamine of the original protein molecule will be represented by glutamic acid.

Since the amount of amide nitrogen in glutamine is equal to the α-amino nitrogen, the same correction is applied in line 7 to the actual glutamine amide nitrogen that was formed in the leaves, in order to allow for the glutamine that may have been derived directly from the protein by enzymatic hydrolysis, the corrected value being the quantity of glutamine amide nitrogen that must have been formed in some other way.

The whole of the glutamine in the tissue will, of course, be found in the cold water soluble extract from the leaves and will therefore contribute to the α-amino nitrogen present in this fraction. A correction for this must be found in order to obtain the quantity of amino nitrogen in the extract that was not altered in any way during the culture period and which belongs to amino acids other than glutamine. Since glutamine yields 180 percent of its amide nitrogen as gas in the Van Slyke apparatus, the corrected value for the soluble amino nitrogen other than glutamine is found by subtracting 180 percent of the glutamine amide nitrogen (line 4) from the soluble amino nitrogen (line 5). The difference is shown in line 8. If nothing had happened to the amino nitrogen derived from the protein subsequent to its liberation, line 8 should agree with line 6, both being quantities of amino nitrogen belonging to amino acids other than glutamic acid, the one being the amount calculated from the analysis of the protein that was decomposed, the other being the amount found in soluble form in the tissues. The difference between these two values is shown in line 9 as the amino nitrogen transformed.

According to the hypothesis of Schulze, this is the nitrogen that should have been converted into ammonia by oxidative deamination and subsequently, in part, resynthesized into glutamine. Two moles of ammonia are required to form one mole of glutamine. Hence the actual quantity of nitrogen involved in the synthesis of glutamine is obviously twice the value in line 7; this is given in line 10. In line 11, the gain in free ammonia is added and the result is compared, in line 12, with the quantity of amino nitrogen transformed, shown in line 9. There is a discrepancy of only 0.095 gm. at 165 hours, and of only 0.037 gm. at approximately 200 hours, the data in the latter case being probably more trustworthy since the results of the analysis of three separate samples were averaged to give the final figures. The small magnitude of the discrepancy is clear evidence that the hypothesis of Schulze applies in the present case as it was found to do in the tobacco plant.

The above argument is seriously complicated by the correction for the glutamine that may have been derived from the protein by enzymatic digestion. If this correction is disregarded, the increase in soluble amino nitrogen at 200 hours corrected for glutamine is, as before, 0.202 gm. but

the amino nitrogen transformed is obtained by subtracting this from line 2. The difference is 0.543 gm. The total nitrogen of the glutamine increase is twice line 4 or 0.474 gm. and, if the free ammonia increase of 0.191 gm. is added, the sum is 0.665 gm. The difference between this value and the 0.543 gm. of amino nitrogen transformed is 0.122 gm. Thus, if the correction for the glutamine derived directly from the protein is omitted, the agreement between the sum of the ammonia and glutamine nitrogen, on the one hand, and the quantity of amino nitrogen transformed on the other, is not as good, although, when the complex nature of the chemical operations and the possibilities of error in the analyses are considered, even this degree of agreement with theory is not to be despised. In both cases, there is evidence that the quantity of ammonia found is somewhat greater than would have been produced merely by oxidative deamination of α-amino acids. This presents no theoretical difficulty. The protein contains nitrogen in a number of different forms and it is quite possible that nitrogen other than peptide nitrogen was converted ultimately to ammonia; in fact Chibnall (20) is disposed to believe that the whole of the protein nitrogen is potentially available for this purpose. Arginine, for example, after oxidative deamination may have been further oxidized and the guanidino group transformed so as to yield a little ammonia, Urea is a conceivable intermediate product of this reaction and, if urease is present in the tissues, this would be promptly converted to ammonia. The intermediary metabolism of histidine may also give rise to ammonia.

The results in general, therefore, confirm the conclusion drawn from the experiments with tobacco leaves. The quantity of nitrogen that underwent metabolic change, as calculated from the ammonia and amide nitrogen values, agrees substantially with that provided on the hypothesis that the amino acids derived from protein digestion underwent oxidative deamination.

It is very interesting to note that the rhubarb plant possesses an amide metabolism closely analogous to that of the tobacco plant. According to the views of Prianischnikow, the synthesis of amides in plants is a mechanism provided to maintain the concentration of ammonia below a toxic level and data in support of this view have been presented by him, by Mothes, and by other investigators. Evidence secured in this laboratory shows that this mechanism is called promptly into play in the tobacco, beet, and tomato plants and, under normal conditions, apparently operates with great efficiency. The ammonia nitrogen found in the tissues of these species is usually very low—in the tobacco leaf, for example, being of the order of 0.8 percent of the soluble nitrogen ((85), calculated from data of Table 20), and in the tomato leaf about 1 percent of the soluble nitrogen ((82), calculated from data of Table VI). After being cultured in darkness for 143 hours, the ammonia of tobacco leaves may rise to as high as 10 percent of the soluble nitrogen though smaller values are more usual. The rhubarb leaf, however, appears to be able to tolerate far higher relative proportions of ammonia than these other species. The data in Table 8 show the proportion of the soluble nitrogen present as ammonia in the fresh tissue and after culture for approximately 213 hours (average of last three observations). The results for the free ammonia of samples D may be rejected for technical reasons already discussed; the results for samples E, the ammonia in this case being determined after direct extraction from the tissues with cold

water, indicate a striking relationship. Nearly one-half the soluble nitrogen of the blade and nearly nine-tenths of that of the petiole were present as ammonia. For the whole leaf, the relationship was approximately 73 percent. During culture, however, although the ammonia in the blade tissue increased materially and that in the petiole slightly, the ratio of ammonia to soluble nitrogen was greatly depressed so that for the whole leaf it became approximately 35 per cent. If, however, there had been no synthesis of amides, the ammonia present at the end of the culture period would have been much greater. The increase in glutamine amide nitrogen (Samples E whole leaf) was 0.237 gm. (Table 7); twice this is 0.474 gm. and, in the absence of amide synthesis, the total ammonia would have been at least 1.06 gm. The mean soluble nitrogen of the last three samples is 1.70 gm., hence

TABLE 8. CONCENTRATION OF AMMONIA NITROGEN IN TERMS OF SOLUBLE NITROGEN IN RHUBARB LEAF TISSUE

Figures not otherwise designated are grams per kilo of fresh weight.

	Soluble N		Ammonia N		Ammonia N as percent of soluble N	
	Fresh leaf	After 213 hrs.	Fresh leaf	After 213 hrs.	Fresh leaf	After 213 hrs.
Blade D	0.333	1.36	0.041	0.261	12.3	19.2
Petiole D	0.365	0.601	0.073	0.163	20.0	27.1
Whole leaf D	0.698	1.96	0.114	0.425	16.3	21.7
Blade E	0.199	0.991	0.088	0.246	44.2	24.8
Petiole E	0.346	0.702	0.310	0.343	89.5	48.9
Whole leaf E	0.545	1.70	0.398	0.589	72.9	34.6

the ammonia alone would have made up 62 percent of the soluble nitrogen. This is only a little less than the ratio of ammonia to soluble nitrogen in the whole leaf at the start of the experiment. Obviously, therefore, most of the newly formed soluble nitrogen must have passed through the stage of ammonia, and the amide synthesis that occurred greatly reduced the ultimate proportion of ammonium ions present. Furthermore the amide synthesizing mechanism in this series of samples was promptly brought into action[1] and, although it does not operate in rhubarb with the effectiveness that it does in the tobacco leaf with respect to maintenance of the ammonia at a very low level, it clearly has an important function, which may perhaps be best expressed as being analogous to that of a buffer provided to prevent rapid fluctuations in the ammonium ion concentration. The picture presented by the amide metabolism is essentially similar in both species; in the rhubarb leaf it is merely displaced further up the scale since this plant appears to be able to tolerate ammonia in far higher concentrations than tobacco.

Comparison of Figures 11 and 13 shows that amide synthesis was most active in the interval from 72 to 165 hours and that this was also the interval during which ammonia was being produced with greatest rapidity.

[1] The series of samples to be described in Part II behaved somewhat differently in this respect. The evidence suggested that the glutamine synthesizing mechanism came into effective operation only after many hours of culture despite the fact that the ammonia concentration was high even at the start.

Examination of the behavior during the first 72 hours shows that the quantity of free ammonia in the whole leaf diminished from 0.398 to 0.296 or by 0.102 gm. per kilo. The glutamine amide nitrogen in this period (Samples E) rose from 0.014 to 0.073 gm. or by 0.059 gm. If our hypothesis of amide metabolism is correct, the ammonia that disappeared should have been converted into glutamine, half of it being employed to form the α-amino group and half the amide group. The correspondence in amount is strikingly close to that required; 0.102 gm. of ammonia should yield 0.051 gm. of amide nitrogen whereas the quantity actually found was 0.059 gm. There was no significant change in the apparent asparagine amide nitrogen in this interval.

Although the agreement is very close when the calculations for the whole leaf are considered, examination of the individual figures for petiole and blade shows a less striking agreement. The ammonia in the blade tissue was at a minimum at 48 hours but had returned to the original value in 72 hours; the ammonia in the petiole was at a minimum at 72 hours but shortly thereafter increased phenomenally, probably owing to transport from the blade. Glutamine amide nitrogen increased significantly, however, in both tissues throughout the first 72 hours and the transport of soluble substances from blade to petiole during this period renders the interpretation of the changes in quantity in the two tissues difficult. The most important inference from the behavior of the ammonia and the glutamine during the early period of culture is, however, that the mechanism which produces ammonia did not begin to operate with notable effectiveness until after the lapse of 72 hours, while the mechanism which produces glutamine was operative from the start. This means that the substances from which ammonia is readily produced only became available in significant amounts after 72 hours and this in turn correlates with the behavior of the protein. According to the hypothesis that has been suggested, the ammonia arises from oxidative deamination of amino acids produced by proteolytic digestion; from the data for protein nitrogen, it is clear that significant amounts of amino acids were produced only after the lapse of 72 hours. All the data that bear upon this point are therefore consistent with the hypothesis.

"Extra" Ammonia Nitrogen

It has been pointed out, in connection with the determination of the ammonia nitrogen, that the values obtained by analysis of the dried tissues were invariably lower than those obtained by analysis of water extracts of the tissues prepared after cytolysis with ether (E samples). The operation of drying these tissues, particularly the petioles, led to what appeared to be serious losses of ammonia. It has been suggested above that we have here to do with a reaction between ammonia and some other component of the tissue that occurs during the drying process.

This phenomenon was also encountered in our studies of tobacco leaf tissue (85, p. 794 and p. 810). The apparent losses were in that case small and either set of ammonia values could be used to calculate the amide nitrogen without producing serious discrepancies in the results. Furthermore it was found that, if extracts of the dried tissues were hydrolyzed with normal acid in order to decompose the asparagine and were then subsequently

boiled with normal alkali for 5 minutes, an additional quantity of ammonia was liberated which corresponded closely in amount with the ammonia apparently lost during the drying operation. This ammonia produced by an extra alkaline hydrolysis was designated "extra ammonia nitrogen". The only reason we have for suggesting that it may represent the ammonia that disappeared during drying is the correspondence in amount. Other suggestions, for example that it represents a small residuum of asparagine that had not been hydrolyzed under the conditions adopted for asparagine hydrolysis, or that it represents some unknown normal constituent present in small amounts which is stable to acid hydrolysis but is decomposed by mild alkaline hydrolysis, are also plausible.

The disconcerting discrepancy between the ammonia values for the dried and for the water extracted rhubarb tissues, particularly the petiole, led us to turn to the determination of the so-called "extra" ammonia nitrogen in the hope that some explanation of this discrepancy might be found. Examination of the dried blade tissue by this entirely empirical method showed that little or no ammonia is produced by a short normal alkali hydrolysis applied after hydrolysis with normal acid: rhubarb blade tissue yields no significant quantity of "extra" ammonia nitrogen. Study of the data in Table 13, or of the curves in Figure 11, shows that, for the blade tissue, the discrepancy between the ammonia values for the D and the E samples is not in fact very great, and this observation therefore appears to support the hypothesis advanced in Bulletin 399 with regard to the nature of this form of nitrogen.

The petiole tissue, on the other hand, yielded an appreciable quantity of "extra" ammonia nitrogen, and this remained unchanged during the period of culture; the average value being 0.083 ± 0.011 gm. Examination of the difference between the ammonia values obtained from the D and E samples (Table 13) shows that this varied in an irregular manner between the limits 0.273 and 0.111. Although the "extra" ammonia nitrogen may represent a part of this quantity, there is clearly no possibility of accounting for the whole of the ammonia that disappeared during the drying of the petiole tissue in terms of conversion into a product that is stable to acid but is decomposed by mild alkali hydrolysis.

It is interesting also to note that the "extra" ammonia nitrogen of the petiole is in all cases materially higher than the apparent asparagine amide nitrogen (Table 13). As has been pointed out, there is probably no asparagine present in this tissue at all, at least in the early stages of culture,[1] and the evidence therefore suggests the presence from the beginning of appreciable quantities of substances other than amides that yield ammonia under suitable conditions of hydrolysis. Further information on the qualitative composition of rhubarb plant tissue is very much to be desired.

ORGANIC ACIDS

pH of Extracts

The acidity of the successive samples of rhubarb leaf tissue as determined on the cold water extracts of the fresh material (E samples) is plot-

[1] It is possible that a little asparagine may be produced directly from the protein, but the proportion of aspartic acid yielded by leaf proteins is low, and in the present case, nearly all the asparagine from this source would be expected to be liberated in the blade tissue. This matter is discussed further in Part III.

ted in Figure 14. Although there are minor irregularities, particularly with the blade tissue, the data as a whole show that the acidity diminished materially as culture proceeded. The blade tissue was initially at a reaction not far from pH 4.0 but had reached pH 5.5 at the expiration of 261 hours. The petiole tissue, which was initially at a reaction not far from pH 3.0, likewise became progressively less acid and finally reached pH 4.0. These changes may be accounted for in two ways: oxidation of organic acids to non-acidic end-products, or progressive neutralization by means of basic organic products of metabolism. As will shortly appear, both of these reactions probably occurred.

The magnitude of the change in acidity during the first 100 hours of culture, while the leaves were still in relatively healthy condition, is quite appreciable if the data from the probably more reliable E series are considered, and suggests that a wide variation in reaction may occur under normal conditions. This is borne out by observations on freshly collected normal leaves—we have found blade tissue samples collected at the same time that showed reactions from pH 3.4 to 4.1 and petiole tissue from 2.7 to 3.4. It seems, therefore, that the rhubarb leaf may show considerable normal variation in reaction.

Total Organic Acids

The changes in the total organic acidity of the dried leaf samples during culture of rhubarb leaves are shown in Figure 15. Over the entire period of culture, there was a loss of some 20 percent of the organic acids of the blade and of about 36 percent of those of the petiole; calculated as malic acid (1 m.e. = 0.067 gm.) this loss is of the order of magnitude of 8.5 gm. of organic solids or nearly half of the loss of organic solids that occurred during the culture period (see Figure 3). This must not be interpreted to mean that the organic acids that disappeared were converted into volatile products that escaped from the tissue. The estimate is introduced merely to emphasize the order of magnitude of the change that occurred with relationship to the organic solids of the tissues.

The behavior of the organic acids of rhubarb leaves is entirely different from that of tobacco leaves. In that tissue, although striking changes in the relative quantities took place, the changes were of the nature of interconversions and the total organic acidity changed to only a minor extent during culture in the dark.

Detailed examination of the curves for rhubarb leaves indicates that decomposition of the organic acids did not assume significance until the lapse of more than 50 hours—previous to this there appeared to have been a small amount of transport of organic acids from blade to petiole—but thereafter the organic acids, particularly of the petiole, underwent rapid conversion to non-acidic products. This observation correlates in general with the change in acidity that occurred although, as will be shown, neutralization by means of ammonia also played a part in the change in reaction.

The much more extensive absolute and proportional losses of organic acids from the petiole than from the blade is a somewhat unexpected phenomenon. If oxidation plays the dominant role here, one might expect this

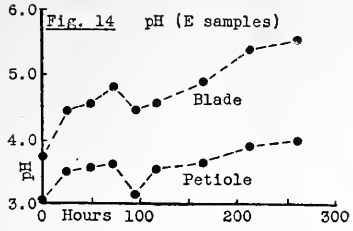


Fig. 14 pH (E samples)
6.0
5.0
4.0
3.0
pH
Blade
Petiole
0 Hours 100 200 300

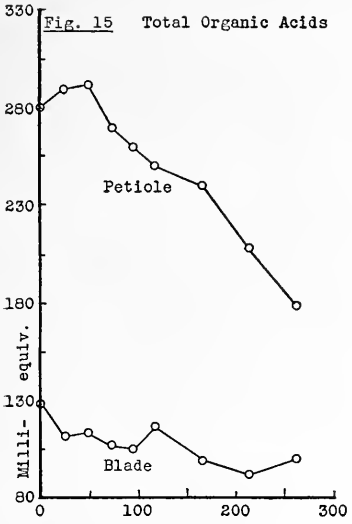


Fig. 15 Total Organic Acids
330
280
230
180
130
80
Milli- equiv.
Petiole
Blade
0 100 200 300

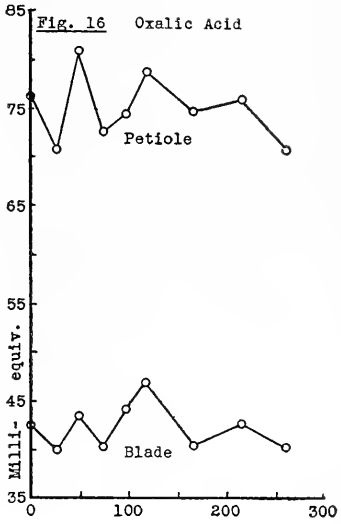


Fig. 16 Oxalic Acid
85
75
65
55
45
35
Milli- equiv.
Petiole
Blade
0 100 200 300

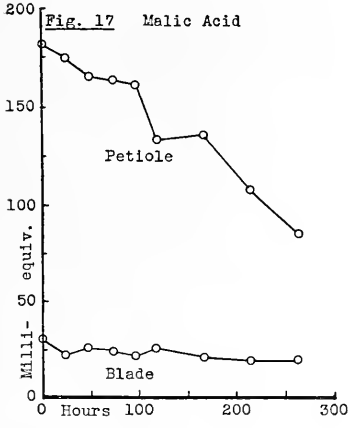


Fig. 17 Malic Acid
200
150
100
50
0
Milli- equiv.
Petiole
Blade
0 Hours 100 200 300

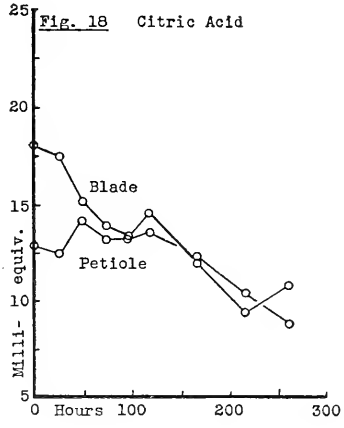


Fig. 18 Citric Acid
25
20
15
10
5
Milli- equiv.
Blade
Petiole
0 Hours 100 200 300

to occur more readily in the blade tissue, with its high ratio of surface to weight and adaptation to reactions in which gas exchange through surfaces takes place. On the contrary, however, by far the greater change occurred in the relatively massive tissue of the petiole. There are analogies in this connection to the rates of loss of organic solids from tobacco leaves and tobacco stalks (86).

Oxalic Acid

The behavior of the oxalic acid is shown in Figure 16. The petiole contained nearly twice as much oxalic acid as the blade tissue, resembling in this respect the younger leaves described in a previous paper (66), but the quantity present changed very little during culture. Analysis of the data by the method of least squares shows that the oxalic acid of the petiole decreased by 3 milliequivalents and that of the blade increased by 4 milliequivalents over a period of 300 hours, that is, in the whole leaf, there was no significant change whatever. There is a possibility that a little oxalic acid migrated from petiole to blade, in the direction of the concentration gradient, but the quantity is probably within the limits of error of the determinations. It is clear, therefore, that oxalic acid is not extensively involved in the organic acid metabolism of rhubarb leaves during culture in water in darkness.

The quantity of oxalic acid in the blade tissue of rhubarb is of the same order of magnitude when expressed in terms of the whole leaf as that present in tobacco leaves (85, Table 20), both being close to 40 milliequivalents per kilo. But, if the comparison is made in terms of blade tissue only, it is much higher. Since the mean ratio of the weight of the blade to the weight of the whole fresh leaf is 0.323 (p. 12), the average oxalic acid content of 42.3 milliequivalents per kilo of whole leaf is equivalent to 131 milliequivalents per kilo of blade tissue. Thus the oxalic acid content of rhubarb blade tissue in terms of equal masses is about three times as great as that of tobacco leaves. The concentration of anhydrous oxalic acid in the blade tissue (42.3 milliequivalents=1.90 gm.) is 4.8 percent of the organic solids at the start and 5.6 percent at the end, the apparent increase being entirely due to the loss of organic solids other than oxalic acid chiefly by respiration.

Although the quantity of oxalic acid in the petiole of the rhubarb leaf is approximatelyt wice that in the blade (average, 75.1 milliequivalents per kilo of whole leaf), the concentration in terms of fresh weight is lower, being 111 milliequivalents per kilo of petiole tissue. The actual amount, 3.38 gm., was 7.4 percent of the organic solids of the petiole at the start and 9.9 percent at the end of the culture period, the change again being due to loss of solids other than oxalic acid.

Because of the material loss of malic acid that occurs during culture of rhubarb leaves in darkness, the relative proportion of oxalic acid in terms of the total organic acidity increased. Thus, at the beginning of the culture, oxalic acid made up 33 percent of the acids of the blade and 27 percent of those of the petiole; after 261 hours of culture, the proportion of oxalic acid had increased to 40 percent of the acids in the blade and to 39 percent in the petiole, although the actual amount in the respective tissues had scarcely changed at all.

The oxalic acid of tobacco leaf is all, or nearly all, present in a form insoluble in water; from 10 to 20 percent only of the oxalic acid can be extracted from the dried tissue with hot water, while extraction of fresh tissue with cold water after ether cytolysis usually fails to bring any detectable quantity of oxalic acid into solution. Inasmuch as the reaction of tobacco leaf tissue is close to pH 5.4, a reaction at which about 95 percent of oxalic acid is present as the divalent ion, and calcium is present in considerable amounts, this behavior is to be expected.

The greater acidity of rhubarb leaf tissue, however, materially alters the behavior of the oxalic acid. About 40 percent of the oxalic acid present in fresh blade tissue can be extracted by cold water, while as much as 64 percent of that of the petiole may be soluble. The dissociation curve of oxalic acid would lead one to expect that, at the respective reactions of the blade and petiole, 29 percent and 7 percent of the oxalic acid would be present as divalent ions, that is, in the present case, the blade should contain 12.4 milliequivalents of divalent oxalate ion and the petiole 5.3 milliequivalents. The calcium oxide content of the blade of the initial fresh leaf sample was 0.92 percent of the crude dry weight, or the equivalent of 14.4 milliequivalents of oxalic acid. This agrees fairly closely with calculated quantity of oxalic acid present as divalent ion in this part of the leaf. The proportion of the oxalic acid that remained insoluble after thorough extraction with cold water was, however, materially greater, being 25.8 milliequivalents. There is every reason to suppose that insoluble oxalic acid is usually present as calcium oxalate, but in this case there was only about half as much calcium present as is necessary to account for the insolubility.

A better agreement between the proportions of calcium and of insoluble oxalic acid was obtained from the analysis of the petiole tissues. The initial D sample of petiole contained 1.45 percent of calcium oxide, which is the equivalent of 27.1 milliequivalents of oxalic acid. The corresponding E sample contained 49.5 milliequivalents of soluble and 27.3 milliequivalents of insoluble oxalic acid. Clearly, therefore, the whole of the insoluble oxalic acid may have been present as calcium salt, although, from the dissociation curve, one would expect only 5.3 milliequivalents of divalent oxalate ion. It is evident that the relationships between the oxalic acid, the calcium, and the reaction of these tissues require far more thorough investigation than we have as yet been able to give them.

Malic Acid

Figure 17 shows the behavior of the malic acid of rhubarb leaves during culture in darkness. The malic acid of the blade is only one-sixth of that of the petiole at the start and careful examination of the curve suggests that the change during the first 100 hours of culture is relatively small and, even at the end of the experiment, the total loss of malic acid from the blade amounted to only 5 to 7 milliequivalents. This is a fairly large proportion of the malic acid present—roughly one-quarter—but, compared with the change that occurred in the petiole, it is very small.

The malic acid of the petiole rapidly diminished in amount even from the beginning, and the total loss over the entire period was approximately 97 milliequivalents or slightly more than half the malic acid present. A mathematical analysis of the data, on the assumption that the change was

a straight line function of time, indicates the loss to have been 94.4 milliequivalents or 50.6 percent of the initial quantity present. This represents 6.3 gm. of malic acid, and the change as a whole approaches the order of magnitude of the loss of malic acid observed when tobacco leaves are cultured in water under similar conditions. The striking difference is, however, that this loss occurred in the petiole; the loss from the rhubarb blade tissue was much smaller. Furthermore, in the tobacco leaf, the loss of malic acid could be almost quantitatively accounted for in terms of a conversion into citric acid. In the rhubarb petiole, the loss can be accounted for almost entirely in terms of a loss of organic acidity; the malic acid that disappeared was apparently converted into non-acidic products.

It has in many cases been possible to show analogies between the behavior of various constituents of rhubarb petiole and that of similar constituents of tobacco stalks. This cannot be done in the present case. The malic acid of tobacco stalks *increased* from about 31 to 41 milliequivalents per kilo during culture in darkness for 330 hours. The behavior of the malic acid of rhubarb petiole is therefore entirely different from that of the malic acid in the tobacco plant.

Citric Acid

Citric acid makes up a relatively small part of the organic acids in rhubarb leaves, but its behavior during culture is different from that of either the malic or the oxalic acid. The data plotted in Figure 18 show that the citric acid of the blade diminished continuously from approximately 18 milliequivalents to approximately 10 milliequivalents during the culture period. Whether this was due to oxidation to non-acidic end-products, or whether a part of the change may be accounted for by transport to the petiole, cannot be determined from the present data. The total organic acids of the blade diminished by about 28 milliequivalents but the sum of the loss of citric, oxalic, and of malic acids accounts for only about 20 milliequivalents.

The citric acid of the petiole increased slightly during the early stage of the culture. The change was small and scarcely beyond the experimental error of the determinations; significant loss did occur, however, after the lapse of 165 hours. The temporary increase in citric acid, in the early stage of the culture, if real, may equally well have been brought about by transport from the blade or by the conversion of a little malic acid into citric acid in the same manner as was found to occur in tobacco leaf. The magnitude of the change is too small, however, to permit definite conclusions. The important point is that the citric acid of the petiole changed very little in spite of the relatively enormous diminution in the malic acid. Synthesis of citric acid from malic acid in this tissue during culture in darkness, if it occurred at all, can be at most a minor phenomenon.

Unknown Acids

When the sum of the oxalic, malic, and citric acids is deducted from the total organic acidity, there is in each case a balance due to the presence of organic acids other than these three. The values found are somewhat irregular because they contain the combined errors of the individual determinations, but have interest in that they show the order of magnitude of

this quantity. The data are given in Table 13: In the blade, the unknown acids made up a relatively constant quantity, the average being 27.5 ± 4.7 milliequivalents. The trend of the values suggests a slight loss during the greater part of the culture period, but no definite conclusion can be drawn.

The values for the petiole are more irregular than those for the blade but are quite low, the average being 16 milliequivalents with individual values from 8 to 30 milliequivalents. There is no obvious trend, and it may be concluded that the unknown acids do not enter actively into the metabolism. In any case they make up less than 10 percent of the organic acidity of the petiole.

Distribution of Organic Acidity

The relative proportions of the several acids in terms of percentage of the total organic acidity are summarized in Table 9. There is little change in the distribution of the acids in the blade save in the oxalic acid. It will be recalled that the actual quantity of oxalic acid did not change significantly at all, but relatively, it increased from 33 to 40 percent of the total acids during the entire period. In the petiole, the oxalic acid likewise remained unchanged in actual amount, but, owing to the large change in malic acid, it increased relatively from 27 to nearly 40 percent of the whole, while the malic acid decreased from 65 percent of the total acidity to 46 percent. The citric acid both in blade and petiole maintained a nearly constant ratio to the whole.

Table 9. Distribution of Organic Acids in Rhubarb Leaves

Figures are percentages of the total organic acidity respectively of the blade and petiole.

	Blade			Petiole		
Hours	0	96	261	0	96	261
Malic acid	23.8	22.1	20.7	65.0	63.0	46.3
Oxalic acid	33.4	42.4	40.3	27.4	28.3	39.5
Citric acid	13.9	12.9	10.8	4.6	5.1	4.9
Unknown acid	28.9	22.7	28.2	3.0	3.1	9.3

Relationship Between pH and Acid Composition

The changes in the pH of cold water extracts obtained from the samples after ether cytolysis, plotted in Figure 14, obviously reflect the changes in the amounts of organic acids that occurred. It is necessary to consider another important factor, however, before any attempt can be made to account for these changes in reaction. The formation of ammonia during the culture period was sufficiently extensive to require consideration and this factor, fortunately, is readily estimated. To what extent other products of the decomposition of the protein may enter into the acid-base balance is, however, a matter of conjecture.

In Table 10 are given the data necessary to calculate the equivalents of monovalent base which must be in combination with organic acids at the reactions of the blade and petiole respectively, at the start and at the end of

the culture period on the assumption that simple equilibrium relationships prevail. The hydrogen ion activities are taken from the calculated straight line which best represents the observations of pH in the extracts of the E samples. The oxalic acid values for the petiole are also taken from the calculated straight lines. In Table 11 are given the quantities of monovalent base that would be expected to be combined with the several ions of the organic acids at the respective reactions of the two tissues as calculated from the dissociation constants.

Table 10. The Organic Acid and Ammonia Content of Rhubarb Leaves Before and After Culture in Darkness for 261 Hours

Figures are milliequivalents per kilo of whole leaf.

	Hours	Malic acid	Citric acid	Oxalic acid	Unknown acids	Ammonia	pH
Blade	0	30.4	17.9	42.6	36.5	6.3	4.08
	261	20.8	10.8	40.4	28.4	19.1	5.52
Petiole	0	182	12.8	76.3	8.0	22.1	3.25
	261	85	8.8	73.6	14.7	28.3	4.00

If attention is confined for the moment to the malic and citric acids, it is clear that the reaction of the blade tissue at the termination of the experiment may be accounted for if 10.6 equivalents of monovalent base are added to the system which has changed in composition as shown in Table 10. The actual increase in ammonia was 12.8 equivalents and, accordingly, the change in reaction of the blade tissue is to this extent accounted for. There is no obvious reason, however, for the exclusion of the oxalic and the unknown acids from this calculation and, when these are included, 38 equivalents more of monovalent base would be required to bring about the change in reaction that actually occurred. Presumably an appreciable part of the oxalic acid is present as insoluble calcium salt, but the quantities of soluble oxalic acid actually found in the extracts of the blade tissue do not conform at all well with the proportion of the total oxalic acid calculated as divalent ion at the reactions of the blade at the start and at the end of the culture experiment. Furthermore, if the actual quantities of soluble oxalic acid (Table 13) are employed in the calculation, more than 32 equivalents of monovalent base are still required to bring about the change observed.

The situation in the petiole is equally puzzling. If again the malic and citric acid alone are considered, 8.9 milliequivalents of monovalent base must be added. The actual increase in ammonia was 6.2 equivalents. When oxalic acid and the unknown acids, calculated on the assumption that they resemble malic acid in strength, are included, 22 additional equivalents of base would be required.

It is quite obvious, therefore, that the observed change in reaction of the tissues cannot be accounted for simply in terms of change in amounts of the individual organic acids and the addition of ammonia on the assumption of equilibrium relationships. The balance sheet of the ions suggests that other changes must also have taken place which rendered considerable

TABLE 11. MONOVALENT BASE BOUND BY ORGANIC ACIDS OF RHUBARB TISSUES BEFORE AND AFTER CULTURE IN DARKNESS FOR 261 HOURS

Figures not otherwise designated are milliequivalents per kilo of whole leaf.

Acid		Before culture			After culture			Δ
		% dissociation of acid	m.e.	Monovalent base equivalent m.e.	% dissociation of acid	m.e.	Monovalent base equivalent m.e.	m.e
				BLADE				
Malic	as M″	9	2.7	5.4	71	14.7	29.4	
	as M′	71	21.6	21.6	28	5.8	5.8	
Citric	as C‴	4	0.7	2.1	50.5	5.4	16.2	
	as C″	33	5.9	11.8	42	4.5	9.0	
	as C′	54	9.6	9.6	6.8	0.7	0.7	
Total				50.5			61.1	10.6
Oxalic	as O″	29.4	12.5	25.0	92	37.1	74.2	
	as O′	70.4	30.0	30.0	8	3.2	3.2	
Unknown	as U″	9	3.2	6.4	71	20.0	40.0	
	as U′	71	25.9	25.9	28	7.9	7.9	
Total				87.3			125.3	38.0
				PETIOLE				
Malic	as M″	11	1.8	3.6	7.2	6.1	12.2	
	as M′	33	61.0	61.0	69.5	59.0	59.0	
Citric	as C‴	0.6	0.1	0.3	3	0.26	0.78	
	as C″	7	0.9	1.8	29	2.5	5.0	
	as C′	52	6.4	6.4	57	5.0	5.0	
Total				73.1			82.0	8.9
Oxalic	as O″	6.0	0.4	0.8	26	19.1	38.2	
	as O′	92	70.0	70.0	74	54.4	54.4	
Unknown	as U″	1	0.08	0.16	7.2	1.0	2.0	
	as U′	33	2.6	2.6	69.5	1.0	1.0	
Total				73.6			95.6	22.0

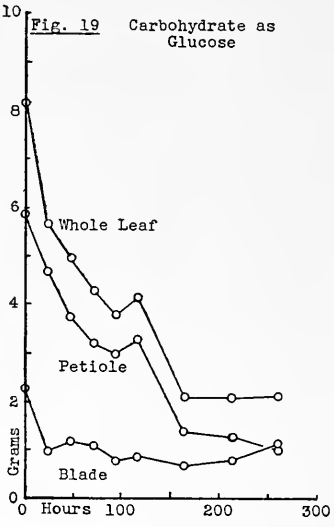
quantities of base available. What these changes may have been does not appear from the data at present available, but it seems highly probable that a realignment of the inorganic basic constituents may have occurred along with the increase in available organic base and the diminution in total organic acidity. The main purpose of the present investigation will have been served if the terms of this problem are more clearly defined. The marked loss of malic acid during the culture of rhubarb leaves in the dark recalls the behavior recorded by Allsopp (5). During the summer season, all the organic acids in the rhubarb shoot increased enormously, but, beginning in September, there was a sharp decrease, the malic acid fall being "in excess of any gain in the acids of either leaf or rhizome."

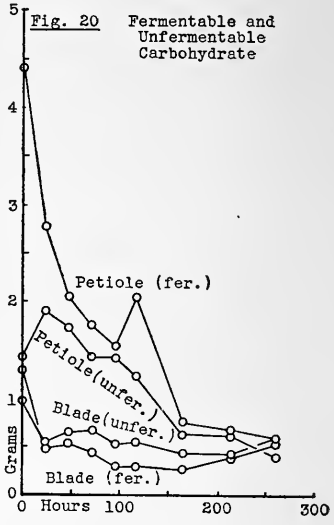
Accordingly, conversion of malic acid into some other product or products must occur at this time, and Allsopp suggests carbohydrates or carbon dioxide as possibilities. The present study indicates that malic acid

is readily oxidized in this tissue. It may be supposed that, under normal conditions with alternations of light and darkness, carbohydrates are chiefly respired, but as the leaves become older and possibly less efficient photosynthetically, and the periods of darkness become longer, the burden falls more and more heavily upon the acid. A loss of malic acid such as Allsopp observed is therefore to be anticipated. This possibility is discussed more fully in Part III.

CARBOHYDRATES

Figure 19 shows the change in reducing carbohydrate, calculated as glucose. At the start, the petiole contained more than twice as much carbohydrate as the blade resembling in this the stalk of the tobacco plant. A rapid loss occurred during the early hours of the culture. The behavior of





the blade carbohydrate suggests that approximately equal amounts of substances of two degrees of stability were involved; one of these appears to have been very rapidly oxidized, the other being far more resistant, much of it remaining until the end of the culture period. About half of it had disappeared after the lapse of 100 hours, but the portion that remained after 160 hours subsequently changed very little. The presence of this more resistant part of the carbohydrate is even more clearly shown by the curve for the whole leaf.

The fermentable carbohydrate data are plotted in Figure 20, together with those for the unfermentable carbohydrate. The rapid loss of fermentable sugar from both tissues is evident as is the obviously greater stability of the unfermentable part. The curve for the fermentable carbohydrate of the blade shows that the whole of this part of the sugar is not equally readily oxidized; more than half disappeared within the first 24 hours, but subse-

quently it diminished slowly and, in the last stage of the culture when the blade tissue was brown and almost completely collapsed, the fermentable sugar increased slightly. It seems improbable that the increase was due to transport from the petiole, but it may be suggested that complex carbohydrates or possibly glucosides present in this rapidly autolyzing tissue were decomposed with the production of minor quantities of fermentable sugar.

The fermentable sugar of the petiole behaved in a manner that resembles that of the tobacco stalk very closely. By no means all of it was oxidized, but the quantity present diminished to a very low level, much lower indeed than that in the tobacco stalk. There was no evidence of an increase at the end of the culture period as was observed in the blade.

The unfermentable carbohydrate of the blade decreased sharply in the first 24 hours but thereafter remained substantially constant. In the petiole, however, this fraction at first increased significantly, possibly because of transport from the blade, and then underwent a slow and steady decomposition at a rate parallel to that of the fermentable carbohydrate. The chemical nature of this part of the carbohydrate is not known, but substances of a similar general behavior have been observed not only in tobacco but also in other plant species (71).

It is of interest to compare the loss of carbohydrate with the loss of organic solids. The essential data are collected in Table 12. In the blade, the loss of carbohydrates was 1.14 gm. whereas the loss of organic solids was 6.1 gm. If the losses of malic and citric acid are added to that of the carbohydrate, the total is 2.25 gm. which is only 37 percent of the loss of organic solids. Other constituents of the blade must therefore have been oxidized to volatile products during the culture period.

Table 12. The Changes in Carbohydrates, Organic Acids, Protein and Organic Solids of Rhubarb Leaves, During Culture in Water in Darkness, Which Have a Bearing on the Respiration

Figures are grams per kilo of whole leaf.

	Blade			Petiole		
	0 hrs.	261 hrs.	Change	0 hrs.	261 hrs.	Change
Total carbohydrate	2.28	1.14	1.14	5.87	1.00	4.87
Malic acid	2.04	1.39	0.65	12.2	5.69	6.51
Citric acid	1.15	0.69	0.46	0.82	0.56	0.26
Total			2.25			11.64
Protein residues estimated			3.32			
Total			5.57			
Organic solids	40.0	33.9	6.10	45.8	33.9	11.9

Consideration of this leads at once to the question of the fate of the products of digestion of the protein which were deaminized with the production of ammonia. If it be assumed that these amino acid residues were in large part further oxidized to carbon dioxide and water, a fairly reasonable account can be given of the loss of organic solids from the blade tissue

of these leaves. The blades at the start contained 1.46 gm. of protein nitrogen. The nitrogen content of this protein is not known, but Miller (53) has presented evidence for believing that purified preparations of grass proteins in general contain about 16 percent of nitrogen. In other words, the conventional factor 6.25 may be used without serious error to calculate the protein content of grass blade tissues from the protein nitrogen. If this be granted, and it is further assumed that the protein of rhubarb blades is similar in nitrogen content, the quantity of protein present can be estimated to have been 9.13 gm. at the start and 2.49 gm. at the end of 213 hours of culture, that is to say, 6.64 gm. of protein were digested. There is no factor known to convert this quantity of protein to the weight of the residual carbon compounds that remain after the protein is digested and the α-amino nitrogen is removed by oxidative deamination. However, hydrolysis of the protein involves addition of water usually to the extent of 12 to 18 percent of the protein digested. The water may be in part obtained from other components so that no substantial change in weight of the tissue is necessarily involved. Oxidation may also possibly proceed, at least to some extent, anaerobically and, since an amino group is replaced by oxygen, there is again no necessarily significant change in weight of the tissues; if molar oxygen enters, an increase is to be expected. Accordingly the products of hydrolysis and of the subsequent first stage of oxidation will probably weigh about the same or a little more than the protein digested. Further oxidation, however, undoubtedly leads to loss of weight of the tissue solids through loss of volatile products.

The data are fairly closely fitted if it is arbitrarily assumed that 50 percent of the protein was oxidized to volatile products.[1] The quantity of solids involved is then 3.32 gm. and, if this is added to the combined carbohydrate and organic acid loss of 2.25 gm., the total loss becomes 5.6 gm. which is not far from the actual loss (6.1 gm.) of organic solids.

An account of the losses of solids from the petiole tissue involves no assumptions regarding the protein since the change in protein was negligible. The sum of the carbohydrate loss, and of the malic and citric acid losses, is 11.6 gm., in remarkably close agreement with the actual loss of organic solids (11.9 gm.). Furthermore, if the losses of carbohydrate and the two acids at each stage of the culture be plotted, the curve agrees within 0.5 gm. or less, with the exception of one point, with a similar curve for the loss of organic solids. Thus not only the total loss, but the rate of loss of organic solids from the petiole is quantitatively accounted for if it be assumed that the carbohydrate and organic acids that disappeared were oxidized to volatile end-products.[2]

In the tobacco leaf (85), the loss of carbohydrate accounted for only a part of the total loss of organic solids from the leaves, and there was no loss of organic acidity. No conclusions could be drawn with respect to the nature of the substances oxidized save that substances other than carbohydrates furnished a large part. In the stalk (86), however, the loss of carbohydrate and the loss of organic solids corresponded very closely.

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[1] This estimate is revised and more fully discussed in Part III. The data there presented show that the changes in the organic acids were probably associated with the formation of glutamine and that the loss due to respiration thus may have fallen even more heavily upon the protein than is here assumed.

[2] That respiration may have drawn upon still other components of the leaves is shown in Part III. The actual process was more complex than is indicated by the data for organic solids discussed here.

SUMMARY

Samples of young but fully developed rhubarb leaves were subjected to culture in distilled water in continuous darkness. Duplicate samples were analyzed at the start, and successive samples were removed for analysis at intervals up to 261 hours. Two sets of samples were employed, the leaves of one set being divided into blade and petiole and dried for analysis, the others being similarly divided and separately cytolyzed with ether and extracted with cold water, both extract and residue being analyzed. From determinations of the various forms of nitrogen, of the organic acids and the carbohydrates, the behavior of many of the components of the leaf were ascertained and inferences have been drawn with respect to the nature of certain of the chemical changes that occurred.

The behavior of the blade tissue was entirely different from that of the petiole. Although transport of a part of certain constituents of the blade into the main veins and petiole was observed, it was possible in many cases to distinguish between reactions that occurred chiefly in the blade or chiefly in the petiole.

During the first 72 hours, the water content of the leaves was increased above the initial value and the tissues were unusually turgid. At 96 hours, loss of water from the blade and chlorophyll destruction became apparent, as shown by an increasing area of yellow color which progressed inward from the margins of the leaves. The petioles and main veins, however, remained fully turgid for more than 200 hours, although the blade tissue had become shriveled and brown at this time.

The leaves may be regarded as having been in a fully healthy condition for at least 72 hours, but the subsequent collapse of the blade tissue was rapid. The petioles remained in good condition for nearly 200 hours.

Both blade and petiole lost organic solids steadily from the first, owing to transformation into volatile products (respiration). The loss from the petiole was both greater and more rapid than that from the blade, possibly because it remained in a healthy condition much longer, and the total loss in the entire period amounted to nearly one-quarter of the organic solids present.

There was no change in the total nitrogen, but conversions of one form of nitrogen into another were extensive. The protein of the blade changed very little for 48 hours, but then underwent rapid digestion, more than two-thirds having disappeared in 165 hours. Subsequently, however, there was little further change. The protein of the petiole was initially less than one-quarter of that of the blade, and very little evidence of digestion was found until the lapse of more than 200 hours.

The nitrogen of the digested protein was converted into water soluble substances, and a small part of these was transported to the petiole. A significant difference was found between the soluble nitrogen of each sample as determined by hot water extraction of the dried tissue and of the soluble nitrogen of the corresponding samples by determination of the nitrogen of the cold water extract after cytolysis with ether. This difference was greatest for the petiole, and the values for ammonium nitrogen were the ones chiefly affected. The tissues behaved as if a considerable part of the ammonia were lost during the operation of drying. There was no loss of

total nitrogen, however, and the behavior was therefore interpreted to indicate the transformation of a part of the ammonia into some other form of nitrogen during the operation of drying the samples. Accordingly ammonia nitrogen values obtained on dried tissue, particularly of the petiole, may be seriously in error. Furthermore a difficult question is raised with respect to the meaning of the term "soluble nitrogen" as applied to the analysis of plant tissues.

Examination of the forms of soluble nitrogen gave evidence in support of Schulze's early hypothesis of nitrogen metabolism according to which amino acids, derived from the digestion of the protein, are to a considerable extent deaminized, the ammonia produced being largely employed in the formation of one or another of the amino acid amides glutamine and asparagine. In rhubarb leaves, glutamine only is produced, no evidence for the presence of significant amounts of asparagine having been found at any stage of the culture period.

The rhubarb leaf differs from the tobacco leaf in certain respects in connection with the nitrogen metabolism. In the first place, the proportion of the nitrogen present as ammonia is very much higher in normal leaves than is usually found in tobacco. During culture, the ammonia increases materially although a considerable part of it is converted into the nitrogen of glutamine. In the tobacco plant, on the other hand, asparagine is formed during culture of the leaves in darkness, glutamine being produced in significant amounts only during culture in light. The quantity of α-amino nitrogen which disappeared from the rhubarb leaves could be accounted for with considerable precision in terms of the total nitrogen of the glutamine that was synthesized, and of the newly formed ammonia. Thus the general picture of the protein metabolism in rhubarb leaves resembles that of tobacco leaves; the differences are that glutamine alone is involved, and that ammonia is present in far higher concentrations at all stages.

Interpretation of amide synthesis in rhubarb in terms of Prianischnikow's hypothesis of ammonia detoxication is not especially helpful. It is difficult to see why a slight increase in the ammonia above the 15 percent of the total nitrogen originally present should give rise to damage to the tissues. Nevertheless, the amide metabolism reduced the proportion of ammonia materially during the first 72 hours of culture and, even at the end of 261 hours, the ammonia amounted to only 25 percent of the total nitrogen. If no glutamine had been synthesized, the final value would have been about 44 percent of the total nitrogen. Thus the presence of an amide-synthesizing mechanism greatly restricted the quantity of ammonia formed and tended to prevent rapid fluctuations in the proportion present. The amide synthesizing mechanism therefore behaved somewhat in the manner of a buffer and to this extent the idea implied by Prianischnikow's term may represent an actuality in this plant.

The hydrogen-ion activity of extracts from the rhubarb leaf decreased during culture. The blade tissue changed from pH 4.0 to 5.5 and the petiole from pH 3.0 to 4.0 in 261 hours. This decrease in acidity was accompanied by a diminution in the quantities of organic acids present and by an increase in the ammonia. The loss fell almost entirely upon the malic acid, about half of the initial quantity of which disappeared during the culture period. The changes in citric acid were of a minor nature, and the oxalic acid was apparently not affected.

Examination of the data for organic solids, for soluble carbohydrates, and for malic acid showed that the entire loss of organic solids from the petiole could be accounted for as the oxidation of soluble carbohydrates and of malic acid to volatile products. Thus one of the important functions of malic acid in the rhubarb petiole during culture is to provide a part of the material employed for respiration. Malic acid and soluble carbohydrates disappeared in quantities roughly in the ratio of 3 to 2.

In the blade tissue, only about two-thirds of the loss of organic solids could be accounted for as a loss of malic acid and of soluble carbohydrates. If it be assumed, however, that 50 percent of that part of the blade proteins that underwent digestion was subsequently oxidized to volatile end products, a fairly accurate accounting of the loss can be made. It will be shown in Part III, however, that an even larger part of the protein was probably involved in this loss of solids. This assumption implies that the amino acids that underwent deamination were subsequently completely oxidized and provides a useful hypothesis of the ultimate fate of the proteins of the blade.

Attempts to correlate the changes in hydrogen-ion activity with the changes in the individual organic acids and in the ammonia were unsuccessful. It is obvious that considerably more base must be provided to account for the change, and it is suggested that an extensive reorientation of the acid and basic components of the tissues must have occurred.

The soluble carbohydrates of both blade and petiole diminished rapidly, probably chiefly due to respiration, but the loss fell much more heavily upon the fermentable carbohydrate than upon the unfermentable. A part of this fraction, the chemical nature of which is unknown, was rapidly oxidized during the first 24 hours, but the remaining unfermentable carbohydrate subsequently changed very little.

No direct information was obtained with respect to the nature of the carbon compound which combines with ammonia to produce glutamine, but an hypothesis, based upon the analytical data already given, to account for this synthesis is to be presented in Part III.

TABLE 13. COMPOSITION OF RHUBARB LEAVES

Figures not otherwise designated are grams per kilo of fresh weight of whole leaves

Hours		0	24	48	72	96	117	165	213	261
Sample[1]										
Fresh weight										
B	D	324	336	351	334	296	262	153	91.4	55.6
P	D	676	726	706	703	712	699	692	655	599
WL	D	1000	1062	1057	1037	1008	961	845	746	654
B	E	315	365	353	349	317	289	172	72.5	47.6
P	E	685	717	745	708	729	719	711	668	605
WL	E	1000	1082	1098	1057	1046	1008	883	740	652
Total solids										
B	D	43.8	40.8	43.7	39.5	37.2	39.6	37.5	35.8	37.9
P	D	52.3	51.8	52.2	48.7	48.4	47.6	47.6	42.1	40.8
WL	D	96.1	92.6	95.9	88.2	85.6	87.2	85.1	77.9	78.7
Water										
B	D	280	295.3	306.8	294.9	258.9	233.0	115.4	55.6	17.9
P	D	624	673.9	654.1	654.4	663.5	651.4	644.0	612.5	558.3
WL	D	904	969	961	949	922	884	759	668	576
Organic solids										
B	D	40.0	37.1	39.6	35.9	33.6	35.7	33.8	32.1	33.9
P	D	45.8	45.3	45.1	42.1	41.8	41.1	41.0	35.4	33.9
WL	D	85.8	82.4	84.7	78.0	75.4	76.8	74.8	67.5	67.8
Inorganic solids										
B	D	3.76	3.68	4.11	3.59	3.64	3.90	3.70	3.71	3.99
P	D	6.42	6.49	7.14	6.61	6.56	6.52	6.59	6.68	6.87
WL	D	10.18	10.17	11.25	10.20	10.20	10.42	10.29	10.39	10.86
Total nitrogen										
B	D	1.99	2.02	1.97	1.78	1.80	1.88	1.85	1.75	1.78
P	D	0.809	0.746	0.841	0.963	0.975	0.991	1.03	0.967	0.945
WL	D	2.80	2.77	2.81	2.74	2.775	2.87	2.88	2.717	2.725
B	E	1.99	1.94	1.62	1.74	1.79	1.60	1.65	1.48	1.60
P	E	0.786	0.732	0.809	0.864	1.03	0.993	1.03	1.02	0.982
WL	E	2.78	2.67	2.43	2.60	2.82	2.59	2.68	2.50	2.58
Protein nitrogen (nitrogen of alcohol and hot-water extracted residue of dried tissue)										
B	D	1.46	1.46	1.42	1.09	0.791	0.588	0.381	0.395	0.419
P	D	0.349	0.327	0.397	0.344	0.355	0.347	0.373	0.360	0.301
WL	D	1.81	1.79	1.82	1.43	1.15	0.935	0.754	0.755	0.720
Protein nitrogen (total nitrogen minus nitrogen of dried tissue soluble in water at 80°)										
B	D	1.66	1.67	1.55	1.23	0.861	0.687	0.421	0.484	0.414
P	D	0.443	0.433	0.438	0.447	0.438	0.366	0.424	0.354	0.359
WL	D	2.10	2.10	1.99	1.68	1.30	1.05	0.845	0.838	0.773

[1] B represents blade, P petiole, and WL whole leaves; D represents samples that were dried, E samples that were extracted with cold water after treatment with ether.

TABLE 13—Continued

Hours		0	24	48	72	96	117	165	213	261
Sample[1]										
Protein nitrogen (total nitrogen of residue from cold water extract)										
B	E	1.787	1.696	1.378	1.210	1.039	0.722	0.510	0.622	0.595
P	E	0.390	0.385	0.413	0.399	0.415	0.378	0.356	0.305	0.265
WL	E	2.177	2.081	1.791	1.609	1.454	1.100	0.866	0.927	0.860
Amino nitrogen of protein after hydrolysis										
B	D	0.953	0.935	0.800	0.759	0.551	0.359	0.250	0.235	0.258
P	D	0.214	0.203	0.224	0.191	0.205	0.203	0.196	0.184	0.144
WL	D	1.167	1.138	1.024	0.950	0.756	0.562	0.446	0.419	0.402
Amide nitrogen of protein after hydrolysis										
B	D	0.091	0.097	0.093	0.079	0.057	0.047	0.036	0.038	0.039
P	D	0.022	0.023	0.024	0.021	0.022	0.023	0.025	0.024	0.022
WL	D	0.113	0.120	0.117	0.100	0.079	0.070	0.061	0.062	0.061
Humin nitrogen of protein after hydrolysis										
B	D	0.118	0.101	0.105	0.089	0.073	0.064	0.071	0.071	0.071
P	D	0.051	0.053	0.060	0.050	0.052	0.049	0.065	0.051	0.052
WL	D	0.169	0.154	0.165	0.139	0.125	0.113	0.136	0.122	0.123
Soluble nitrogen of protein after hydrolysis										
B	D	1.29	1.37	1.23	0.994	0.752	0.551	0.335	0.356	0.349
P	D	0.297	0.274	0.337	0.294	0.304	0.298	0.308	0.308	0.249
WL	D	1.59	1.64	1.57	1.29	1.06	0.849	0.643	0.664	0.598
Amino nitrogen in percent of protein nitrogen										
B	D	65.4	63.9	56.2	69.4	69.6	61.0	65.5	59.5	61.6
P	D	61.8	62.2	56.6	55.4	57.7	58.5	52.6	51.2	47.7
Amide nitrogen in percent of protein nitrogen										
B	D	6.27	6.63	6.55	7.25	7.26	7.97	9.54	9.72	9.35
P	D	6.41	6.99	6.08	6.00	6.32	6.55	6.69	6.59	7.38
Humin nitrogen in percent of protein nitrogen										
B	D	8.10	6.92	7.35	8.15	9.17	10.8	18.7	18.0	16.9
P	D	14.7	16.2	15.1	14.6	14.5	14.1	17.5	14.3	17.3
Soluble nitrogen in percent of protein nitrogen										
B	D	89.8	93.6	86.6	91.0	95.1	93.7	87.8	90.1	83.3
P	D	85.3	83.8	84.9	85.4	85.5	85.9	82.5	85.7	82.7
Soluble nitrogen										
B	D	0.333	0.348	0.423	0.553	0.935	1.19	1.43	1.27	1.37
P	D	0.365	0.314	0.404	0.516	0.537	0.624	0.604	0.613	0.586
WL	D	0.698	0.662	0.827	1.069	1.472	1.814	2.03	1.88	1.96
B	E	0.199	0.240	0.245	0.529	0.748	0.877	1.14	0.862	1.00
P	E	0.346	0.348	0.395	0.470	0.617	0.616	0.671	0.718	0.716
WL	E	0.545	0.588	0.640	0.999	1.365	1.49	1.81	1.58	1.72

[1] B represents blade, P petiole, and WL whole leaves; D represents samples that were dried, E samples that were extracted with cold water after treatment with ether.

TABLE 13—Continued

Hours	Sample[1]	0	24	48	72	96	117	165	213	261
Soluble amino nitrogen										
B	D	0.088	0.117	0.145	0.236	0.415	0.666	0.708	0.702	0.705
P	D	0.089	0.074	0.107	0.125	0.119	0.182	0.156	0.178	0.197
WL	D	0.177	0.191	0.252	0.361	0.534	0.848	0.864	0.880	0.902
B	E	0.082	0.113	0.123	0.288	0.400	0.490	0.647	0.506	0.564
P	E	0.058	0.052	0.052	0.178	0.178	0.211	0.196	0.207	0.186
WL	E	0.140	0.165	0.175	0.466	0.578	0.701	0.843	0.713	0.750
Soluble "peptide" nitrogen										
B	D	0.085	0.065	0.085	0.085	0.104	0.097	0.112	0.103	0.124
P	D	0.060	0.071	0.095	0.124	0.151	0.090	0.100	0.067	0.049
WL	D	0.145	0.136	0.180	0.209	0.255	0.187	0.212	0.170	0.173
Soluble amino nitrogen corrected for glutamine										
B	D	0.036	0.077	0.098	0.146	0.201	0.281	0.323	0.313	0.360
P	D	−0.013	0.029	0.046	0.035	−0.001	0.105	0.046	0.083	0.107
WL	D	0.023	0.106	0.144	0.181	0.200	0.386	0.369	0.396	0.467
B	E	0.066	0.051	0.084	0.198	0.218	0.124	0.274	0.153	0.200
P	E	0.049	0.041	0.027	0.133	0.142	0.134	0.097	0.122	0.101
WL	E	0.115	0.092	0.111	0.331	0.360	0.258	0.371	0.275	0.301
Ammonia nitrogen										
B	D	0.041	0.042	0.041	0.066	0.118	0.169	0.279	0.266	0.239
P	D	0.073	0.050	0.046	0.082	0.100	0.140	0.128	0.176	0.186
WL	D	0.114	0.092	0.087	0.148	0.218	0.309	0.407	0.442	0.425
B	E	0.088	0.085	0.055	0.091	0.153	0.201	0.258	0.213	0.267
P	E	0.310	0.233	0.220	0.205	0.333	0.266	0.288	0.344	0.397
WL	E	0.398	0.318	0.275	0.296	0.486	0.467	0.546	0.557	0.664
Ammonia nitrogen after hydrolysis at pH 7.0										
B	D	0.069	0.064	0.067	0.116	0.237	0.382	0.495	0.524	0.486
P	D	0.130	0.075	0.080	0.131	0.167	0.184	0.189	0.229	0.236
WL	D	0.199	0.139	0.147	0.247	0.404	0.566	0.684	0.753	0.722
B	E	0.097	0.098	0.076	0.141	0.254	0.348	0.466	0.408	0.469
P	E	0.315	0.239	0.234	0.228	0.353	0.309	0.343	0.392	0.444
WL	E	0.412	0.337	0.310	0.369	0.607	0.657	0.809	0.800	0.913
Ammonia nitrogen after hydrolysis with 1 N acid										
B	D	0.076	0.074	0.081	0.122	0.252	0.419	0.526	0.576	0.517
P	D	0.093	0.078	0.083	0.118	0.136	0.187	0.170	0.218	0.234
WL	D	0.169	0.152	0.164	0.240	0.388	0.606	0.696	0.794	0.751
B	E	0.115	0.106	0.098	0.163	0.276	0.386	0.523	0.461	0.491
P	E	0.353	0.259	0.263	0.261	0.400	0.334	0.392	0.424	0.488
WL	E	0.468	0.365	0.361	0.424	0.676	0.720	0.915	0.885	0.979
Glutamine amide nitrogen										
B	D	0.029	0.022	0.026	0.050	0.119	0.214	0.216	0.258	0.247
P	D	0.057	0.025	0.034	0.050	0.067	0.043	0.061	0.053	0.050
WL	D	0.086	0.047	0.060	0.100	0.186	0.257	0.277	0.311	0.297
B	E	0.009	0.013	0.021	0.050	0.101	0.148	0.207	0.196	0.202
P	E	0.005	0.006	0.014	0.023	0.020	0.043	0.055	0.047	0.047
WL	E	0.014	0.019	0.035	0.073	0.121	0.191	0.262	0.243	0.249

[1] B represents blade, P petiole, and WL whole leaves; D represents samples that were dried, E samples that were extracted with cold water after treatment with ether.

TABLE 13—Continued

Hours		0	24	48	72	96	117	165	213	261
Sample[1]										
Asparagine amide nitrogen										
B	D	0.007	0.010	0.014	0.006	0.016	0.037	0.031	0.052	0.031
P	D	−0.037	0.003	0.003	−0.013	−0.031	0.003	−0.019	−0.011	−0.002
WL	D	−0.030	0.013	0.017	−0.007	−0.015	0.040	0.012	0.041	0.029
B	E	0.018	0.008	0.022	0.022	0.022	0.038	0.057	0.053	0.022
P	E	0.038	0.020	0.028	0.032	0.047	0.026	0.049	0.032	0.044
WL	E	0.056	0.028	0.050	0.054	0.069	0.064	0.106	0.085	0.066
Ammonia nitrogen after 1 N acid hydrolysis followed by 1 N alkali hydrolysis										
P	D	0.192	0.156	0.154	0.196	0.216	0.281	0.265	0.298	0.304
"Extra" ammonia nitrogen										
P	D	0.099	0.078	0.071	0.078	0.081	0.095	0.095	0.079	0.069
Difference between ammonia nitrogen values										
P	E-D	0.273	0.183	0.174	0.123	0.233	0.126	0.160	0.168	0.111
Total amide nitrogen										
B	D	0.036	0.032	0.040	0.056	0.134	0.251	0.248	0.310	0.278
P	D	0.020	0.028	0.037	0.036	0.036	0.046	0.042	0.042	0.049
WL	D	0.056	0.060	0.077	0.092	0.170	0.297	0.290	0.352	0.327
B	E	0.026	0.021	0.043	0.072	0.123	0.185	0.264	0.248	0.224
P	E	0.043	0.026	0.042	0.056	0.067	0.069	0.105	0.080	0.092
WL	E	0.069	0.047	0.085	0.128	0.190	0.254	0.369	0.328	0.316
pH										
B	D	4.36	4.55	4.48	4.53	4.58	4.90	5.02	5.41	5.41
P	D	3.42	3.56	3.52	3.58	3.60	3.57	3.65	3.79	4.16
B	E	3.73	4.44	4.55	4.80	4.44	4.56	4.91	5.39	5.54
P	E	3.06	3.50	3.57	3.62	3.15	3.55	3.65	3.89	4.01
Total organic acids in milliequivalents										
B	D	128	111	113	107	105	117	99	92	100
P	D	280	290	292	270	259	250	240	208	179
WL	D	408	401	405	377	364	367	339	300	279
Malic acid in milliequivalents										
B	D	30.4	23.3	27.1	24.9	23.2	26.0	21.8	20.3	20.8
P	D	182	175	167	165	163	134	137	108	85
WL	D	212	198	194	190	186	160	159	128	106
Citric acid in milliequivalents										
B	D	17.9	17.5	15.2	13.8	13.3	14.6	12.0	9.4	10.8
P	D	12.8	12.5	14.2	13.2	13.4	13.5	12.3	10.4	8.8
WL	D	30.7	30.0	29.4	27.0	26.7	28.1	24.3	19.8	19.6

[1] B represents blade, P petiole, and WL whole leaves; D represents samples that were dried, E samples that were extracted with cold water after treatment with ether.

TABLE 13—Concluded

Hours / Sample[1]		0	24	48	72	96	117	165	213	261
Oxalic acid in milliequivalents										
B	D	42.6	40.0	43.6	40.4	44.4	46.9	40.4	42.7	40.4
P	D	76.8	70.8	81.0	72.7	74.6	78.9	74.8	75.9	70.8
WL	D	119	111	125	113	119	126	115	119	111
Total known acids in milliequivalents										
B	D	91	80.8	85.9	79.1	80.9	87.5	74.2	72.4	72.0
P	D	272	258	262	251	251	226	224	194	165
WL	D	363	339	348	330	332	314	298	266	237
Unknown organic acids in milliequivalents										
B	D	36.5	30.1	26.8	28.2	23.9	29.6	24.5	19.6	28.2
P	D	8.0	31.8	29.1	18.5	7.8	23.3	15.4	14.6	14.7
WL	D	44.5	61.9	55.9	46.7	31.7	52.9	39.9	34.2	42.9
Soluble oxalic acid in milliequivalents										
B	E	16.8	16.2	14.6	21.3	24.3	24.7	23.1	16.3	19.5
P	E	49.5	30.5	43.8	41.0	51.0	45.7	49.4	39.7	31.1
Soluble oxalic acid as percent of total oxalic acid										
B	$\frac{E}{D}$	39.0	40.4	33.5	52.7	54.7	52.7	57.2	38.3	48.4
P	$\frac{E}{D}$	64.5	43.2	54.0	56.4	68.4	57.9	66.0	52.4	43.9
Total reducing carbohydrate as glucose										
B	D	2.28	1.02	1.20	1.13	0.810	0.868	0.700	0.802	1.14
P	D	5.87	4.68	3.77	3.20	3.00	3.30	1.42	1.31	1.00
WL	D	8.15	5.70	4.97	4.33	3.81	4.17	2.12	2.11	2.14
Fermentable carbohydrate as glucose										
B	D	1.31	0.484	0.536	0.449	0.298	0.309	0.267	0.388	0.537
P	D	4.41	2.78	2.04	1.76	1.55	2.06	0.774	0.688	0.596
WL	D	5.72	3.26	2.58	2.21	1.85	2.37	1.04	1.08	1.13
Unfermentable carbohydrate as glucose										
B	D	0.983	0.540	0.663	0.685	0.512	0.559	0.433	0.414	0.603
P	D	1.46	1.90	1.73	1.44	1.45	1.24	0.644	0.618	0.404
WL	D	2.44	2.44	2.39	2.13	1.96	1.80	1.08	1.03	1.01
Total carbon[2]										
B	D	19.63	18.26	19.46	17.23	16.31	17.11	15.77	14.79	15.79
P	D	20.52	19.68	20.03	19.43	18.96	18.76	18.65	16.15	15.28
WL	D	40.15	37.94	39.49	36.66	35.27	35.87	34.42	30.94	31.07
Insoluble carbon (carbon of alcohol extracted residues)[2]										
B	D	11.00	11.00	11.67	9.67	8.76	8.42	8.00	7.99	8.27
P	D	9.66	9.66	10.46	9.39	9.63	9.44	10.12	9.40	9.08
WL	D	20.7	20.7	22.1	19.1	18.4	17.9	18.1	17.4	17.3

[1] B represents blade, P petiole, and WL whole leaves; D represents samples that were dried, E samples that were extracted with cold water after treatment with ether.

[2] These data are discussed in Part III.

Chemical Investigations of the Rhubarb Plant

II. Chemical Changes that Occur in Leaves During Culture in Water in Light and in Darkness and also in Glucose Solution in Darkness

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INTRODUCTION

The experiments described in Part I of this Bulletin dealt with the behavior of the chief components of excised rhubarb leaves subjected to culture in water in complete darkness. Particular attention was devoted to the changes in the proteins, the amino acids and amides, and to the organic acids and soluble carbohydrates. The presence of an amide-synthesizing mechanism was established and the general nitrogen metabolism was found to follow the outlines established long ago by Schulze both for seedlings and for leaves.

The primary object of the experiments was to discover to what extent the metabolism of the rhubarb leaf in culture differs from that of the tobacco leaf. The most obvious chemical difference between the two species is in the acidity of extracts from the leaves. Rhubarb leaves normally contain a high concentration of organic acids, especially in the petiole, and possess a strongly acid reaction; tobacco leaves on the other hand, although they may contain an even higher concentration of organic acids, are much more nearly neutral in reaction. Another striking difference is in the concentration of ammonium ions normally present in the tissues. The tobacco leaf possesses an amide-synthesizing mechanism which, under the conditions of culture employed, was called into play immediately on the appearance of more than minute traces of ammonia. As a result the concentration of ammonium ions in the tissues was maintained at a very low level indeed until irreversible changes coupled with destruction of the pigments and decomposition of the cell contents supervened. Rhubarb leaves, on the other hand, normally contain a considerable part of the soluble nitrogen in the form of ammonium ions, and, although amide synthesis began promptly under the conditions of culture in darkness described in Part I, and much of the nitrogen of the blades was thereby converted into the amide glutamine, the ammonium ion concentration was only temporarily and slightly depressed and a remarkably high relative concentration was ultimately attained. Rhubarb leaves seem able to tolerate concentrations of ammonia far greater than those usually found in tobacco leaves.

On the other hand, more recent experience has shown us that the conditions which determine whether or not rapid amide synthesis shall take place are probably considerably more complex than would appear from the

observations on rhubarb leaves and tobacco leaves quoted. Although the presence of ammonium ions in a concentration that may be perhaps regarded as abnormally high for a given species is doubtless one of the factors involved, and may even be the most important factor, other conditions also play a part. Thus, in tobacco leaves from plants grown in culture solutions that provided nitrogen largely in the form of ammonium ions, there was found an unusually high proportion of ammonia nitrogen but the concomitant enrichment in amides was not especially striking. And the metabolism of glutamine in the rhubarb leaves to be described below possesses features that differentiate it from that observed in the leaves described in Part I.

The results of the study of the behavior of rhubarb leaves during culture in darkness induced us to inquire next into the effect of light. Furthermore the significant position occupied by the carbohydrates in the general metabolism suggested that culture in a solution that contained glucose might give results that would assist in the interpretation of the observations. Three initially identical series of samples of rhubarb leaves were therefore subjected to culture under the following conditions:

DW culture: in continuous darkness in water
DG culture: in continuous darkness in 0.3 M (5.4 percent) glucose solution
LW culture: in continuous light in water

The results of the previous work indicated that sufficient information regarding the major changes in chemical composition could still be secured if analyses were made at fairly widely spaced intervals of time. Accordingly, samples were removed for analysis at 25 hours, in order to find the nature of the changes that were initiated promptly, at 93 hours when the leaves were beginning to become yellow, and at 165 hours when the blades were becoming moribund.

PREPARATION OF MATERIAL

The leaves were collected June 7, 1937. The field had been fertilized with chicken manure in the spring and the crop growing on the old, well-established rhizomes was vigorous. Heavy rain fell the night before the collection, although the preceding day had been clear, and the leaves were still moist when they reached the laboratory. The moisture was carefully blotted away, and samples of 10 and of 20 leaves each were selected with the same precautions as before. The petiole was cut square at the base and the leaves were supported in earthenware crocks. Duplicate 20-leaf and 10-leaf samples were reserved for immediate analysis, three 20-leaf and three 10-leaf samples were placed in a greenhouse with provision for illumination at night, and six 20-leaf and six 10-leaf samples were placed in the dark room. The cultures in light and half the cultures in the dark were carried out in distilled water, the other samples in the dark in 0.3 M (5.4 percent) glucose solution. The 10-leaf samples were provided so that water extracts of the petioles might be prepared, the blade tissue being dried. Both petiole and blade tissue of the 20-leaf samples were dried.

The general behavior of the leaves is shown in Table 14. The leaves in water in the light remained in a healthy condition longer than those in darkness and chlorophyll degeneration was delayed. It is especially interesting and significant that the same is true of the leaves cultured in

glucose in the dark and we have recently observed an analogous phenomenon with tobacco leaves. On the basis of appearance alone, one might infer that the glucose supplied in the dark culture played a part similar to that of the carbohydrates presumably synthesized in the leaves cultured in light.

The symbols employed to designate the various sets of samples are for the most part self-explanatory; F refers to the fresh leaf samples prepared for analysis at the start, and the symbols DW, DG, and LW refer to the conditions of culture respectively in darkness in water, in darkness in glucose, and in light in water. All the 20-leaf samples were dried for analysis and accordingly require no symbol to denote this. The 10-leaf samples, however, received in addition the key letter E to denote that the petioles were extracted with cold water after cytolysis with ether.

Table 14. General Behavior of Rhubarb Leaves in Culture

Hours	Water in darkness (DW)	Glucose in darkness (DG)	Water in light (LW)
24	Turgid	Turgid	Leaves turgid, a few petioles split.
93	Trace of yellowing on margins but still turgid; petioles very turgid.	Only one leaf definitely yellow on margin; blades and petioles turgid.	Yellowing on margins, one leaf all yellow, somewhat flaccid; petioles less turgid.
165	Blades flaccid and largely yellow extending from margins; petioles turgid, no infection.	Less flaccid than leaves in water but yellow color similar; petioles turgid, no infection.	All blades yellow with greenish cast and nearly all flaccid; petioles turgid, no infection.

The fresh weights of the samples before culture are shown in Table 15 together with the ratio of the weight of the blade to the total weight. The standard error of the fresh weights of the 20-leaf samples was ± 1.4 percent, of the 10-leaf samples ± 2.7 percent. These leaves were considerably larger and heavier than those collected in 1936 but the organic solids were lower and the ratio of the weight of the blade tissue was smaller; the mean ratio was 0.273 ± 0.014, or ± 5.1 percent.

The cultures were managed and the tissues were prepared for analysis essentially as before, but in addition, data were obtained on the respective quantities of water and of glucose solution absorbed by the leaves. To this end, a measured amount of water or solution was placed in the crocks and the quantity that remained 48 hours later was determined. The solution was then renewed and the culture was continued. The glucose solutions were analyzed for glucose after being removed. As controls on the rate of evaporation, similar crocks that contained the same amount of water or of glucose solution were placed beside the culture crocks. The data in Table 16 give a general idea of the rate of absorption of water under the conditions of the experiments.

Table 15. Weights and Blade Weight Ratios of the Samples of Rhubarb Leaves Collected in 1937

20-leaf samples	Initial weight gm.	Ratio: blade / whole leaf	10-leaf samples	Initial weight gm.	Ratio: blade / whole leaf
F1	3509	0.266	FE1	1796	0.278
F2	3433	0.289	FE2	1865	0.290
LW1	3510	0.246	LWE1	1726	0.268
LW2	3468		LWE2	1801	
LW3	3431		LWE3	1735	
DW1	3458	0.290	DWE1	1726	0.277
DW2	3552		DWE2	1730	
DW3	3403		DWE3	1700	
DG1	3500	0.267	DGE1	1725	0.261
DG2	3490		DGE2	1737	
DG3	3537		DGE3	1773	
Mean	3481			1756	
Standard error	±47(±1.4%)			±48(±2.7%)	

Although the results were somewhat irregular, the rapid absorption during the first 24-hour period is evident. The rate fell off subsequently, and became very small towards the end of the period studied. The cultures in light absorbed more water than the corresponding cultures in darkness and the transpiration rate was doubtless higher. The corrections for evaporation loss from the solutions were small being of the order of 1 to 2 gm. per hour in light and about 1 gm. per hour in darkness.

Table 16. Water Absorption by Rhubarb Leaves During Culture in Light and in Darkness

20-leaf samples	Successive periods hrs.	Absorption ml. per hr.	10-leaf samples	Successive periods hrs.	Absorption ml. per hr.
LW1	24	34.2	LWE1	24	22.4
LW2	24	34.2	LWE2	24	17.5
	48	7.5		48	2.0
	24	11.2		24	2.0
LW3	24	29.8	LWE3	24	8.9
	48	8.2		48	0.5
	48	4.4		48	1.1
	48	2.9		48	0.0
DW1	24	17.0	DWE1	24	11.8
DW2	24	14.6	DWE2	24	5.8
	72	4.9		72	1.9
DW3	24	14.1	DWE3	24	6.3
	96	3.6		96	0.9
	48	1.6		48	0.5

The data for the rate of absorption of glucose in darkness are summarized in Table 17. The successive periods are shown in Column 2 and it is to be understood that sample DG2, for example, had been absorbing solution for 26 hours before the solution was changed and analyzed. This fresh lot of solution was analyzed after 43.8 hours and replaced, and the third lot was analyzed after another 24.1 hours. A similar procedure was followed for the other samples.

Table 17. Absorption of Glucose Solution by Rhubarb Leaves Cultured in Darkness

Concentration of glucose 55.7 gm. per liter.

Sample	Number of leaves	Successive periods	Total absorption	Total glucose absorbed	Ratio: $\frac{\text{gm. glucose}}{\text{ml. solution absorbed}}$	Absorption
		hrs.	ml.	gm.		ml. per hr.
DG1	20	24.8	300	16.78	0.0559	12.1
DG2	20	26	310	17.53	0.0565	11.9
		43.8	290	16.20	0.0558	6.6
		24.1	100	6.2	0.0619	4.1
DG3	20	26.3	340	19.86	0.0584	12.9
		43.8	310	15.80	0.0509	7.0
		47.0	130	7.0	0.0538	2.7
		49.2	80	6.4	0.0794	1.6
DGE1	10	24.8	220	13.27	0.0602	8.8
DGE2	10	26.0	170	11.13	0.0654	6.5
		43.8	180	9.10	0.0505	4.1
		24.1	50	3.1	0.0619	2.0
DGE3	10	26.6	210	12.80	0.0609	7.9
		43.8	180	9.30	0.0516	4.1
		47.0	60	2.4	0.0400	1.3
		49.2	70	3.3	0.0459	1.4

The total amount of solution absorbed by all samples was 3000 ml. and the total quantity of glucose that disappeared from the residual solutions was 170.2 gm.; accordingly the ratio of glucose used to solution absorbed was 0.0567. This agrees so closely with the actual composition of the solution, 0.0557 gm. per ml., that there is little doubt that glucose and water entered the tissues in the same ratio as they were supplied. Assurance is also furnished that the results are not seriously obscured by the possibility that glucose in the culture solution was in part destroyed or otherwise changed as, for example, by fermentation. Examination of samples of used solution for gas formation gave uniformly negative results save in the case of the last sample. In this only a trace of gas formation was noted.

It is of some interest to calculate the transpiration rate for these leaves during the first 25 hours of the experiment when water or glucose solution was being absorbed in considerable amounts. It is to be anticipated that

the rate at which water is given off by the leaves in the uniform conditions with respect to temperature and humidity in the dark room would be less than in the higher and fluctuating temperature and humidity of the greenhouse. The DW leaves absorbed water at the average rate of 45.7 ml. per hour for the entire quantity of leaves in samples 1, 2, and 3. This is at the rate of 105 ml. per kilo of fresh weight in 25 hours. At the end the leaves retained 25 gm. per kilo of water in excess of that present at the start. The average transpiration rate was therefore 80 ml. of water per kilo in 25 hours.

The DG leaves absorbed glucose solution at the average rate of 36 ml. per hour for the entire quantity of leaves in samples 1, 2, and 3. This is equivalent to 82 ml. per kilo in 25 hours. At the end they retained 5 gm. of water per kilo in excess of that present at the start and the transpiration rate was therefore 77 ml. of water per kilo in 25 hours. Thus the transpiration in these two sets of leaves was practically identical.

In 25 hours the three samples in light absorbed water at the average rate of 90.8 ml. per hour. This is equivalent to an absorption of 209 ml. per kilo in the entire period. At the end there was a deficit of 1 gm. of the water present initially and the transpiration rate was accordingly of the order of 208 ml. of water per kilo, or about two and one-half times greater than the transpiration rate of the leaves cultured at a uniform temperature in darkness.

The calculations of the data of the 1937 experiment were carried out in a manner analogous to that used in the 1936 experiment; the analytical methods were essentially the same and the complete data are given in Table 24 on p. 102.

In the following discussion of the results, attention will be directed chiefly to differences in behavior between the leaves cultured under different conditions and to analogies with, or differences from, the behavior of tobacco leaves. The results from the leaves cultured in water in darkness confirmed the previous experiment in most respects and, accordingly, are not described in great detail. The more important changes are shown by the curves plotted in the figures, but frequent reference will be made to the main data table (Table 24) on p. 102. For convenience in discussion the three experiments will often be referred to in the text by the key letters DW, DG, and LW.

WATER AND SOLIDS

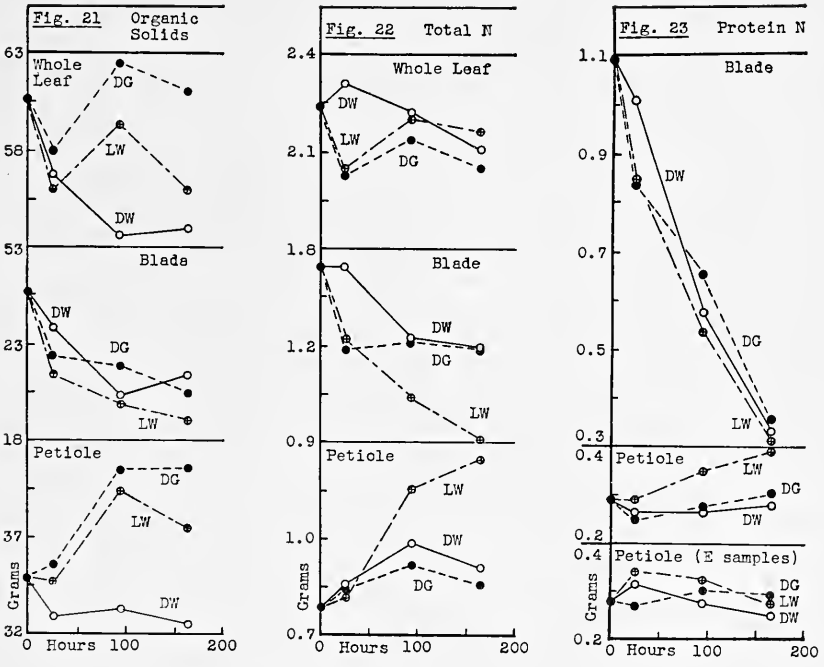
Very little difference was found in the fresh weights of the blade and petiole tissues under the three conditions of culture. The blades in light (LW) and in glucose in darkness (DG) did not increase in weight at first, although the petioles did. In all cases, the blades lost weight rapidly in the interval from 95 to 165 hours, the petioles, however, at the end of the experiment had lost but little. The behavior resembled that observed in the 1936 experiment during the same interval of time, and no clear distinctions can be drawn between the effects of the different conditions of culture.

The blades in the DG and LW experiments lost water slowly at the start, those in the DW experiment gained a little at first; later, all three sets lost water rapidly. The petioles in all three experiments gained some water at first but had lost this together with a little of their initial water at the

end. The changes in the petiole were, however, of minor importance in all cases; those of the blades closely resemble the 1936 series.

It was to be anticipated that the organic solids of the leaves would differ markedly in behavior according as the leaves were cultured in darkness or in light. In our study of tobacco leaves (85) the organic solids were found to increase rapidly in light and to diminish in darkness; photosynthesis in the tobacco leaf was sufficiently extensive to overcome completely the loss due to respiration. Even in tobacco stalks photosynthesis approximately balanced the loss due to respiration (86) so that little significant change in weight took place. Partly owing to the possibility of transport from the blade to the petiole, the changes observed in the rhubarb leaf were, however, different.

Figure 21 shows the behavior; reference to the curves for the whole leaves shows that, during the first 25 hours, all leaves lost small quantities of organic solids; subsequently the LW leaves increased slightly in organic solids but, at the end of 165 hours, had diminished again to the level reached in 25 hours. The DW leaves continued to lose organic solids for 93 hours, but the DG leaves, after the initial drop, increased in organic solids even above the initial weight and retained this increased amount to the end. It would seem that photosynthesis played a significant part during the culture of the leaves in light, while the leaves cultured upon glucose in darkness obviously acquired organic solids from the culture solution.



More detailed examination of the behavior shows that the greater part of the change took place in the petiole. The blade tissues in all three experiments lost weight throughout, somewhat more rapidly in fact from the LW blades than from the DW, the DG blades occupying an intermediate position at the end of 25 hours, and showing less effect than the others at 95 hours. The final values were, however, approximately the same, but the petioles in both DG and LW experiments showed marked gains in organic solids. It is clear from the curves that the initial loss in organic solids from the whole leaves in these two experiments was entirely due to net losses from the blades, the petioles changing but little. The later increases in the petioles are obviously due to acquisition of new organic matter.

The increase in weight of the petiole in the DG experiment may be quite simply accounted for. Glucose was taken up by the petiole and some of it was either retained as such or was metabolized into other non-volatile substances; a little also probably reached the blade. But the increase in organic solids of the petioles of the leaves cultured in light, considered together with the *loss* in weight of the blades, can only mean that substances newly photosynthesized in the blades or their equivalent in original solids must have been transported to the petiole. Although the petiole of rhubarb is provided with a little chlorophyll and may share to some extent in the reactions of photosynthesis, the marked increase in organic solids that occurred in this tissue can hardly be accounted for unless transport from the blades occurred, and the loss from the blades must be due to the combined effects of respiration and of this transport.

The loss of organic solids from the whole leaves in the DW culture was 6.6 gm. from a total of 60.6 gm. Of this, the blades lost a little over 4 gm., the petioles a little less than 3 gm. At a similar point in the 1936 experiment the leaves had lost 11 gm. from a total of 85.8 gm., the blades losing 6.9 and the petioles 4 gm. The general picture is similar in the two experiments, the greater loss in the early stages falling upon the blade tissue. It will be recalled, however, that the *final* loss in the much more prolonged earlier experiment apparently fell considerably more heavily upon the petiole than upon the blade.

A plot of the data in Table 24 for the inorganic solids has not been given because of the individual variations between the samples. There are too few determinations to give conclusive evidence of trends, and the previous experiment indicated clearly that no material interchange of inorganic constituents between petiole and blade occurred during culture in darkness. The mean value of the inorganic solids for the whole leaf tissue of the ten samples was 6.78 $\pm$ 0.31 gm., an uncertainty too great to permit inferences to be drawn with respect to changes.

NITROGENOUS CONSTITUENTS

Total Nitrogen

The data for the whole leaves, plotted in Figure 22, indicate no trend in total nitrogen content. The mean value of the ten samples was 2.15 $\pm$ 0.08 gm. per kilo of original fresh weight. The variation of $\pm$ 4 percent is somewhat higher than that found in the 1936 experiment and the individual samples were apparently somewhat less uniform in original composition.

The curves that show the total nitrogen of the blade and petiole tissue indicate transport of nitrogen from blade to petiole during the course of the experiment. This was particularly marked in the leaves cultured in light, the exchange in this case amounting to approximately 0.5 gm. of nitrogen, or nearly one-quarter of the total nitrogen of the sample. The exchange in the two dark cultures was of the order of 0.1 to 0.2 gm. of nitrogen, or less than 10 percent of the nitrogen of the sample; but its order of magnitude is sufficiently greater than the variation in nitrogen content from sample to sample to make it certain that transport took place.

Independent confirmation of the transport of nitrogen into the petiole is found in the data obtained from the 10-leaf samples, the petioles of which were extracted with cold water after ether cytolysis. In this case, nitrogen determinations were made on both extract and residue after extraction, and the pertinent data are shown in Table 18; they should be compared with those from the petiole in Table 24. Transport was evident in the dark culture, but was pronounced in the leaves cultured in light; and the change is obviously largely due to the acquisition of soluble nitrogen from the blade, although the relative order of magnitude of the change was slightly smaller in this set of samples than in the 20-leaf set. This phenomenon raises questions regarding the effects of illumination the discussion of which must be postponed until further details of the data have been presented. Clearly, however, light promotes the transport of soluble nitrogenous substances from blade to petiole.

Table 18. Nitrogen in Petiole Extract and Residue of 10-Leaf Samples of Rhubarb Leaves Subjected to Culture in Water in Darkness and in Light

Figures are grams per kilo of original fresh weight of whole leaves.

Hours	0	Darkness (Samples DWE) 25	93	165	Light (Samples LWE) 25	93	165
Water soluble N	0.611	0.578	0.532	0.777	0.432	0.777	0.882
Insoluble N	0.282	0.318	0.277	0.242	0.339	0.323	0.273
Total N	0.893	0.896	0.809	1.019	0.771	1.100	1.155

Protein Nitrogen

The behavior of the protein of the blade tissue, shown in Figure 23, was remarkably uniform in all three experiments. In darkness, there seems to have been a slight delay in the initiation of protein digestion, a phenomenon already noted in the 1936 experiment, but, subsequently, digestion proceeded at a fairly rapid rate. The glucose and the light cultures apparently did not show this initial delay in spite of the supply of carbohydrates presumably available to the blade in both cases. The most striking feature of the curves is that the final value in all three experiments is approximately the same—the total hydrolysis of protein in the blade was not significantly influenced by the conditions under which the leaves were cultured.

This result is unlike that obtained with tobacco leaves. In tobacco leaf tissue, protein hydrolysis proceeded at approximately the same rate,

regardless of illumination, for about 100 hours but, subsequently, the rate of hydrolysis of the protein in the illuminated leaves diminished so that, after 143 hours of culture, the leaves in light retained considerably more protein than those cultured in darkness. This behavior was observed in three independent experiments and raises interesting but difficult questions with respect to the mechanism of the regulation of the protein metabolism. In darkness, the protein was digested at a uniform rate; in light, the rate in the later stages was diminished. The illuminated leaves meanwhile had accumulated a large store of newly synthesized organic substances more than half of which was present as soluble reducing sugar. Is the diminution in rate of protein digestion under these circumstances a matter of the demand for energy by the cells being satisfied to an increasing extent by the oxidation of carbohydrates and to a decreasing extent by the hydrolysis of protein and subsequent oxidation of the hydrolysis products? In other words, do the carbohydrates in the tobacco leaf cultured in light spare the proteins when a sufficient amount has been accumulated by prolonged photosynthesis?

However this may be in the tobacco leaf, the conditions in the present samples of rhubarb leaf, with their relatively small proportion of blade tissue, were different. Photosynthesis was not capable of preventing the organic solids in the blade from diminishing rapidly under the combined effects of respiration and of transport to the petiole, and the solids of the whole leaves also diminished. Obviously photosynthesis in these leaves was far less effective than it was in the tobacco leaves studied under similar circumstances.

It is possible that, in the present case, had the culture been prolonged beyond 165 hours, a difference in the extent of the protein hydrolysis may have developed, but this is improbable because, in the 1936 experiment, it was found that hydrolysis of the blade protein ceased entirely after 165 hours of culture when the blades were beginning to become brown. Even after 261 hours of culture, the blades contained as much protein nitrogen as those cultured for the shorter period, and this was true within very narrow limits for two independent sets of samples and for three different methods of estimating the protein content of the tissues. As will be shown in Part III, loss of carbon from the blades ceased at 165 hours, an evidence of the cessation of respiration and of death of the cells, and it appears probable, therefore, that the drain of metabolism on the protein thereupon also ceased.

The protein content of the petiole was much smaller than that of the blade and the changes that took place during culture in darkness were so small as to be scarcely discernible by the methods of analysis employed. It was found, in the 1936 experiment, that significant digestion of protein in the petiole did not occur until after 200 hours of culture in darkness, an observation consistent with the fact that the petioles retained their turgidity and were in an apparently wholesome condition for the greater part of the culture period. The present experiments in which the leaves were cultured in darkness confirm the previous observation, but the leaves cultured in water in light behaved differently. The apparent protein content of the petioles *increased* significantly, the total change being of the order of magnitude of 0.08 gm. of protein nitrogen, or more than one-quarter of the protein nitrogen originally present.

Attention has already been directed to the marked transport of soluble nitrogen into the petiole during culture in light, and it would appear that a part of this newly acquired nitrogen was employed for the regeneration of protein in the petiole. The analytical method employed measured the increase of a form of nitrogen that was rendered insoluble by successive treatment of the dried tissue with hot alcohol and hot water. There is adequate evidence to indicate that most of the nitrogen that behaves in this way is protein, but that all of it is protein cannot be asserted.

In order to obtain additional evidence that this newly acquired insoluble form of nitrogen belonged to protein, samples of the petiole tissue were subjected to acid hydrolysis and the amino nitrogen was determined. At the start there was 0.296 gm. of protein nitrogen of which 0.161 gm., or 54.4 percent, was found to be amino nitrogen after complete hydrolysis. At 93 hours there was 0.345 gm. of protein nitrogen of which 0.186 gm., or 53.9 percent, was amino nitrogen after complete hydrolysis. Thus at this stage the increase in potential amino nitrogen was strictly proportional to the increase in protein nitrogen. The data at 165 hours were less satisfactory, there being only 0.180 gm. of amino nitrogen formed in spite of the increase in protein nitrogen to 0.387 gm. Nevertheless, in view of the inadequacies in the methods of analysis as applied in this case, a closer agreement could perhaps scarcely be anticipated. The important point is that the increase in apparent protein nitrogen was accompanied by an increase in hydrolyzable amino nitrogen.

The results of the analysis of the 10-leaf samples, the petioles of which were extracted with cold water after ether cytolysis, did not indicate an increase in protein during culture in the light. The data are also plotted in Figure 23 for comparison. The method of preparation of the tissue—a thorough extraction with cold water with use of the hydraulic press, the pressed residue being ground, again extracted, and finally dried and analyzed for total nitrogen—might not be expected to give values that correspond closely with those obtained from dried tissue that had been extracted successively with hot alcohol and with hot water. The conditions employed were essentially those advocated by Chibnall for the extraction of soluble protein from leaves. On the other hand, however, Chibnall has repeatedly pointed out the critical nature of many apparently unimportant conditions which must be satisfied if one is to obtain success in the extraction of the cell proteins from plant tissues. Our problem was the converse of his; we were interested chiefly in extracting the soluble components and preferred that most of the protein should remain unextracted. Accordingly none of the precautions that he has emphasized as essential were observed and the results show that little, if any, protein passed into solution. The average of the determinations of protein nitrogen in the petioles of the 20-leaf samples cultured in darkness was 0.278 gm., that of the determinations in the parallel 10-leaf samples was 0.281 gm. The samples cultured in glucose in darkness gave a mean value of 0.281, and the parallel 10-leaf samples gave 0.287. Obviously the two methods of analysis yield values for what is essentially the same thing in each case, and the calculation of a mean value is justified by the obvious lack of any significant trend in the data.

The petioles of the leaves cultured in light showed an increase in apparent protein as determined by analysis of the dried tissue, but none was

evident from the analysis of the 10-leaf water-extracted samples. A clear inference is that, whatever the nature of the newly synthesized compound formed in the petiole from nitrogen imported from the blade may have been, it was soluble in cold water. This result does not conflict with the conclusions already drawn; if the substance was in fact protein, as would appear from the analyses of the dried samples, it was rendered insoluble (denatured) by being dried at 80° or by the subsequent treatment with alcohol and hot water. If it was not protein, its formation presents a very puzzling chemical problem.

These results have analogies in the work of Michael (52) who found evidence for translocation of nitrogen from blade to petiole of cultured *Tropaeolum majus* leaves at the time of yellowing. A part of the transported nitrogen contributed to the apparent protein nitrogen of the petiole as determined by a method not unlike that employed in the present work.

The apparent synthesis of protein in the rhubarb petiole during culture in light raises a number of important theoretical questions. The protein content of the petiole was considerably less than that of the blade and it would appear, from the experiments in darkness, that this protein is not normally digested during culture. Possibly this has to do with the fact that the petiole contains an abundance of carbohydrates and of organic acids available for the demands of metabolism and, with the nitrogenous components of its protoplasm at least intact, remains turgid and in an apparently wholesome condition long after extensive protein decomposition has taken place in the blade. Exactly why the products of the decomposition in the blade should migrate to the petiole is not apparent. In darkness they do, however, although to a small extent; but in light, the migration of nitrogen assumes considerable significance.

We may perhaps assume that the products of photosynthesis in some way promote this transport since the movement was most rapid while the blades were still in a fully healthy condition and may be supposed to have been able to synthesize carbohydrates. But if this were the case, that is, if the demands of metabolism in the blade could have been met by these products of photosynthesis, the question at once arises: Why was the protein decomposed at all? This brings up the whole problem of the regulation of protein metabolism in leaves, a problem that is still unsolved but to which Mothes, Paech, and Chibnall have each contributed suggestions, and it will be necessary to defer further discussion until the complete data have been presented (see Part III).

Soluble Nitrogen

The vagueness of the meaning of the term soluble nitrogen was briefly discussed in Part I and it was pointed out, in the case of the blade tissue, that the quantity found is materially affected by the method of extraction and also by the treatment of the tissue before extraction. In general, especially for the blade tissue, less nitrogen was brought into solution by cold water extraction of the cytolyzed tissue than by hot water extraction of the previously dried tissue.

The quantities of soluble nitrogen as determined by hot water extraction of the previously dried rhubarb tissues are plotted in Figure 24 together

with the quantities found after cold water extraction of the cytolyzed petiole tissue of the E samples. The results for the blade should be compared with the rate of digestion of protein plotted in Figure 23. Both series of samples of leaves cultured in darkness showed the formation of soluble nitrogen at a rapid rate that can be closely represented by a straight line. The rate of production of soluble nitrogen in the glucose culture was slightly less than in the water culture, and this conforms to the slight differences in relative rates of digestion of protein in the two cases.

Soluble nitrogen was formed distinctly less rapidly in the blades cultured in light although protein was digested at approximately the same rate. The two phenomena are, however, closely related in this case also. It will be recalled that transport of nitrogen from blade to petiole was especially marked in the light experiment. The data in Table 24 (see Figure 22) suggest that approximately 0.46 gm. of nitrogen was transported. The loss of protein nitrogen during 165 hours of culture was 0.78 gm. and the increase in soluble nitrogen was 0.37 gm. The sum of the transported nitrogen and of the increase in soluble nitrogen is therefore 0.83 gm., which agrees closely with the loss of protein nitrogen. Accordingly, the main features of the reactions are clear; the protein underwent rapid digestion to soluble products, of which somewhat more than half migrated to the petiole in the case of the leaves cultured in light. These relationships may perhaps be better appreciated from the data in Table 19 which also show that calculations for the two experiments conducted in darkness lead to a similar result although the amount of nitrogen transported was much smaller under these conditions. It is clear, therefore, that the reduced rate of accumulation of soluble nitrogen in the blades of the leaves cultured in light, as compared with those cultured in darkness, is a result of the more rapid transport of nitrogen to the petiole.

Table 19. Relations Between Digestion of Protein, Increase in Soluble Nitrogen and Transport of Nitrogen from Blade to Petiole in Rhubarb Leaves During Culture for 165 Hours

Figures are grams per kilo of original fresh weight.

	DW	DG	LW
Increase total N of petiole	0.12	0.07	0.46
Increase soluble N of blade	0.70	0.60	0.37
Sum	0.82	0.67	0.83
Decrease protein N of blade	0.76	0.73	0.78

The changes in the soluble nitrogen of the petiole shown in Figure 24 likewise reflect the transport of nitrogen from the blade. In the DW experiment, the soluble nitrogen increased by 0.22 gm. in 93 hours and thereafter diminished slightly; in the DG experiments it increased nearly as much in 24 hours, but subsequently diminished so that the overall change was a matter of only about 0.1 gm. Reference to Figure 23 shows that the production of soluble nitrogen from the petiole protein should be very small

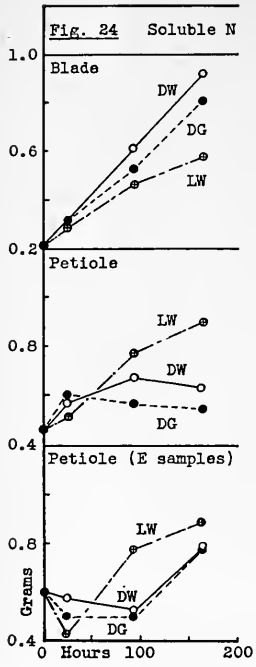


Fig. 24 Soluble N
Blade
Petiole
Petiole (E samples)
DW
DG
LW
Grams
0 Hours 100 200

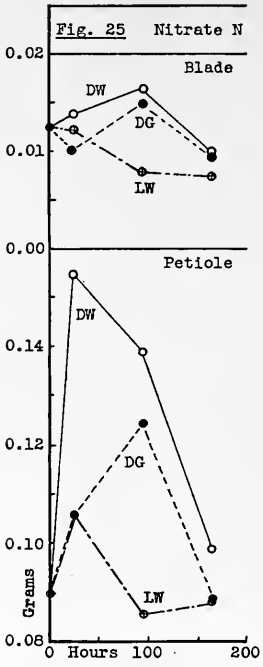


Fig. 25 Nitrate N
Blade
Petiole
DW
DG
LW
Grams
0 Hours 100 200

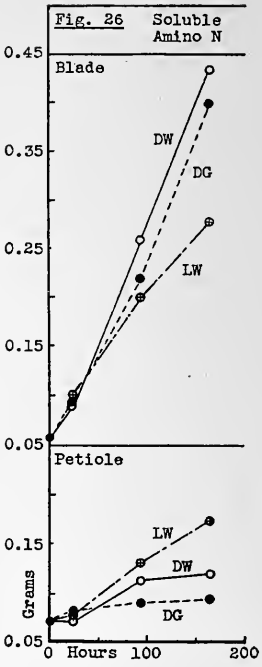


Fig. 26 Soluble Amino N
Blade
Petiole
DW
DG
LW
Grams
0 Hours 100 200

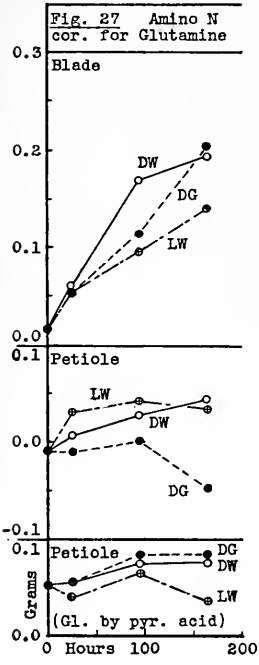


Fig. 27 Amino N cor. for Glutamine
Blade
Petiole
Petiole
(Gl. by pyr. acid)
DW
DG
LW
Grams
0 Hours 100 200

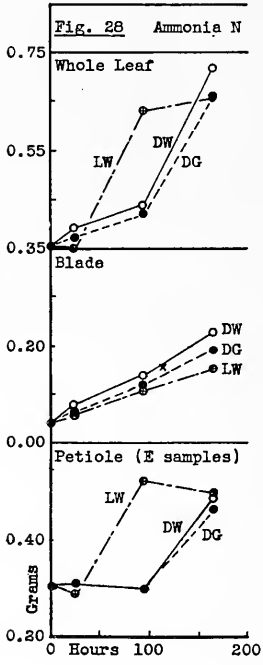


Fig. 28 Ammonia N
Whole Leaf
Blade
Petiole (E samples)
DW
DG
LW
Grams
0 Hours 100 200

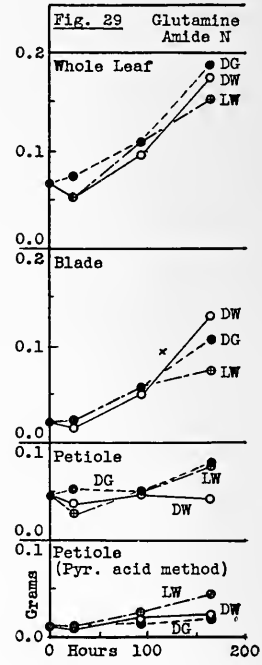


Fig. 29 Glutamine Amide N
Whole Leaf
Blade
Petiole
Petiole
(Pyr. acid method)
DG
DW
LW
Grams
0 Hours 100 200

in these two experiments since the overall change in protein content is scarcely detectable. There is an indication of a drop in protein nitrogen of the order of 0.04 gm. in the first 24 hours, but the subsequent change, if any, was in the direction of regeneration of protein. The data that show the increases in soluble and also in total nitrogen are summarized in Table 20 and indicate a satisfactory agreement between the two quantities in two of the three experiments. In the LW experiment, however, the increase in total nitrogen of the petiole was greater than the increase in soluble nitrogen. It is apparent that a part of the imported soluble nitrogen was converted into a form that is insoluble in hot water and the data for soluble nitrogen are therefore in accord with the conclusions already drawn with respect to the synthesis of protein in the petioles of the LW series.

Table 20. Comparison of the Increase in Total Nitrogen and the Increase in Soluble Nitrogen of Rhubarb Petioles During Culture for 165 Hours

Figures are grams per kilo of original fresh weight.

	DW	DG	LW
Increase soluble N	0.170	0.088	0.439
Increase total N	0.121	0.071	0.457

The quantities of soluble nitrogen found in the petioles of the 10-leaf E samples are also plotted in Figure 24. The analytical data represent the nitrogen that remained in solution after the cold water extracts of the cytolyzed tissue had been heated to coagulate the traces of protein they contained. Attention has been called, in connection with the 1936 experiment, to the fact that the ammonia in such extracts is usually very much greater than that found in hot water extracts of previously dried tissue and, accordingly, discrepancies between the curves which show soluble nitrogen, as determined in these two ways, are to be expected. It happens that the two sets of results for the LW experiment check closely, with the exception of the samples taken at 25 hours. All three sets of samples show a marked increase in soluble nitrogen over the entire period of culture but, probably owing to the peculiar behavior of the ammonia during drying of the D samples, there is little agreement in detail, and the data serve chiefly to call attention to the fundamental difficulty involved in the determination of soluble nitrogen.

Nitrate Nitrogen

Although rhubarb leaves may accumulate and store relatively enormous proportions of nitrate nitrogen when grown under favorable conditions (21), the leaves discussed in Part I contained none, nor was any found after they had been cultured in darkness. The presence or absence of nitrate in the tissues appears to be largely a matter of availability in the soil, and wide variations in the quantity present in different samples are to be expected. The rhubarb leaves employed in the present experiments contained a relatively high proportion of nitrate, particularly in the petiole, and the behavior of this during culture is shown by the curves plotted in Figure 25.

The nitrate nitrogen of the blade was initially quite low, of the order of 1 percent of the total nitrogen, or 6 percent of the soluble nitrogen. During culture in water in darkness it *increased* from an initial value of 12 mg. to 16 mg. per kilo of whole leaf in 93 hours and then diminished to approximately 10 mg. at 165 hours. The samples cultured in glucose in darkness likewise showed a slight increase at 93 hours, followed by a decrease to 10 mg. at the end. The samples cultured in light, on the other hand, showed a steady decrease to a final level of about 7 mg. These quantities are small; the changes are not particularly striking, and doubt as to the validity of the conclusion that an increase in nitrate content occurred in darkness might readily arise. The petiole, however, displayed an analogous series of changes but the amount of nitrate present initially was high, and the changes during culture were of a magnitude that leaves little question as to what happened. The petioles of the leaves of the DW series contained 90 mg. of nitrate nitrogen at the start, 150 mg. after 25 hours, and 139 mg. after 93 hours, but the nitrate nitrogen had returned almost to the initial value at 165 hours. The petioles cultured in glucose solution showed a slower rate of increase, but the nitrate nitrogen reached 124 mg. in 93 hours and likewise dropped to the initial value at 165 hours. On the other hand, the petioles of the leaves cultured in light showed only a minor increase to 106 mg. in 24 hours and subsequently the nitrate diminished to somewhat below the initial value. It is difficult to escape the conclusion that synthesis of nitrate took place in the petioles of these rhubarb leaves during culture in darkness.

This phenomenon is by no means unique. It was first observed in this laboratory (84) in connection with experiments in which tobacco leaves were cultured in water in darkness, or, alternatively, were allowed to dry slowly in air (cured) in dim, diffuse light. In the former case, increase in nitrate promptly occurred, followed by a decrease; in the latter, the increase in nitrate was much slower and no decrease was noted up to the termination of the experiment at a time soon after the leaves had become brown. Confirmation of these observations has been obtained in subsequent studies (85), although the relative changes observed were somewhat smaller in order of magnitude; nevertheless the conclusion appeared justified that synthesis of nitrate usually takes place during the early phases of the culture of tobacco leaves in darkness. It did not, however, occur during culture in light; on the contrary, reduction of nitrate occurred under these conditions.

The increase in the nitrate content of the rhubarb petiole makes all the more interesting the failure to detect any significant change in the nitrate content of tobacco stalks denuded of leaves during culture in darkness (86). It is possible that the presence of the leaf blade tissue is essential. This can be determined only by experiments in which stalks with their leaves still attached are subjected to culture.

A similar phenomenon has recently been observed by McKee and Lobb during experiments with leaves of Swiss chard and tomato (50). They noted an increase in nitrate that amounted to 25 percent or more of the initial quantity during culture in water in darkness and also during slow drying of the leaves in air in darkness.

Pearsall and Billimoria (61) have also recorded an instance of nitrate synthesis in leaves cultured in darkness. When apical segments of daffodil

(*Narcissus pseudonarcissus*) leaves were floated for 60 hours in complete darkness in a culture medium that contained ammonium chloride, they observed an absorption of about 16 mg. of nitrogen per 10 gm. of tissue from the solution, and a production of nearly 1 mg. of nitrate nitrogen. The tissue was originally free from nitrate[1] and they point out that the process of reduction of nitrate to ammonia via nitrite is, under these circumstances, reversible. The phenomenon of nitrate synthesis in detached leaves may therefore be of general significance. Nightingale (58) has reported that tomato plants that had "no external supply of nitrogen and no nitrate in their tissues", when placed in darkness, failed to yield a test for nitrate at any subsequent time. This experiment is not, however, strictly analogous to culture in water in the dark of detached leaves that already contain a certain amount of nitrate, and his statement was not intended to raise a question as to the validity of the observations referred to above.

The explanation of this phenomenon is difficult and will probably not be made until the chemistry of the early phases of the metabolism of nitrate in plants is better understood. There are certain features of the reaction that are doubtless significant. In the first place, synthesis of nitrate apparently does not usually occur unless the tissues are already provided with an appreciable concentration of nitrate. This follows from our failure to observe nitrate in the rhubarb leaves studied in 1936, from Nightingale's observation already mentioned and from Spoehr and McGee's observations on sunflower leaves (75) quoted by Nightingale, but Pearsall and Billimoria's experiment with daffodil leaves apparently provides an exception to this rule.

Second, synthesis has been observed only in leaf blades or in petioles with blades attached when these are cultured in water or in nutrient solution in complete darkness. In light, on the other hand, utilization of nitrate begins promptly, presumably owing to reduction in the presence of the products of photosynthesis. The very small temporary increase of nitrate observed in the petioles in our own experiment with rhubarb leaves cultured in light emphasizes the significance of this generalization.

Third, synthesis usually occurs promptly while the cultured leaves are still in a healthy condition and the pigments are for the most part intact. As the chlorophyll begins to decompose, reduction of nitrate supervenes, and the final level reached when the leaves are mostly brown is almost invariably about the same or only a little lower than that in the initial sample. In other words, the equivalent of the newly-formed quantity of nitrate is again consumed. This behavior is less conspicuous when leaves are allowed to dry in air at ordinary temperature in darkness; in our early experiments with tobacco leaves the additional nitrate remained unchanged for many hours (84). McKee and Lobb's data show cases in which it remained, others in which a part was reduced, but the return to the initial level in cultured leaves suggests as a possibility that the newly formed substance is in fact not nitrate, but some other substance which is first formed and then utilized during culture in the dark. A partial answer to this question may be found in the method of analysis employed.

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[1] Professor Pearsall in a private communication states that the possibility that traces were present is not precluded.

This method depends upon a property of nitric acid that is usually overlooked, namely that it can be quantitatively extracted along with the organic acids by ether in a continuous extraction apparatus, provided the sample is acidified with sulfuric acid. Sulfuric acid is not extracted under these conditions. The acids are transferred to aqueous alkaline solution, ether is removed, and the solution is boiled with dilute sulfuric acid and reduced iron powder. The ammonia thereby produced from the nitric acid is determined in the customary manner by distillation with alkali. The method thus makes use of several highly specific properties of nitric acid, and nitrous acid is the only substance that is known to behave in a similar way. On the other hand, if the new substance formed in the leaves is, in fact, nitrous acid rather than nitric, the physiological problem involved is equally interesting and important. Although there is little doubt that reduction of nitrate takes place via nitrite, we have obtained no evidence that nitrous acid is present, and the survival of significant amounts of this substance in a tissue that contains large quantities of amino nitrogen and that has been dried for analysis at 80° is questionable on purely chemical grounds.[1] Qualitatively, therefore, the observations appear to be firmly established.

It is possible that the reaction is connected with an already established equilibrium between nitrate and its immediate metabolic products. It is conceivable that much of the nitrate in nitrate-rich samples of leaf tissue is present simply in storage as a reserve to be drawn upon when occasion demands. Such a quantity as that in the rhubarb petioles under discussion —20 percent of the soluble nitrogen—almost certainly represents an excess over the immediate needs of the plant. But, in tissues that are metabolizing at all, some of the nitrate must be chemically linked with intermediate products of unknown nature. If it be supposed that one of the substances in the chain of reactions by means of which nitrate is normally utilized is rendered available from some other source under the conditions that prevail in the tissue during culture in darkness, a reversal of the equilibria might be expected to occur that would result in an enrichment with nitrate.

It may be useful to draw attention to the relative order of magnitude of the nitrate nitrogen in these samples of rhubarb leaves. The initial sample contained 2.24 gm. of total nitrogen, 0.67 gm. of "soluble nitrogen" and 0.103 gm. of nitrate nitrogen. The nitrate nitrogen was thus 4.6 percent of the total nitrogen and 15.4 percent of the soluble nitrogen. The increase in nitrate nitrogen after 25 hours of culture in water in darkness was 0.066 gm., or 1.4 percent of the total nitrogen, and 4.3 percent of the soluble nitrogen of the whole leaf. Nearly all of the newly formed nitrate, however, arose in the petiole and the change involved the transformation of 11.4 percent of the soluble nitrogen in this organ into nitrate. Thus the series of reactions whereby nitrate was produced were, from the quantitative point of view, very important. No single change in the nitrogenous components in the petiole that took place within 25 hours was nearly so extensive.

There was a large increase in "soluble nitrogen" in the petiole —0.109 gm.—and also an increase in total nitrogen of 0.067 gm., due to transport

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[1] Pearsall and Billimoria have brought forward much evidence that loss of nitrogen from leaves of daffodil during culture either in light or in darkness takes place through the interaction of amino nitrogen with nitrous acid formed as an intermediate in the equilibrium reactions whereby nitrate is reduced to ammonia.

from the blade, so that reactions in which substantial quantities of nitrogen were involved may have occurred. But no evidence was secured of the origin of the 0.065 gm. of nitrogen in the form of newly synthesized nitrate and it seems unlikely that such evidence will become available until very complete information has been obtained with respect to the forms of nitrogen in this tissue.

Amino Nitrogen

The rate at which amino nitrogen accumulated in the rhubarb leaves is shown in Figure 26. The curves for the blade resemble in general appearance and in relative position to each other those for the rate of production of soluble nitrogen shown in Figure 24, and indicate that a considerable part of the soluble nitrogen consists of amino nitrogen. The data cannot be interpreted strictly to represent the rate of production of α-amino acids from protein digestion, however, since a part of the amino acids were oxidized with the production of ammonia, and glutamine was synthesized as the result of a side reaction. A correction can be calculated for the effect of the glutamine, but certain assumptions must be made.

Glutamine, when subjected to the action of nitrous acid in the Van Slyke apparatus, yields 180 percent of its amino nitrogen as gas. Thus the amino nitrogen values plotted in Figure 26 are all too high because most of the amide nitrogen of the glutamine is included in them. It has already been shown in Part I that a large part of the nitrogen found as glutamine in cultured leaf tissues arises from a series of reactions in which amino acids, derived from the hydrolysis of proteins in the tissue, are oxidatively deaminized and the ammonia produced enters into reaction with a non-nitrogenous precursor to form glutamine. If this is true, both amide and amino groups of the newly formed glutamine originated for the most part from α-amino groups of amino acids, and it would thus appear that the curves in Figure 26 do, in fact, closely represent the rate at which amino nitrogen was rendered available by protein hydrolysis. There are several errors in this assumption. In the first place, only 80 percent of the glutamine amide nitrogen reacts in the Van Slyke apparatus under standard conditions. Hence the figures should be increased by 20 percent of the glutamine amide nitrogen in each case. Secondly, some of the glutamine may have arisen directly from the protein by hydrolysis of peptide bonds, the amide groups remaining intact, and at present we are not in a position to determine how large a proportion this may have been; and finally, no allowance is made for the ammonia, produced by deamination, which was not employed for glutamine synthesis.

In order to obtain a picture of the rate of production of amino nitrogen during the culture of rhubarb leaves, it seems best to correct the quantity actually found for the contribution made by the glutamine, and two methods may be used. One may subtract 180 percent of the glutamine amide nitrogen present in each sample; in this case the difference will represent amino acids, other than glutamic acid, which survived deamination. The curve that shows the course of this reaction will then represent the difference between the rate of production of α-amino nitrogen exclusive of glutamic acid and the rate of deamination of the amino acids.

On the other hand, one may subtract 80 percent of the amide nitrogen of glutamine; the curve will then represent the sum of the rates of produc-

tion of amino acids including glutamic acid, diminished by the rate of deamination of amino acids. Both methods of correction have advantages, but the first yields a result the significance of which is perhaps more easily appreciated.

The corrected data are plotted in Figure 27 and suggest an interesting effect of the carbohydrate in the glucose culture in darkness, and of the products of photosynthesis in the water culture in light, namely a slight retardation of the rate of production of amino nitrogen during the early phases of culture. This effect was maintained to the end of the experiment in the light culture but was not maintained in the DG culture.

The amino nitrogen of the petiole, shown in Figure 26, shows a contrast in behavior to that of the blade. In the first place, the quantities present are small as are also the changes in the two dark cultures. Consideration of these curves in relation to those of Figure 23, which show that the protein nitrogen of the petiole underwent very little, if any, change, and also in relation to the curves of Figure 24, which show the increase in soluble nitrogen of the petiole, indicates clearly that the more or less regular increases in amino nitrogen in the petiole were largely due to transport of soluble nitrogen compounds from the blade. From this it is clear that not only nitrogen but carbon was transported. The nature of these imported substances can be in part inferred from the data for glutamine.

Glutamine amide nitrogen was determined in the petiole by two methods of which one may be presumed to give maximal values, the other minimal values. If the soluble amino nitrogen of the petiole is corrected for the nitrogen gas that would arise from the decomposition of glutamine, as was done for the blade tissue, the data shown in Table 24 and plotted in Figure 27 are obtained. Correction by the results of one of the methods gives negative quantities for the initial value and for several of the other observations, particularly in the DG series. The data indicate that only very small quantities of amino nitrogen other than glutamine were present in the petiole at the end of the experiment, and that transport was particularly prompt in the light culture. Correction by the results of the other method gives values that are consistent with the view that the petiole contained a quantity of amino nitrogen, other than glutamine, of the order of 50 mg. at the start, and that this changed but little during culture under any of the conditions. Thus the increase in amino nitrogen of the petiole, indicated by the curves of Figure 26, is probably almost exclusively due to the import of glutamine from the blade. Whichever of these interpretations is correct, it is evident that transport of amino acids other than glutamine into the petiole was a minor phenomenon. This conclusion is of some importance in connection with the evidence for the transport of carbon from the blade to the petiole discussed in Part III.

Ammonia Nitrogen

The difficulty of obtaining reliable determinations of the nitrogen present as ammonium ions in rhubarb leaf tissue has been discussed in Part I. It was there shown that values secured from *blade* tissue that had been dried before extraction with water were somewhat lower than values obtained from parallel samples that were extracted with cold water after cytolysis with ether. The differences were not large, however, and, accordingly, in

the interest of economy of time, cold water extracts of the blade tissue in the present series of experiments were not prepared, reliance being placed upon the analysis of the dried blade samples.

Independent evidence that this was justified was secured from the examination of a large sample of leaves collected at the same time. This material was separately subjected to culture for 114 hours in darkness, the blades being then removed and examined for ammonia and amides. The extraction was carried out with cold water by the ether cytolysis technic, and the quantity of ammonia found was equivalent to 0.158 gm. per kilo of whole leaves weighed at the start of the experiment. This point falls almost exactly on the line plotted for the ammonia of the blade tissue in the DW experiment and is indicated by a cross in Figure 28. The control sample analyzed at the start showed 0.049 gm. per kilo, also in close agreement with the DW value. Further details of this experiment are mentioned in Part I and have been given in full elsewhere (79).

The results of the analysis of petiole tissue by the two methods were so widely different in the earlier work as to necessitate preparation of cold water extracts of the petioles in the present case, if trustworthy data were to be secured. Comparison of the figures for ammonia nitrogen of the petioles of the D and E samples, shown in Table 24, makes this abundantly clear, and it is obvious that, in this experiment as well as in the 1936 experiment, grave losses of ammonia were encountered during the drying of the petioles. It was assumed in Part I that the losses were due to a reaction between ammonia and some constituent of the petiole to form substances not decomposed under the conditions employed for the determination of the ammonia. It was further noted that these hypothetical substances are not decomposed by mild acid hydrolysis nor to any great extent by subsequent mild alkaline hydrolysis.

A full discussion of this phenomenon cannot be entered upon until more extensive data have been accumulated. But it is of some interest to point out that the "protein" nitrogen of the E samples, that is the total nitrogen of the tissue residue of the petiole after thorough extraction with cold water, differs from the more reliable value for protein nitrogen determined in the dried samples by a quantity that in only one case exceeds 0.030 gm. for the leaves cultured in darkness. The discrepancy in the ammonia in all cases save one is at least 0.150 gm. Thus, whatever this hypothetical substance may be, it passed into the cold water soluble fraction and did not contribute to the insoluble nitrogen which is reckoned as "protein" nitrogen in the analysis of the E samples. In the case of the samples cultured in light, in two out of three cases the soluble nitrogen as determined in the D and the E samples was in close agreement, although the ammonia values differed widely. Accordingly, here also the hypothetical condensation product of ammonia produced during drying must have been soluble in water.

Examination of the data for ammonia as determined in the three sets of dried petiole samples shows that the quantity increased in both DW and LW cultures but did not increase in the DG culture. The parallel sets of samples that were extracted with cold water showed a marked increase in ammonia at the end in each case. Hence, whatever the mechanism may have been whereby ammonia was removed during drying, this mechanism

was far more effective in the leaves of the glucose culture than in the others. As will appear later, the carbohydrates in the DG culture either increased slightly or remained with little change throughout the course of the experiment. In the other cultures the carbohydrates, with the exception of sucrose, diminished sharply. Thus the samples subjected to drying in the one case contained fairly high concentrations of carbohydrates; in the others, low concentrations. It is therefore possible that the difference between the behavior of the ammonia in the dried leaves of the DG culture, as compared with the other two, may have been due to the effect of carbohydrates or metabolites of carbohydrates present in unusually high concentrations in these particular samples.

The data for the ammonia content of the blade tissue plotted in Figure 28 indicate an increase which began during the first 24 hours and continued subsequently at a steady rate in each case. The increase was greatest in the DW experiment and least in the LW, the DG experiment occupying an intermediate position. The change involved a substantial fraction of the nitrogen of the blade. In the DG culture, the increase in ammonia at the end was 0.190 gm. or 13 percent of the total nitrogen of the blade and 17 percent of the protein nitrogen present at the start. The magnitude of this value is proof that some of the ammonia, at least, must have arisen from deamination. In the LW culture the increase was 0.094 gm. or almost exactly half as great. The production of ammonia is therefore one of the major interconversions of nitrogen that occurs, and, as will become clear from the behavior of the glutamine, even these figures do not fully indicate its probable magnitude in the blade.

The ammonia in the petioles of both series cultured in darkness was initially high, amounting to 39 percent of the total nitrogen and 50 percent of the soluble nitrogen in the tissue. The absolute quantity present underwent no significant change for 93 hours but, in the interval between 93 and 165 hours, it increased until it reached 62 percent of the soluble nitrogen. The sudden increase in ammonia nitrogen, which began at about the time the blade tissue lost its turgidity and showed signs of chlorophyll degeneration, is reflected in the figures for soluble nitrogen in the petiole. The increases in ammonia in the DW and DG experiments (E samples) in the interval mentioned were respectively 0.185 and 0.160 gm.; the increases in soluble nitrogen in the same period were 0.255 and 0.284 gm. It seems clear therefore that much of the increase in soluble nitrogen was due to the advent of ammonia either produced from previously insoluble nitrogenous substances or transported from the blade. The changes (decreases) in insoluble nitrogen in the E samples during this interval were small—of the order of 0.03 gm.—and, accordingly, the evidence points clearly to the transport of ammonia from the blade as the major factor in the increase in ammonia found in the petiole.

The behavior of the ammonia in the petioles of the LW series (E samples) was somewhat different from that in the leaves cultured in darkness. There was no material change for 24 hours, but subsequently the ammonia increased by nearly 68 percent of the initial amount, and then decreased slightly in the last interval between analyses. The increase in ammonia at 93 hours was 0.213 gm.; the increase in total nitrogen of the petiole of the D samples at the same point was 0.367 gm. Transport of ammonia therefore accounts for much of the increase at this point.

The decrease in ammonia in the LW leaves in the last period between analyses is of interest because of its relation to the change that occurred at that time in what was apparently soluble protein. The data for the D samples indicates an increase in protein in the petiole that amounted to 0.05 gm. of nitrogen at 93 hours and 0.09 gm. at 163 hours. This change did not show in the data for the water insoluble nitrogen in the E samples because, as has been pointed out above, the hypothetical newly formed protein was soluble under the conditions employed for the extraction of the tissue. The increase in apparent protein nitrogen in the petiole is probably associated with the decrease in ammonia nitrogen that took place at the same time and the picture presented by the data is consistent and fairly clear. Translocation of nitrogen, largely in the form of ammonia, from blade to petiole was greatly stimulated under the influence of light, and a part of this nitrogen, including some of the ammonia, was apparently transformed into protein. For further discussion of this point, see Part III.

The curves for the ammonia nitrogen of the whole leaf, plotted in Figure 28, possess a few features that help to clarify the interpretation of the changes. With respect to the whole leaf, ammonia formation began promptly in darkness, but was delayed in light. This ammonia was derived from protein decomposition and the changes in protein nitrogen already discussed suggest that hydrolysis in the first 24 hours was at least as rapid in the LW experiment as it was in the DG, and was somewhat more rapid than in the DW experiments. The temporary delay in ammonia formation in the LW experiment suggests, therefore, that oxidative deamination did not assume significance in the first few hours of the culture in light. Later, however, it accelerated and an appreciable part of the ammonia produced in the blade was transported to the petiole. In darkness, the net production of ammonia was delayed until the blades had undergone quite extensive irreversible changes, but it then became very rapid. The total production of ammonia in dark culture was restricted somewhat both in rate and amount in the experiment in which glucose was supplied.

It must be emphasized that the values for ammonia nitrogen plotted in Figure 28 represent net rates of ammonia production rather than absolute rates. Although ammonia was formed by oxidative deamination of amino acids, part of it was utilized in the synthesis of glutamine and, in the light culture, probably also for the formation of protein in the petiole. The curves therefore merely reveal the difference in rate between production and utilization.

Amide Nitrogen

Although glutamine appears to be the only amide synthesized in the blades of rhubarb leaves during culture in darkness (79), the hydrolytic method employed for the determination of the amides nevertheless usually shows the presence of a small proportion of a substance that yields ammonia only on acid hydrolysis, and which accordingly appears in the calculations as asparagine. This observation clearly indicates the limitation which must be placed upon the interpretation of indirect analytical methods. In the present case, the values for apparent asparagine amide nitrogen will be regarded merely as an unknown form of nitrogen and no qualitative interpretation will be placed upon them.

The data for glutamine amide nitrogen are plotted in Figure 29. The DW experiment closely confirms the results obtained in 1936 in general outline, and further confirmation of the accuracy of the extraction and analytical methods employed was found also in the results of the large-scale isolation experiment already mentioned. The quantity of glutamine amide nitrogen present at the start in that experiment was 0.019 gm. per kilo; after 114 hours of culture in darkness, it was 0.094 gm. per kilo. This point is indicated by a cross in Figure 29; its position suggests that the curve should be represented as rising somewhat more steeply than is there shown, a conclusion that is also supported by the shape of the curve for glutamine amide nitrogen, obtained from the more detailed experiments of 1936. Inspection of the curves in Figure 29 shows that, in the DW and DG experiments, glutamine formation in the blade did not become established until after the lapse of 24 hours, but subsequently proceeded rapidly in both, although the final value reached in 165 hours was somewhat higher in the DW than in the DG culture. The data for ammonia nitrogen (Figure 28) indicate that large quantities of ammonia were present in both sets of samples, and the general theory of amide metabolism would lead one to expect an immediate stimulation of amide formation in the DG culture. On the contrary, the quantity of glutamine indicated in the blade at 165 hours was distinctly less in the glucose culture than in the dark culture. The curve for the whole leaf, however, shows that the total amount of glutamine formed was practically the same in the two experiments, and it is clear that the smaller apparent synthesis in the blade was due to translocation to the petiole.

When the present experiments were planned it had seemed probable, from analogy with experiments with seedlings (74) described in the literature, that glucose would bring about a definite stimulation of amide synthesis in the dark. The observation that no definite effect was produced raises an interesting question regarding the nature of the non-nitrogenous precursor necessary for this synthesis. As will appear later, the concentration of carbohydrates in the tissues of the DG leaves was maintained throughout the period of culture at nearly the initial level, whereas the carbohydrates in the DW leaves diminished rapidly. The failure of this condition to promote the formation of glutamine indicates that the reactions are far more complex than would appear from the early seedling work and further discussion will be presented in Part III.

The behavior of the rhubarb leaves cultured in light differed but little from those cultured in darkness. Glutamine synthesis proceeded at a rate indistinguishable from that of the others for 93 hours but subsequently fell somewhat behind. The curve for the whole leaf shows that translocation of glutamine to the petiole occurred both in the LW and DG series, but the final value for the whole leaf in the LW series was definitely less than that in either of the dark cultures, and it is clear that even the products of photosynthesis do not necessarily stimulate the formation of glutamine.

In the rhubarb leaf, therefore, the conditions are somewhat in contrast to those in the tobacco plant. In tobacco leaves, glutamine was synthesized in substantial quantity only during culture in light; in darkness asparagine alone was formed. The inference was drawn that the necessary precursor of glutamine was produced in the tobacco leaf by photosynthetic reactions,

but no definite clue as to its chemical nature was secured. Furthermore, the apparent connection with the products of photosynthesis suggested that glucose might possibly be effective if used in sufficient concentration and led to the test of glucose in the present experiment. The observation that neither glucose itself nor the products of photosynthesis are able to stimulate glutamine formation in rhubarb leaves may indicate that the selection of an organism that normally contains a high concentration of ammonia in which to study amide metabolism was not judicious. The argument in favor of rhubarb leaves for the test is, however, that the complications involved by the formation of asparagine are avoided and moreover, weight for weight, rhubarb blade tissue is more efficient than tobacco leaf in the synthesis of glutamine. Data collected in Table 21 show this clearly. The glutamine amide nitrogen synthesized per kilo of blade tissue (exclusive of the original fresh weight of the petiole) is materially greater in rhubarb than in the tobacco leaf both in darkness and in light in the period of culture during which the leaves were still in relatively good condition. Owing to the very rapid synthesis of asparagine in tobacco leaves in darkness, the total amide increases considerably more than in rhubarb blade tissue, but this is not so in light.

Table 21. Comparison of Amide Synthesis in Rhubarb Leaf Blade Tissue and in Tobacco Leaves During 93 Hours and 95 Hours Respectively of Culture in Water

	Glutamine		Total amide	
	Darkness	Light	Darkness	Light
Rhubarb blades, gm. per kilo of whole leaf	0.030	0.036	0.036	0.044
Rhubarb blades, gm. per kilo of blade tissue[1]	0.109	0.132	0.132	0.161
Tobacco leaves, gm. per kilo of whole leaf [2]	0.038	0.071	0.288	0.116

[1]Calculated from mean blade-weight ratio of fresh leaves of 0.273 (Table 15).
[2]Data in Bulletin 399 (85).

In rhubarb blades cultured in light for 165 hours, practically the same quantity of glutamine was formed as in tobacco leaves cultured in light for 190 hours (85), but the total amide synthesis was somewhat greater in the latter tissue. This comparison is not entirely fair, however, inasmuch as transport of glutamine out of the blade of the rhubarb occurred to a significant extent during the later hours of the experiment, and amide synthesis had gone on in the tobacco leaves for a full day more.

The determination of glutamine in rhubarb petiole tissue is especially difficult not only because the proportion present is very small but there is reason to suspect that the results are somewhat in error owing to interference from other nitrogenous constituents of unknown nature. The values obtained by the usual hydrolysis method are plotted in Figure 29 but are presented with some reservation; they are at best maximal values. For this

reason, attention will be directed merely to the facts that very little change occurred during culture in water at any time in darkness, nor for 93 hours in the other two experiments. An increase in the glutamine content occurred in these during the last interval, probably largely due to transport from the blade. Whether the changes actually represent the behavior of glutamine itself or not, the values do represent the behavior of a nitrogenous constituent which yields ammonia under carefully controlled conditions of mild hydrolysis, and the failure of glutamine to migrate to any notable extent into the petiole in the DW experiment confirms the results of the 1936 investigation.

Considerable study has been devoted to the problem of the behavior of the glutamine in rhubarb petiole tissue, and the recent development of a new method to determine this substance made it possible to obtain some measure of confirmation of the results just mentioned. The new method takes advantage of a highly specific property of glutamine; this substance is converted into pyrrolidone carboxylic acid by hydrolysis at pH 7.0 (82), an acid which can be quantitatively extracted from solution in the presence of excess of sulfuric acid by ethyl acetate. Determination of the increase in amino nitrogen brought about by hydrolysis with normal hydrochloric acid furnishes a measure of the quantity of glutamine originally present. The extraction is carried out in a rocking apparatus of the Widmark type in which the solvent alternately flows back and forth through a wide bore tube that connects two Erlenmeyer flasks, one of which contains dilute alkali, the other the acidified solution under investigation. The alkali collects the organic acid extracted by the solvent, and the solution is subsequently freed from solvent, acidified, and boiled with normal hydrochloric acid for a few hours. Extensive tests of this technic on pure glutamine and on tissues known to contain glutamine have given satisfactory results, and the method promises to furnish a valuable check upon the hydrolytic method. Full details will be published elsewhere.

The results secured by the application of the new method to the rhubarb petiole are given in Table 24 and are plotted at the bottom of Figure 29 where they may be compared with the results by the hydrolytic method. In general, one might expect the new method to yield low results with a tissue that contains so little glutamine as rhubarb petiole, and we feel that the actual behavior of the glutamine would be more accurately shown by curves intermediate between the two sets shown in Figure 29. The pyrrolidone carboxylic acid method clearly reveals an increase of glutamine in the petioles of the LW culture, but whether this is due to synthesis *in situ* or to transport cannot be decided at once.

"Asparagine" Amide Nitrogen

When the amide nitrogen due to asparagine is calculated in the customary way from the hydrolysis data, small and irregularly disposed values are found for the rhubarb blade tissue and relatively large negative values are found for the petiole. The data are given in Table 24. This result conforms to what would be anticipated from the failure of a careful study of the amides of rhubarb blade to reveal any evidence for the presence of asparagine. The small magnitude of the asparagine values for the blade tissue support the view that the glutamine values secured by the hydroly-

sis method are reasonably accurate. The negative values found in the petiole clearly indicate that the glutamine values found by the hydrolysis method are too high.

The glutamine data obtained by the pyrrolidone carboxylic acid method permit the calculation of a second set of data for asparagine amide nitrogen in the petioles; these are also shown in Table 24 and indicate that the apparent asparagine rises and then falls in each case. Such a behavior of asparagine under the conditions of these experiments is unlikely, and the results merely furnish further evidence of the presence of substances in the rhubarb petiole which interfere with the accurate determination of total amide nitrogen by hydrolytic methods.

It cannot be asserted that these tissues contained absolutely no asparagine in the later phases of the culture. The aspartic acid of the protein is probably combined in the intact molecule as asparagine, and it is therefore possible that a little asparagine may arise directly from the protein when this is digested by the intracellular enzymes. But the quantity of such primary asparagine formed should not be large. Miller has shown that grass leaf proteins contain from 3 to 4 percent of their nitrogen in the form of aspartic acid nitrogen[1]; thus the order of magnitude of the asparagine amide nitrogen to be anticipated from the quantity of protein digested in these experiments would be 0.020 gm., and this is, in fact, the order of magnitude of the "asparagine amide nitrogen" values found in the petioles. But most of this asparagine would be formed in the blade and very little of it could be expected to migrate to the petiole. Obviously, therefore, the "asparagine amide nitrogen" values shown in Table 24 are misleadingly high, even upon the most favorable assumptions.

In Part III speculative reasons, based upon a theoretical interpretation of the phenomena of amide synthesis in rhubarb leaves, are given which account adequately for the failure to demonstrate the presence of asparagine in the blades even after prolonged culture in darkness and in spite of the possibility that some asparagine may have arisen directly from the protein.

Amide Metabolism

According to the hypothesis of Schulze, the increase in amide nitrogen during culture of leaves is due to the formation of asparagine and glutamine from ammonia produced by the deamination of amino acids. It should therefore be possible to show a relatively close agreement between the quantities of nitrogen involved. The data obtained from the 1936 experiment conformed reasonably well with the predictions from this hypothesis, and it is of interest to carry out similar calculations for the present experiments.

Determinations of the amino nitrogen produced from the residual protein in the tissues were not made, but a close approximation to the quantity of amino nitrogen lost from the protein can be obtained by the use of the factor 0.76 for the amino nitrogen of leaf proteins after complete hydrolysis. This factor is an average value for the ratio of hydrolyzable amino to total nitrogen in a series of purified leaf proteins and was communicated to us by Professor Chibnall.

[1] Revisions in the methods of dicarboxylic amino acid analysis recently made in Chibnall's laboratory indicate that these early estimates of aspartic acid are somewhat low.

The relevant data are collected in Table 22 which shows the conditions both at 93 and at 165 hours. Inasmuch as transport of soluble nitrogen from blade to petiole occurred to a certain extent in each case, values for the whole leaf only are included. The ammonia values from the E samples of the petiole are employed. The calculations were carried out in the same manner as those of Table 7, Part I, but the presence of nitrate in this series of samples introduces a slight complication. As has been shown, the nitrate increased during culture in the dark and then decreased. Although no assumption has been made with regard to the origin of the newly formed nitrate, it is highly probable that the nitrate that disappeared was converted into ammonia. Accordingly an allowance must be made for ammonia from this source. If the value for the nitrate at 165 hours is subtracted from the maximal value attained at a previous stage in the culture, an estimate can be obtained of the quantity of ammonia derived from the reduction of nitrate.

The figures for the conditions at 165 hours are shown in the last three columns of the table. Line 7 shows the amino nitrogen that should have been produced from the protein, corrected for the glutamic acid, which, if

Table 22. Relationship Between Changes in Protein, Amino, Nitrate, and Ammonia Nitrogen in Whole Rhubarb Leaves Cultured Under Different Conditions for 93 and for 165 Hours

Figures are grams per kilo of original fresh weight of whole leaves.

	Conditions of culture	93 hours DW	93 hours DG	93 hours LW	165 hours DW	165 hours DG	165 hours LW
1	Protein N loss	0.540	0.465	0.504	0.783	0.731	0.691
2	α-Amino N liberated from protein (1 × 0.76)	0.410	0.353	0.383	0.595	0.556	0.525
3	Ammonia N gain (E)	0.087	0.067	0.279	0.463	0.304	0.301
4	Glutamine amide N gain	0.031	0.040	0.039	0.106	0.119	0.084
5	Soluble amino N gain	0.245	0.180	0.202	0.425	0.365	0.321
6	Nitrate N reduced	0.014	0.000	0.022	0.061	0.040	0.022
7	Protein amino N corrected for glutamine (2 – 8% of 1)	0.367	0.316	0.343	0.532	0.498	0.470
8	Glutamine amide N gain corrected for glutamine from protein (4 – 8% of 1)	–0.012	0.003	–0.001	0.043	0.061	0.029
9	Soluble amino N corrected for glutamine (5 – 180% of 4)	0.189	0.108	0.132	0.234	0.151	0.170
10	Amino N tranformed (7 – 9)	0.178	0.208	0.211	0.298	0.347	0.300
11	Amino N transformed + nitrate N reduced (10 + 6)	0.192	0.208	0.233	0.359	0.387	0.322
12	Total N of synthesized glutamine (Twice 8)	–0.024	0.006	–0.002	0.086	0.122	0.058
13	Glutamine N + ammonia N (12 + 3)	0.063	0.073	0.277	0.549	0.426	0.359
14	Difference (11 – 13)	0.129	0.125	–0.044	–0.190	–0.039	–0.037

our assumptions are correct, would have been liberated as the amide glutamine in the leaf cells. Line 8 shows the glutamine amide nitrogen gain corrected for this same quantity of glutamine of presumably direct protein origin. Line 9 gives the soluble amino nitrogen corrected for all the glutamine, whatever its origin, it may have contained. If nothing had happened to the amino nitrogen after it was set free from the protein, line 9 should agree with line 7. The difference in line 10 indicates the order of magnitude of the quantity of amino nitrogen that was destroyed as such and transformed into something else. This quantity is actually more than half of the amino nitrogen derived from the protein, and the total quantity of nitrogen the fate of which must be accounted for according to the hypothesis, is obtained by adding the loss of amino nitrogen (line 10) to the loss of nitrate nitrogen. This is done in line 11.

If it be assumed that this nitrogen is converted into ammonia which in turn contributes to the nitrogen of glutamine, the last four lines of the table show the relationships between the quantities that would occur. The differences between the calculated values shown in the last line illustrate the degree with which the data conform to the hypothesis. When the experimental errors of the several determinations, together with the assumptions involved, are considered, it is clear that the results of the DG and LW series at 165 hours agree satisfactorily and furnish further support to the conclusions drawn from the studies of the amide metabolism of tobacco leaves. The exception provided by the DW series is entirely due to an unusually high value for the ammonia nitrogen in the petioles of a single sample.

It is to be noted that, in each case, the leaves at 165 hours contained an excess amount of ammonia. If oxidation of basic amino acids beyond the stage of deamination, for example decomposition of arginine with the production of urea and subsequent hydrolysis of this by urease, occurred, the production of such an excess can be readily understood.

The conditions at 93 hours provide a contrast to those at 165 hours. The protein nitrogen loss is, of course, less and an estimate of the possible quantity of glutamine that may have arisen directly from this protein in each case (8 percent of line 1) agrees remarkably closely with the actual increase in glutamine amide nitrogen. The figures for glutamic acid nitrogen from the protein are respectively 0.043, 0.037, and 0.040 gm.; those for glutamine amide nitrogen gain are 0.031, 0.040, and 0.039 gm. Consideration of these figures indicates that it is quite possible that no glutamine whatever was synthesized *de novo* in these leaves during the first 93 hours; all the glutamine present at this time may have arisen directly from the protein. This possibility is of particular importance inasmuch as it indicates that the synthesis of glutamine in rhubarb leaves is not invariably initiated immediately in spite of the presence of a considerable concentration of ammonia. In the leaves described in Part I, synthesis probably began promptly; there was an immediate diminution in the ammonia present and the E samples showed a definite increase in glutamine from the beginning, that is, at a time when protein digestion had scarcely become established (see Part I, Table 13). On the other hand, the present data indicate that nearly all the glutamine present at the expiration of 93 hours may have arisen directly from the protein, and synthesis of glutamine, as

distinct from liberation from the protein, was delayed for many hours. This suggests that the glutamine synthesizing mechanism is controlled by several factors of which the ammonia concentration is only one, and a full discussion of the significance of this point will be given in Part III.

The observations on the distribution of the nitrogen at 93 hours in the DW and DG experiments, shown in Table 22, reveal another interesting relationship. Line 10 shows that approximately half the α-amino nitrogen presumably liberated from the protein and corrected for glutamine derived from the protein had been transformed into something else. In the DW experiment the quantity was 0.178 gm., in the DG experiment 0.208 gm. The calculations in the last four lines show that, respectively, 0.129 and 0.125 gm. of this nitrogen had not been transformed into glutamine nor into ammonia. Nevertheless, at 165 hours, this nitrogen, together with a considerable additional amount, was present as glutamine nitrogen or as ammonia. This is evidence that, at the 93-hour point, an appreciable quantity of nitrogen was present in some intermediary stage of metabolism; it was neither amino nitrogen, ammonia nitrogen, nor glutamine nitrogen although, later, it was detected in one or another of these forms.

The LW experiment provides a distinct contrast since both at 93 hours and at 165 hours a fairly adequate account can be given of the fate of the amino nitrogen that disappeared—it was undoubtedly present as glutamine nitrogen or as ammonia.

In Table 23 are collected data for the ammonia and also for the soluble nitrogen in the whole leaves at the beginning and end of the three experiments. The concentration of ammonia in percentage of the soluble nitrogen is also shown and it is evident that this remained practically constant in the DW experiment and diminished somewhat in the DG and LW experiments in spite of the marked increase in ammonia that occurred. If there had been no formation of glutamine, the ammonia present at the end would in each case have been greater by twice the amount of amide nitrogen formed. The quantities of ammonia that would have been present in this circumstance on the assumption that all of the glutamine arose from synthesis are shown in the next to the last line and the resultant concentrations are given in the last line of the table. In each case there would have been a large increase in the proportion of ammonia nitrogen and it is clear that amide formation operates to reduce this proportion under all conditions of culture.

Table 23. Concentration of Ammonia Nitrogen in Rhubarb Leaves in Terms of Soluble Nitrogen

	Fresh leaf	After 165 hrs. DW	DG	LW
Soluble N, gm.	0.673	1.54	1.36	1.48
Ammonia N, gm.	0.353	0.816	0.657	0.654
percent of soluble N	52.4	53.0	47.2	44.2
Calculated ammonia N in absence of amide synthesis, gm.	0.353	1.028	0.881	0.854
percent of soluble N	52.4	66.4	64.7	57.7

The 1937 DW experiment presents a contrast to the 1936 experiment in the proportion of ammonia present in the fresh leaf. In the earlier samples the ammonia made up 72.9 percent of the soluble nitrogen but was reduced to 34.6 percent at the end of 213 hours of culture. If there had been no amide synthesis, the final proportion of ammonia nitrogen would have been 66 percent. Amide synthesis was decidedly more effective in reducing the ammonia in that experiment than in the present one, a result possibly of the higher proportion of ammonia available from the start.

ORGANIC ACIDS

The change in hydrogen ion activity of the petiole and blade tissue that occurred is shown in Figure 30. The blade had a reaction close to pH 4.0 at the start, but became less acid as the culture progressed. The change was considerably greater in darkness than in light, but there were minor differences in behavior between the two groups of leaves cultured in darkness. The increase in pH was somewhat retarded by glucose at first although the final value attained was the same as that of the leaves in water. The petioles, however, showed little evidence of change in acidity until after 93 hours; there was then an increase of about 0.2 pH units from the initial value of 3.0. The changes in general confirm the inferences made from the more detailed study of the leaves examined in 1936 although they were somewhat smaller in order of magnitude.

Total Organic Acidity

The data plotted in Figure 31 show that the organic acids in the blade amount to about one-quarter of those in the whole leaf and that they diminished by about one-eighth of the amount initially present during culture. The increase at the end of the DW culture is not in agreement with the behavior of the blades of the leaves cultured in darkness in 1936. A continuous loss was observed in 1936 throughout a series of samples, but no observations were made in the present case between 93 and 165 hours.

There is a definite contrast in the behavior of the petioles in the three experiments. The DW petioles lost a small amount of acid as they did in the 1936 experiment, but the petioles in the DG and LW cultures both gained significant amounts of acid, the DG petioles retaining it until the end of the period of investigation, the LW petioles ultimately losing most of the increased amount. The significance of these changes can perhaps be better appreciated from the curves for the whole leaves since the possible effects of transport from the blade are thereby eliminated. The DW leaves lost total acid for 93 hours but thereafter changed but little. This result is not in agreement with the more detailed observations of the 1936 experiment. Both DG and LW leaves increased in total organic acids, the former retaining most of their gain to the end, the latter ultimately returning to the initial value. It would seem, therefore, that the prompt increases shown in the petioles in these two experiments are to only a small extent, if at all, the result of translocation from the blades. Definite synthesis of organic acids took place in both cases early in the experiment. The increase was relatively small and perhaps could not be expected to become apparent on the pH curve although there is a suggestion of this at the 25-hour point. In the later stages, the production of ammonia was doubtless an important

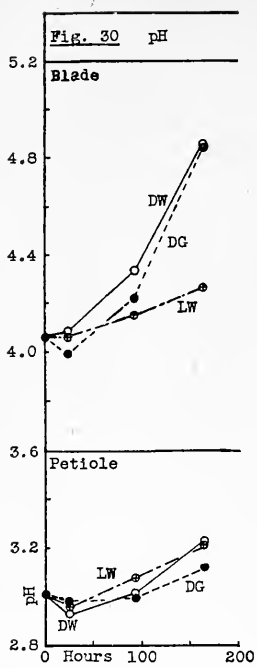


Fig. 30 pH
Blade
Petiole
pH
5.2
4.8
4.4
4.0
3.6
3.2
2.8
DW
DG
LW
0 Hours 100 200

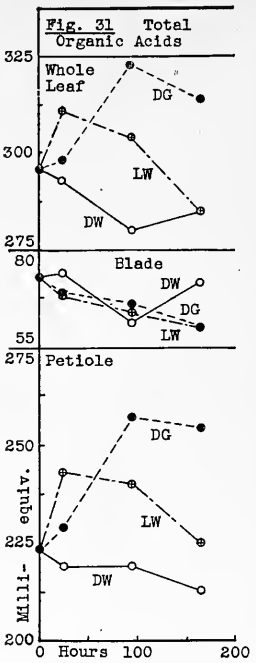


Fig. 31 Total Organic Acids
Whole Leaf
Blade
Petiole
Milli-equiv.
325
300
275
80
55
250
225
200
DW
DG
LW
0 Hours 100 200

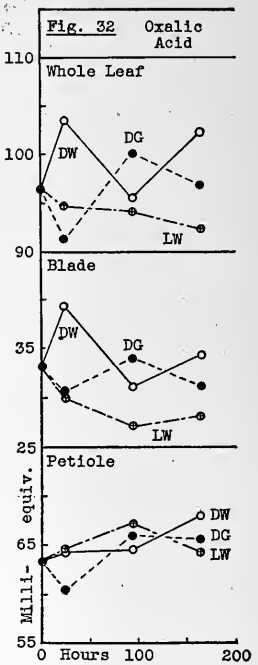


Fig. 32 Oxalic Acid
Whole Leaf
Blade
Petiole
Milli-equiv.
110
100
90
35
25
65
55
DW
DG
LW
0 Hours 100 200

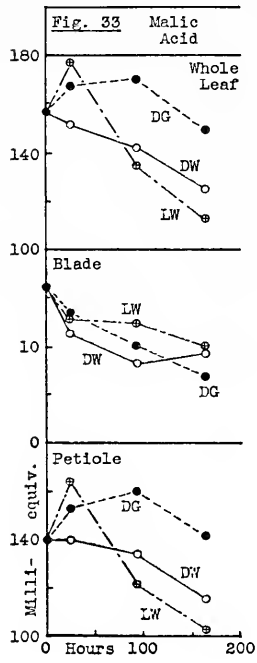


Fig. 33 Malic Acid
Whole Leaf
Blade
Petiole
Milli-equiv.
180
140
100
10
0
DW
DG
LW
0 Hours 100 200

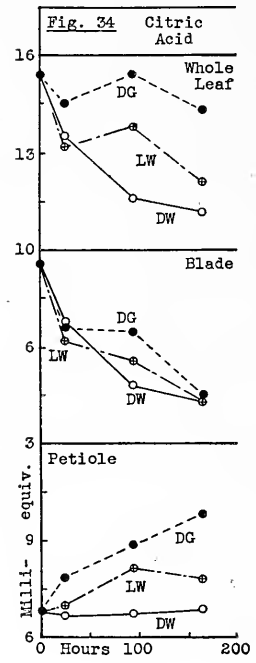


Fig. 34 Citric Acid
Whole Leaf
Blade
Petiole
Milli-equiv.
16
13
10
6
3
9
DW
DG
LW
0 Hours 100 200

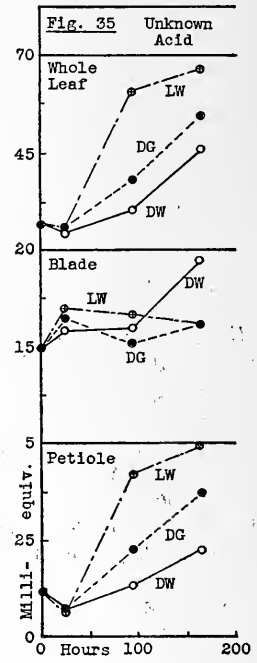


Fig. 35 Unknown Acid
Whole Leaf
Blade
Petiole
Milli-equiv.
70
45
20
15
5
25
0
DW
DG
LW
0 Hours 100 200

factor in the decrease of hydrogen-ion activity, as the relationships between total acid and acidity became increasingly complex.

An increase in organic acids in the leaves cultured in darkness in glucose solution, and in the leaves cultured in light with opportunity for photosynthesis, is extraordinarily interesting in its suggestion of a metabolic relationship between carbohydrates on the one hand and organic acids on the other. Ruhland and Wetzel (70) have particularly emphasized the possibility that organic acid synthesis in rhubarb leaves is related to the protein metabolism, organic acids and ammonia being supposed to be formed in equivalent amounts by deamination of amino acids. As has been shown above, the protein metabolism was scarcely, if at all, influenced by the conditions under which the leaves were cultured; the changes were very largely confined to the blade tissue, and the ammonia produced was in part converted into glutamine. Evidence that the carbon residues that remained in the blade, after deamination of the amino acids, were disposed of to a considerable extent by oxidation (respiration), will be presented in Part III. On the other hand, the increase in the organic acids took place chiefly in the petiole and was especially marked in the DG experiment in which glucose was absorbed by this organ from the culture solution. The change was prompt in all cases and, in the LW series, was complete before protein decomposition had become at all extensive. These observations, therefore, point far more convincingly to a genetic relationship between carbohydrates and organic acids than they do to one between proteins and organic acids.

Oxalic Acid

The behavior of the oxalic acid is plotted in Figure 32. The data show the same type of irregularity that was encountered in the 1936 experiments but also lead to the conclusion that, if any change at all occurred, it was small, and the evidence does not permit the conclusion that the conditions of the culture affected the oxalic acid either of blade or petiole in any significant way. The behavior is similar to that observed in tobacco leaves; oxalic acid apparently does not actively share in the changes that take place.

There is a suggestion, from the curve for the petiole in the DW experiment, that a little oxalic acid may have been transported from the blade to the petiole, and the somewhat more satisfactory data for the LW experiment suggest a similar migration in this case also. The quantity involved is less, however, than the order of magnitude of the variations noted in the other experiments, so that no emphasis can be placed on this possibility.

Malic Acid

Figure 33 shows the changes that occurred in the quantity of malic acid. The leaves contained initially about three-quarters of the amount found in the 1936 samples and the distribution between blade and petiole was somewhat different. In the 1936 leaves, the petioles contained about six times as much malic acid as the blades; in the present leaves, the petioles contained about nine times as much. The behavior of the malic acid in the DW culture was similar to that observed under the same conditions in 1936; in the blade it decreased by about one-third of the initial amount in 165 hours, in the petiole by about one-sixth, but the loss was actually far

larger in the petiole than in the blade, being 24 milliequivalents against approximately 7 milliequivalents in the latter tissue.

Culture in light brought about similar changes in the malic acid in the blade although the total change was smaller and the rate of loss was less. In the petiole, however, there was a striking difference; during the first 24 hours the malic acid increased by about 16 percent of the amount initially present. The increase was 24 milliequivalents, or approximately 1.5 gm., of malic acid. Subsequently, however, it rapidly diminished so that the later values were appreciably lower than those observed during culture in the dark.

The prompt effect of light upon the malic acid content of the petioles raises a number of interesting questions. In the first place, if this substance is produced from carbohydrates already present, the data for reducing carbohydrates in the petioles (Table 24) show that there is hardly enough of this type of material present to account for the quantity of malic acid formed. During the first 25 hours the loss from the petiole of reducing carbohydrate calculated as glucose was a trifle over 1 gm., and the loss from the whole leaf was only 1.3 gm. The loss of organic solids from the whole leaf was of the order of 4 gm., a quantity as large as the loss during the same time in the parallel dark experiment where photosynthesis could play no part. Accordingly, the production of malic acid during the first 25 hours of culture in light seems to have been a phenomenon specifically promoted by light in which substances already present in the tissues, or newly synthesized, were converted to malic acid. There was a net increase in total organic acids in the whole leaves of 15 milliequivalents and the increase in total organic acids in the petioles was 20 milliequivalents. The increase in malic acid in the whole leaves was 21 milliequivalents. The data as a whole are therefore consistent with the view that malic acid was synthesized from some non-acidic constituent during the early stages of culture in light. The subsequent drop, which amounted to 64 milliequivalents, or 4.1 gm. in the whole leaf, occurred along with a diminution of about 26 milliequivalents of total acidity and 2.4 gm. of organic solids. It would thus appear that the malic acid was, in part at least, converted into non-acidic products.

The behavior of the malic acid in the leaves cultured in glucose in darkness differs materially from those cultured in similar circumstances in water. In the blades, malic acid dropped steadily, although somewhat slowly, but the final value reached was lower than in either of the other series. Glucose thus only temporarily delayed the change. It is impossible to tell whether the decrease represents chemical change or transport to the petiole, but the evidence from analyses of the petiole is definite. In the petioles of the leaves in glucose solution, malic acid was promptly synthesized, the increases being 13 milliequivalents in 25 hours and 20 milliequivalents (1.3 gm.) in 93 hours, and accordingly far larger than could be accounted for by transport from the blade. This increase is parallel with an increase of 27 milliequivalents of total acidity and of 2 gm. of organic solids in the same time. These effects are in marked contrast to the rapid loss of malic acid, total acidity, and organic solids in the DW experiment, and clearly indicate a specific effect of glucose upon the metabolism of the organic acids in the leaves. Whether this effect is direct or not, that is, whether

the incoming glucose was transformed in part into malic acid, or whether its presence permitted the leaves to synthesize malic acid from components which would otherwise have had some other fate, does not appear. But there is a definite analogy between the behavior of the leaves cultured in glucose in darkness and the leaves cultured in water in light which were presumably able to produce carbohydrates by photosynthesis. This analogy is most striking in the early phase of the experiment and its failure to extend throughout can perhaps be best understood in the light of the data for organic solids (Figure 21). The most that can be said for the photosynthetic activity of these leaves is that the loss of organic solids was delayed and minimized. Although photosynthesis may have been active during the period (93 hours) when the blades were still in reasonably good condition, it did not prevent actual loss of organic solids. Furthermore such increase as occurred is shown only in the petiole, an evidence of transport of new as well as of original material from the blade tissue. On the other hand, the glucose substantially increased the organic solids of the petiole although it had little effect upon the solids of the blade. Thus one would anticipate that evidences of metabolic change would be more clearly distinguishable in the petiole tissue and this is, in fact, what is found.

The increase in malic acid of the whole leaves brought about by the products of photosynthesis on the one hand and by glucose in darkness on the other recalls the observation of Steinmann (76) that the titratable acidity of rhubarb increases during the day, an observation which he interpreted as an indication that acids are formed by photosynthesis. The change amounted to 9 percent of the morning acidity. The increase in malic acid observed in the present case during 25 hours of continuous illumination was about 13 percent of the initial value, while the increase during culture in glucose solution in darkness for the same time was 7 percent. These data refer, however, to actual malic acid rather than to titratable acidity, and the correspondence in order of magnitude to the data of Steinmann is perhaps fortuitous. The important point is that malic acid metabolism in rhubarb leaves appears definitely to be related to that of the carbohydrates.

The changes observed in rhubarb leaves are the exact reverse of those that have been recorded as characteristic of plants of the succulent type. In these, there is a prompt increase of acidity when the plants are placed in darkness and this has been held to be due to the synthesis of malic acid (11). The curve which shows the behavior of the titratable acid of *Sedum prealtum* leaves (11, p. 148) during culture in darkness is very closely similar to the curve for malic acid in rhubarb leaves (Figure 33) cultured in light. It is clearly impossible, therefore, to make general statements about the behavior of malic acid in leaves during culture. Unfortunately the information in the literature with regard to the behavior of the acidity of the leaves of succulent plants, usually referred to as the "crassulacean type of acid metabolism", deals almost exclusively with determinations of titratable acidity, the interpretation in terms of malic acid being founded on the assumption that this is the acid actually involved. Although this is to some extent probably justifiable, it is obvious that far more factual information than is at present available will be required before the metabolism of malic acid in plants is understood.

Citric Acid

The behavior of the citric acid of the rhubarb leaves is shown in Figure 34. In the DW culture, the citric acid of the blade diminished by about 4 milliequivalents; that of the petiole remained constant. This agrees with the results of the 1936 experiment. The change, although absolutely small and also small in relation to the changes in the malic acid, is large in relation to the quantity of citric acid present initially, being approximately one-quarter of this. It does not, however, necessarily represent a loss in total organic acidity. This diminished by about 11 milliequivalents, and the malic acid diminished by 31 milliequivalents during the same period. The unknown organic acids increased by approximately 20 milliequivalents and no decision can be made with respect to the fate of the citric acid that disappeared. It may have been involved in transformations to non-acidic products or into acids in the unknown group for which there are at present no specific analytical methods.

The citric acid in the leaves cultured in light diminished even less than that in the leaves in darkness. There was a slow loss from the blades and a small increase in the petiole. The loss from the whole leaves was only about 3 milliequivalents, and the evidence points to transport from blade to petiole as the chief change. This was more strikingly the case in the leaves of the DG culture. Here there was no significant loss of total citric acid, the disappearance of about 4 milliequivalents from the blades being compensated by the appearance of about 4 milliequivalents in the petiole. Thus, in the presence of carbohydrates, citric acid does not appear to enter into the acid metabolism in the sense that change in the quantity present occurs.

This result is an interesting contrast to the behavior of citric acid in tobacco leaves. In these, during culture in darkness, there is a rapid loss of a large part of the malic acid and a correspondingly rapid and approximately equivalent increase in citric acid.

Unknown Acids

The transformations of the malic acid in rhubarb leaves are accompanied by very significant increases in the quantity of unknown acids present. Although the calculation of the unknown acids by difference gives figures that include the experimental error of all the individual determinations, the curves plotted from the data (Figure 35) are smooth and present clear evidence of the type of reaction that occurred.

In the DW culture, there was little if any significant change during the first 93 hours. In this period the protein diminished by nearly 3 gm. and it is obvious that organic acids of the unknown group were not produced from this source in any appreciable amount. This conclusion is of considerable significance in view of the relationship between protein and organic acid metabolism postulated by Ruhland and Wetzel. In the interval between 93 and 165 hours, the unknown acids of the whole leaves increased from 30 to a final value of 46 milliequivalents. In this period, the malic acid decreased by some 17 milliequivalents and it seems likely that a part was converted into acids of the unknown group.

In the DG series, the unknown acids increased rapidly at a somewhat earlier stage of the culture. At the expiration of 93 hours, the increase was

about 11 milliequivalents and, at 165 hours, about 27 milliequivalents. Whether this represents transformation of malic acid into unknown acids or synthesis from the glucose does not appear definitely, but, in the last interval, 31 milliequivalents of malic acid were replaced by nearly 17 milliequivalents of unknown acid and there was no change in the citric acid. The evidence therefore points very strongly to a partial conversion of malic acid into other acids in the later phases of the culture period.

The LW series furnishes even more impressive evidence to the same effect. The unknown acids increased almost from the first, the total synthesis amounting to nearly 40 milliequivalents. The total organic acids underwent no significant *net* change in this experiment, but malic acid dropped from 177 to 113 milliequivalents, or by 64 milliequivalents, in the period from 25 to 165 hours, and it is obvious that a large part of this malic acid may have been converted into unknown acids. The citric acid was unchanged.

Metabolism of Malic Acid

Certain points regarding the metabolism of malic acid in rhubarb leaves stand out clearly from the foregoing experiments. If we confine our attention first to the early part of the culture period when the leaves were still essentially normally pigmented and turgid, it is clear that culture in water in darkness brought about loss of malic acid, but the addition of glucose to the culture solution, on the other hand, gave rise to a pronounced synthesis that was initiated promptly. Obviously, therefore, light energy has no essential connection with this reaction. One cannot infer, however, that the glucose absorbed by the leaves was in part directly transformed into malic acid; culture upon glucose may have so stimulated the general metabolism that the organism produced malic acid from some other precursor.

It is of some interest to inquire into the relationships between the quantities involved. Data are collected in Table 17 that show how much glucose actually entered these samples of leaves. Sample DG2 was cultured for 93 hours; in this time it absorbed 700 ml. of a solution of 5.57 percent concentration. The absorption of glucose calculated from analyses of the culture solution that remained was 39.9 gm. and the fresh weight of the 20 leaves before culture was 3,490 gm. Accordingly, glucose was taken up to the extent of 11.4 gm. per kilo of original fresh weight in the period of 93 hours. Notwithstanding this absorption of organic matter, the organic solids of the leaves increased only 2 gm., and the actual reducing sugar content changed very little. The malic acid increased 20 milliequivalents, or 1.3 gm. Obviously a very considerable quantity of glucose was metabolized and it seems clear that most of it must have been completely oxidized. In the parallel experiment in which leaves were cultured in water, the loss of organic solids was 6.9 gm. If this figure can be accepted as an order of magnitude for the total respiration loss (including loss of water) per kilo of leaves in darkness in 93 hours, the fate of the glucose becomes reasonably clear. Of the 11.4 gm. taken in, the equivalent of about 7 gm. was used in respiration, and of 2 in laying down new organic solids a part of which may have consisted of malic acid. A closer quantitative agreement could perhaps scarcely be expected in experiments of this type. It is to be noted that

these figures refer to the whole leaves. The increase in organic solids of the petiole in this particular sample was 5.7 gm.; the loss from the petioles of the parallel DW experiment was 1.7 gm.; hence 7.4 gm. of substance must be accounted for. There is no way to discover how the incoming 11.4 gm. of sugar was distributed between petiole and blade, but the reducing sugar in the petiole did not change significantly from its initial level of 1.5 gm. although the other carbohydrate constituents did increase slightly. Clearly synthetic reactions in which glucose was involved played an important part in these petioles and the evidence suggests that malic acid was one of the products of the reactions.

This conclusion is of importance in connection with the general relationships of organic acid metabolism. Kostytchev and Tschesnokow (40) as well as Ruhland and Wetzel have held that the plant acids are closely connected with the protein metabolism, but Bennet-Clark (12), in his comprehensive review of the whole problem, has shown that other interpretations of the evidence are possible. Genetic relationships between carbohydrates and the organic acids, particularly malic and citric acid, are inherently probable on purely chemical grounds. Speculations on possible mechanisms involved have been discussed in connection with our studies of tobacco leaves (85) and will be more fully amplified in Part III. The present observations furnish a striking example of stimulation of malic acid production by glucose and indicate the definite possibility of a direct transformation in the petiole of the rhubarb plant.

The analogous stimulation of malic acid production in the petioles of the leaves cultured in light is in a similar manner evidence of a relationship between malic acid and the products of photosynthesis. The curve (Figure 33) shows that the effect was initiated promptly, and, since no samples were collected in the interval between 25 and 93 hours, there is no evidence of how long the increase in malic acid may have continued. It is clear, however, that the rate of transformation of malic acid into other substances must have prevailed over the synthetic reactions soon after the lapse of 24 hours. The very promptness of the synthesis is an argument against the view that the newly formed malic acid arose from protein decomposition products. At the 25-hour point, only 0.3 gm. of protein nitrogen had been digested (Figure 23) in the whole leaves. This involves at most 1.8 gm. of protein and is wholly inadequate to account for the formation of the 1.6 gm. of malic acid which occurred in the same interval if any consideration at all is given to the chemical constitution of the products of amino acid oxidation.

The failure of the petioles in the LW experiment to maintain the high level of malic acid attained at the 25-hour point is probably a reflection of the diminishing efficiency of photosynthesis as culture progressed. Although translocation from the blade, as evidenced by the continuous increase in nitrogen and of organic solids in the petiole, was especially well marked in this experiment, it is evident that photosynthesis was not able to make up for the losses due to respiration. Furthermore it would seem likely that the transformation of malic acid into other products, especially into unknown organic acids, was stimulated by light.

CARBOHYDRATES

The calculation of the quantities of the various forms of carbohydrate found in rhubarb leaf tissue depends upon certain assumptions regarding their nature, and all, save sucrose, are arbitrarily given in terms of glucose as estimated from the reducing power. This was determined after inversion of the sucrose, and also after a sample of the inverted solution had been treated with yeast. From these data the fermentable carbohydrate which includes the sucrose is calculated. Sucrose was determined from the reducing power before and after inversion, and the glucose was calculated from the fermentable carbohydrate by subtracting the glucose equivalent of the sucrose. It should be pointed out, however, that these identifications are purely tentative. The respective sugars behave as if they were glucose and sucrose, but the individual substances have not been isolated, and there is no question that the unfermentable sugar must have been something other than glucose.

The glucose in the blade tissue (Figure 36) was initially low and diminished even in the leaves cultured in glucose solution, although these leaves took up 4.8 gm. of glucose per kilo from the culture solution within 24 hours. Even the 11.4 gm. that had been taken up at the end of 93 hours did not suffice to increase the level of glucose in the blade tissue significantly above that in the leaves cultured in darkness in water. The most that can be said for the direct effect of the glucose in the culture solution upon the carbohydrates in the blade is that it maintained the glucose unchanged for 25 hours.

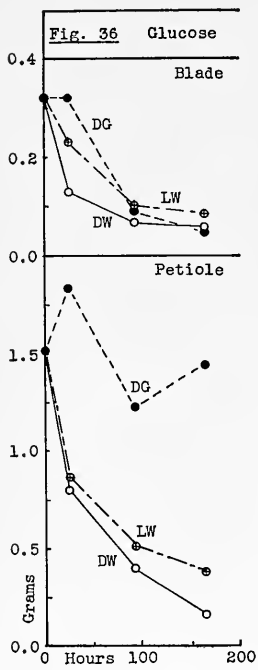
Culture in light also delayed the utilization of glucose slightly for 25 hours, but the ultimate result was the same in all three experiments. The picture is obviously one of rapid oxidation of the simple sugars of the leaf almost as rapidly as carbohydrates were supplied either from the culture solution or by photosynthesis.

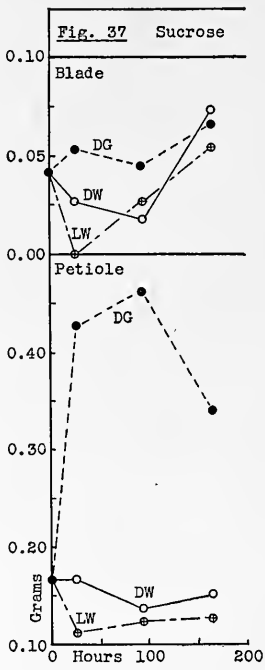
In the petiole, which was initially far richer in glucose than the blade, the changes in the DW and LW leaves followed the same general outline, but culture in glucose solution maintained the level in the petiole essentially unchanged throughout the period studied. It is clear that, in all cases, sugar was rapidly transformed into non-reducing substances and it seems most probable that it was utilized in respiration. This point is more fully discussed in Part III.

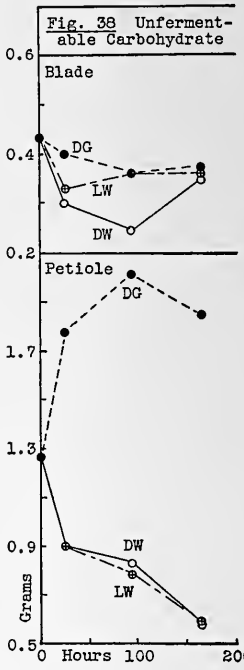
The behavior of the sucrose is shown in Figure 37. In the blade in darkness, sucrose diminished at first but apparently increased again towards the end. The total quantity was, however, very small and the changes have no great importance. In light, the behavior was similar. The blades of the leaves cultured in glucose solution in darkness showed no change in sucrose.

In the petioles, the sucrose underwent no great change during culture in water in darkness, resembling in this the behavior of the sucrose of tobacco stalks under similar circumstances. In light, there was a small and possibly significant loss in the first 25 hours, but thereafter no change. The petioles of the leaves cultured in glucose, however, showed a prompt increase in sucrose. This, although absolutely very small (0.26 gm.), was relatively very large, since it represented an increase of about 200 percent

over the initial sucrose content. Furthermore, the quantity remained at this higher level for more than 100 hours and dropped only a little at the end. Evidently sucrose synthesis like that of malic acid was stimulated at first but, later, the conditions did not favor further increase.







The so-called unfermentable carbohydrate of the blade diminished slightly in the early phase of the culture in darkness and increased slightly at the end. The changes were small and of doubtful significance, and those in the leaves cultured in light were even less pronounced. The leaves cultured in glucose lost still less unfermentable carbohydrate. The quantity of unfermentable carbohydrate in the petiole was initially about three times greater than that in the blades; it diminished sharply during the first 25 hours of culture in water both in darkness and in light and thereafter diminished slowly at rates practically the same in the two experiments. The final value was about half the initial value and this constituent therefore shared to a small extent in the metabolism of the petiole, the total quantity used being of the order of 0.6 gm. The unfermentable carbohydrate in the petioles of the leaves cultured in glucose increased by nearly 0.8 gm. in 93 hours, and thereafter dropped slightly. Apparently glucose absorbed from the culture solution contributed directly or indirectly to the formation of this type of substance.

These results may be contrasted with those obtained with tobacco. In the tobacco leaf, unfermentable carbohydrate was rapidly synthesized

in light but was utilized in darkness. In the rhubarb, the quantity present was not appreciably influenced by light, but was increased by culture in glucose in darkness. In the absence of information as to the identity of the substance or substances that make up this fraction in the two species, speculation on the meaning of these observations is scarcely warranted.

The presence of an unfermentable reducing substance, which enters to some extent into the general metabolism in both rhubarb and tobacco, raises an important question. The behavior of the fraction suggests that it is complex, at least two types of components being probably present. Andreadis, Toole, and Binopoulos (8) have found evidence for the presence of fermentable and also of unfermentable reducing substances in manufactured tobacco of Oriental types and have discussed the possible chemical nature of the unfermentable fraction responsible for what they call the *Restreduktion*. They determined fermentable carbohydrate by means of estimation of the alcohol produced when the sample is treated with yeast—a method to be commended on the grounds of specificity—and invariably found a deficit between the carbohydrate so indicated and that determined by total reduction. This deficit they regarded as being made up of dextrins, flavonol substances and uronic acid and sugar complexes.

Probably the most important inference from the present study of the carbohydrates of rhubarb leaves is the evidence that glucose administered in culture solution was rapidly utilized by the tissues. The samples took in a quantity of the order of about 11 gm. of glucose per kilo while the leaves were still in relatively healthy condition and must have oxidized very nearly an equivalent quantity of organic matter. The glucose had a direct and striking effect upon the organic acids and also upon the carbohydrates, bringing about definite synthesis of a little sucrose or other polysaccharide and also of an appreciable amount of unfermentable reducing material. Furthermore, the general metabolism, as expressed by the quantity of material respired, was definitely stimulated.

SUMMARY

The studies described in Part I of this Bulletin have been extended to a consideration of the effect of continuous light during culture of rhubarb leaves in water and also to the effect of 5 percent of glucose added to the culture solution used in an experiment carried out in darkness As a control, a series of samples was also cultured in water in darkness. The experiment was continued for 165 hours and was conducted in duplicate, 20-leaf samples being employed in one case and 10-leaf samples in the other. Both blade and petiole tissue of the 20-leaf sets were prepared for analysis by being dried. Only the blade tissue of the 10-leaf sets was dried, the petiole being extracted with cold water after cytolysis with ether in order to obtain reliable information with respect to the ammonia and amino nitrogen.

The behavior of the leaves cultured in water in darkness confirmed in many details the observations described in Part I.

Culture in darkness in glucose maintained the leaves in somewhat better condition than the corresponding leaves in water and this was also true for the leaves cultured in light. The glucose entered the tissues in sufficient amounts so that very little loss of the organic solids occurred in

the early stage, and there was a small increase over the initial weight in the later stages. Notwithstanding this, evidence was found that the loss of solids including absorbed glucose from these leaves was actually greater than that from the leaves cultured in water. Culture in light likewise contributed to a maintenance of the organic solids content. The petioles indeed increased in weight due to transport from the blade of newly synthesized organic substances or their equivalent of original solids.

No change in the total nitrogen content occurred, but there was an extensive conversion of protein nitrogen into other forms in all cases. Transport of nitrogen from blade to petiole also took place, this being particularly marked in the light. The protein of the blade tissue underwent digestion to a remarkably uniform extent in all three cases, about two-thirds of it being decomposed in the entire period of culture. The rapid digestion of the protein in light is especially noteworthy inasmuch as the protein of tobacco leaves is much less extensively attacked in light than in darkness under otherwise similar circumstances. The protein of the petiole, on the other hand, changed hardly at all in darkness but increased significantly in light. The behavior suggested that a part of the nitrogen transported to the petiole was employed for the regeneration of protein, but this apparently newly-formed protein was unlike the original protein with respect to solubility.

Examination of the fate of the nitrogen of that part of the protein that underwent digestion showed that a large part of it was converted into ammonia and a smaller part contributed to the formation of glutamine. Some of the glutamine found in the tissues may have arisen directly from the action of proteolytic enzymes on the protein, but part of it was undoubtedly newly synthesized. Calculation showed a close agreement between the amount that would have been expected on the basis of Schulze's views of leaf protein metabolism and the actual amount found. This result confirms the conclusions drawn from the experiment described in Part I and extends these conclusions to experiments under the other conditions of culture.

Culture in glucose in darkness had no stimulatory effect upon the quantity of glutamine synthesized, nor did culture in light. The provision of a large supply of a simple sugar or even of the products of photosynthesis was not sufficient to bring about an increase in amide synthesis, and it is clear that the conditions that must be fulfilled before this takes place are more complex than have been generally supposed. The leaves contained much ammonia initially and the quantity actually or potentially present was increased during the culture period. The synthesis of glutamine was in all cases sufficient to prevent the concentration of ammonia from increasing unduly. Nevertheless the detoxication hypothesis of Prianischnikow, as was noted in the 1936 experiments, is inadequate as an explanation of the behavior.

The samples contained initially an appreciable proportion of nitrogen in the form of nitrate, the greater part being found in the petiole. During culture in darkness, the amount present increased by more than 50 percent of the initial quantity in the case of the leaves in water and by nearly 33 percent of the initial quantity in the leaves in glucose solution. The increase was temporary only, the quantity present at the end of the experiment in each case being approximately that at the start. This phenomenon closely resembles that observed some years ago in this laboratory in tobacco leaves,

and recently by other workers in tomato and Swiss chard leaves. No chemical explanation has been secured. It is a phenomenon associated almost entirely, according to our present knowledge, with culture in darkness of detached leaves that already contain nitrate. The rhubarb leaves cultured in light showed it to a very small extent, and it has not been observed at all in tobacco leaves under these conditions.

The behavior of the organic acids, especially in the petioles, was markedly influenced by the conditions of culture. In water in darkness, there was a loss in total acidity, but, in glucose in darkness and in water in light, there was an increase, although only temporary in the latter case. The close correlation between the organic acidity and the carbohydrate metabolism is obvious.

Oxalic acid changed little if at all in any case, but malic acid diminished, particularly in the petiole in water in darkness. In the glucose experiment, the malic acid diminished in the blade but increased materially in the petiole. Malic acid production was apparently specifically stimulated by glucose, particularly in the petiole. In light, there was also a prompt increase in malic acid in the petiole, again illustrating the relationship of the malic acid to the carbohydrate metabolism. Citric acid diminished in the leaves in water, both in darkness and in light, but apparently did not change in the leaves cultured in glucose solution, save for the transport of a part from the blades to the petiole. The unknown group of acids increased to some extent in all cases.

The carbohydrates in the leaves cultured in water in darkness diminished rapidly, all forms examined, with the exception of the sucrose, being affected. Culture in glucose had the effect of maintaining the carbohydrate supply without serious loss; minor increases of sucrose and of unfermentable carbohydrate were observed in the petioles. In light, the utilization of the carbohydrates was delayed.

Evidence was found to support the view that respiration drew heavily upon the carbohydrates, and possibly to some extent on the organic acids. It is probable that a large part of the products of digestion of the protein of the blade was also completely oxidized.

TABLE 24. COMPOSITION OF RHUBARB LEAVES DURING CULTURE
Figures not otherwise designated are grams per kilo of fresh weight of whole leaves.

Culture conditions		Darkness							Light		
			Water (DW)			Glucose (DG)			Water (LW)		
Hours		0	25	93	165	25	93	165	25	93	165
Sample											
Fresh weight											
B	D	278	289	233	136	267	253	167	246	211	176
P	D	722	732	737	688	735	732	713	747	745	704
WL	D	1000	1021	970	824	1002	985	880	993	956	880
B	E	284	277	242	142	261	265	146	268	221	120
P	E	716	736	713	712	745	695	686	724	685	639
WL	E	1000	1013	955	854	1006	960	832	992	906	759
Total solids											
B	D	28.1	26.4	22.6	23.9	24.3	24.1	22.8	23.4	21.8	21.0
P	D	39.6	37.5	37.7	37.4	39.9	45.2	45.3	39.2	44.1	42.3
WL	D	67.7	63.9	60.3	61.3	64.2	69.3	68.1	62.6	65.9	63.3
Water											
B	D	250	263	210	112	242	229	144	223	189	155
P	D	682	694	699	651	695	687	668	708	701	662
WL	D	932	957	909	763	937	916	812	931	890	817
Organic solids											
B	D	25.6	23.9	20.4	21.4	22.4	21.9	20.5	21.4	19.9	19.1
P	D	35.0	32.9	33.3	32.6	35.6	40.7	40.6	34.7	39.4	37.5
WL	D	60.6	56.8	53.7	54.0	58.0	62.6	61.1	56.1	59.3	56.6
Inorganic solids											
B	D	2.47	2.47	2.17	2.51	1.90	2.26	2.27	2.00	1.85	1.93
P	D	4.57	4.61	4.42	4.83	4.28	4.48	4.68	4.56	4.68	4.82
WL	D	7.04	7.08	6.59	7.34	6.18	6.74	6.95	6.56	6.53	6.75
Total nitrogen											
B	D	1.45	1.45	1.23	1.20	1.19	1.22	1.19	1.22	1.04	0.911
P	D	0.793	0.860	0.993	0.914	0.841	0.918	0.864	0.825	1.16	1.25
WL	D	2.24	2.31	2.22	2.11	2.03	2.14	2.05	2.05	2.20	2.16
Protein nitrogen (nitrogen of alcohol and hot-water extracted residue of dried tissue)											
B	D	1.09	1.01	0.578	0.329	0.836	0.647	0.356	0.844	0.541	0.312
P	D	0.296	0.266	0.272	0.278	0.248	0.278	0.303	0.288	0.345	0.387
WL	D	1.39	1.28	0.850	0.607	1.08	0.925	0.659	1.13	0.886	0.699
Protein nitrogen (total nitrogen of residue from cold water extract)											
P	E	0.282	0.317	0.277	0.248	0.271	0.302	0.291	0.339	0.323	0.273

TABLE 24—Continued

Culture conditions		Darkness						Light		
		Water (DW)			Glucose (DG)			Water (LW)		
Hours	0	25	93	165	25	93	165	25	93	165
Sample										
Soluble nitrogen										
B	D 0.215	0.319	0.611	0.914	0.319	0.532	0.815	0.286	0.473	0.584
P	D 0.458	0.567	0.674	0.628	0.603	0.564	0.546	0.508	0.774	0.897
WL	D 0.673	0.886	1.29	1.54	0.922	1.10	1.36	0.794	1.25	1.48
P	E 0.611	0.578	0.532	0.777	0.510	0.506	0.790	0.432	0.777	0.882
Nitrate nitrogen										
B	D 0.0125	0.0138	0.0164	0.0099	0.0102	0.0150	0.0096	0.0122	0.0079	0.0076
P	D 0.0903	0.155	0.139	0.099	0.106	0.124	0.089	0.106	0.086	0.088
WL	D 0.103	0.169	0.155	0.109	0.116	0.139	0.099	0.118	0.094	0.096
Soluble amino nitrogen										
B	D 0.0562	0.0389	0.261	0.435	0.0958	0.219	0.399	0.102	0.200	0.277
P	D 0.0733	0.0731	0.114	0.120	0.0841	0.0913	0.0963	0.0787	0.132	0.174
WL	D 0.130	0.162	0.375	0.555	0.180	0.310	0.495	0.181	0.332	0.451
Soluble amino nitrogen corrected for glutamine										
B	D 0.0176	0.0611	0.169	0.197	0.0531	0.115	0.204	0.0564	0.0969	0.142
P[1]	D −0.010	0.0066	0.028	0.045	−0.009	0.001	−0.047	0.030	0.042	0.036
P[2]	D 0.054	0.057	0.075	0.077	0.053	0.086	0.084	0.041	0.065	0.037
Ammonia nitrogen										
B	D 0.042	0.078	0.141[3]	0.232	0.066	0.119	0.196	0.062	0.108	0.156
P	D 0.127	0.168	0.190	0.252	0.153	0.121	0.129	0.135	0.221	0.279
WL	D 0.169	0.246	0.331	0.484	0.219	0.240	0.325	0.197	0.329	0.435
P	E 0.311	0.313	0.299	0.484	0.310	0.301	0.461	0.290	0.524	0.498
WL	BD+PE 0.353	0.391	0.440	0.716	0.376	0.420	0.657	0.352	0.632	0.654
Glutamine amide nitrogen by hydrolysis at pH 7.0										
B	D 0.0214	0.0154	0.0510[4]	0.132	0.0237	0.0580	0.108	0.0252	0.0574	0.0752
P	D 0.0465	0.0370	0.0476	0.0418	0.0515	0.0500	0.0794	0.0271	0.050	0.0767
WL	D 0.0679	0.0524	0.0986	0.174	0.0752	0.108	0.187	0.0523	0.107	0.152
"Asparagine amide nitrogen" by hydrolysis with 1 N acid										
B	D 0.002	0.011	0.008	0.030	0.001	0.004	0.000	0.006	0.010	0.022
P	D −0.083	0.000	−0.01	−0.02	−0.090	−0.067	−0.090	−0.026	−0.021	0.015
Total amide nitrogen										
B	D 0.023	0.026	0.059	0.162	0.025	0.062	0.108	0.031	0.067	0.097
P[5]	D 0.0465	0.0370	0.0476	0.0418	0.0515	0.0500	0.0794	0.0271	0.050	0.0767
WL	D 0.070	0.063	0.107	0.204	0.077	0.112	0.187	0.058	0.117	0.174

[1]Glutamine of petiole determined hy hydrolysis method.
[2]Glutamine of petiole determined by pyrrolidone carboxylic acid method.
[3]At 114 hrs., 0.158 from large scale experiment (79).
[4]At 114 hrs., 0.094 from large scale experiment.
[5]Glutamine amide nitrogen figures.

TABLE 24—Continued

Culture conditions		Darkness									
		Water (DW)			Glucose (DG)			Light Water (LW)			
Hours		0	25	93	165	25	93	165	25	93	165
Sample											
Glutamine amide nitrogen by pyrrolidone carboxylic acid method											
P	D	0.0107	0.0092	0.0214	0.0238	0.0110	0.0155	0.0200	0.0123	0.0271	0.0460
"Asparagine amide nitrogen" using lower glutamine data											
P	E	0.011	0.027	0.043	0.014	0.034	0.027	0.013	0.049	0.022	0.027
pH											
B	D	4.06	4.08	4.34	4.86	3.99	4.22	4.85	4.06	4.15	4.27
P	D	3.01	2.93	3.02	3.23	2.98	3.00	3.12	2.96	3.08	3.22
Total organic acids in milliequivalents											
B	D	72.8	74.2	61.3	71.9	68.9	66.3	60.1	68.0	63.6	60.1
P	D	223	219	219	213	229	257	254	243	240	225
WL	D	296	293	280	285	298	323	314	311	304	285
Malic acid in milliequivalents											
B	D	16.2	11.3	8.2	9.4	13.5	10.2	6.9	12.8	12.5	10.2
P	D	140	140	134	116	153	160	142	164	122	103
WL	D	156	151	142	125	167	170	149	177	135	113
Citric acid in milliequivalents											
B	D	8.6	6.8	4.8	4.3	6.6	6.5	4.5	6.2	5.6	4.3
P	D	6.81	6.68	6.78	6.89	7.86	8.93	9.82	7.03	8.16	7.84
WL	D	15.4	13.5	11.6	11.2	14.5	15.4	14.3	13.2	13.8	12.1
Oxalic acid in milliequivalents											
B	D	33.2	39.4	31.2	34.4	30.8	34.1	31.3	30.1	27.0	28.1
P	D	63.4	64.3	64.6	68.0	60.5	66.1	65.6	64.7	67.2	64.3
WL	D	96.6	103.7	95.8	102.4	91.3	100.2	96.9	94.8	94.2	92.4
Unknown acids in milliequivalents											
B	D	14.8	16.7	17.0	23.9	18.0	15.4	17.3	18.9	18.5	17.5
P	D	12.3	7.8	13.4	22.3	7.6	22.7	37.4	6.7	42.2	49.3
WL	D	27.1	24.5	30.4	46.2	25.6	38.1	54.7	25.6	60.7	66.8
Total reducing carbohydrate as glucose											
B	D	0.805	0.457	0.343	0.487	0.780	0.497	0.495	0.563	0.487	0.501
P	D	2.96	1.90	1.32	0.898	4.06	3.71	3.64	1.88	1.44	1.12
WL	D	3.77	2.36	1.66	1.39	4.84	4.21	4.14	2.44	1.93	1.62
Unfermentable carbohydrate as glucose											
B	D	0.434	0.300	0.251	0.353	0.403	0.362	0.375	0.330	0.360	0.362
P	D	1.27	0.920	0.832	0.588	1.78	2.01	1.85	0.896	0.788	0.599
WL	D	1.70	1.22	1.08	0.941	2.18	2.37	2.23	1.23	1.15	0.961

TABLE 24—Concluded

Culture conditions		Darkness									
		Water (DW)				Glucose (DG)			Light Water (LW)		
Hours Sample		0	25	93	165	25	93	165	25	93	165
Fermentable carbohydrate as glucose											
B	D	0.371	0.157	0.092	0.133	0.377	0.135	0.120	0.232	0.126	0.140
P	D	1.69	0.976	0.484	0.310	2.28	1.70	1.79	0.982	0.648	0.519
WL	D	2.06	1.13	0.576	0.443	2.66	1.84	1.91	1.21	0.774	0.659
Sucrose											
B	D	0.042	0.026	0.018	0.073	0.053	0.045	0.066	0.000	0.027	0.054
P	D	0.167	0.168	0.138	0.151	0.427	0.461	0.340	0.113	0.124	0.128
WL	D	0.209	0.194	0.156	0.224	0.480	0.506	0.406	0.113	0.151	0.182
Glucose (fermentable carbohydrate minus 1.05 sucrose)											
B	D	0.327	0.130	0.073	0.057	0.321	0.088	0.051	0.234	0.099	0.083
P	D	1.51	0.800	0.339	0.151	1.83	1.22	1.44	0.864	0.518	0.384
WL	D	1.84	0.930	0.412	0.208	2.15	1.31	1.49	1.10	0.617	0.467
Total carbon[1]											
B	D	12.42	11.62	9.71	9.05	10.74	10.70	9.75	10.35	9.43	8.74
P	D	15.57	14.50	15.04	14.43	15.98	17.88	18.42	15.51	17.50	16.66
WL	D	27.99	26.12	24.75	23.48	26.72	28.58	28 17	25.86	26.93	25.40
Carbon insoluble in 75 percent alcohol[1]											
B	D	7.66	7.22	5.50	5.30	6.23	5.93	5.10	6.21	5.26	4.72
P	D	7.75	7.48	7.54	8.04	7.24	8.30	8.90	7.79	8.90	8.82
WL	D	15.41	14.70	13.04	13.34	13.47	14.23	14.00	14.00	14.16	13.54
Carbon soluble in 75 percent alcohol[1]											
B	D	4.76	4.40	4.21	3.75	4.51	4.77	4.66	4.14	4.17	4.02
P	D	7.83	7.02	7.50	6.39	8.74	9.58	9.52	7.73	8.60	7.84
WL	D	12.59	11.42	11.71	10.14	13.25	14.35	14 18	11.87	12.77	11.86

[1]These data are discussed in Part III.

Chemical Investigations of the Rhubarb Plant

III. Interpretations of the Behavior of the Components During Culture of Detached Leaves Under Various Conditions

Hubert Bradford Vickery and George W. Pucher

INTRODUCTION

The biochemical behavior of plant tissues is still so incompletely understood that the interpretation in terms of specific chemical reactions is necessarily highly speculative. It seemed essential therefore to describe the experimental results in Parts I and II in as objective a way as possible and to include only brief references to chemical mechanisms that may serve to account for the observations. An exception was made in the case of the ammonia metabolism since the data pointed quite convincingly to the general conclusion that a large part of the protein nitrogen is converted into ammonia and much of this ultimately appears as the nitrogen of the amide glutamine. Furthermore, this interpretation is in line with the now classic views of Schulze.

Discussion of the details of the exact mechanism of this as well as of other reactions was deferred, however, since there are definite interrelations between the behavior of the three chief groups of substances—proteins, carbohydrates and organic acids—and it is desirable to deal with these interrelations from a more or less unified point of view.

The great significance of the proteins in leaf metabolism has been admitted since their fundamental share in the composition of protoplasm was first recognized, but Borodin (15) as early as 1878 called attention to the difficulty of perceiving what their functions may be. We owe to Schulze much of the experimental background of our present-day knowledge, but notable advance from the position he had reached fully forty years ago has only recently been made.

Of the individual substances that occur in important proportions in various plant species under certain conditions, the amides asparagine and glutamine, together with ammonia and amino acids, occupy the foremost position among the nitrogenous substances other than the proteins; malic, citric, oxalic, and tartaric acids are the most plentiful of the organic acids, and starch, glucose and sucrose are the commonest of the carbohydrates. Consideration of the chemical structure of these substances emphasizes certain relationships that can hardly be without meaning. Aspartic and glutamic acids are universally present among the decomposition products of proteins, the latter frequently in very large relative amounts. It would seem that the influences that make these components of the protein so plentiful must be in some way related to those that concern the respective amides

found dissolved in plant saps. The chemical relationship of asparagine to malic acid is clear and was, in fact, pointed out by Piria nearly a century ago (62). The relationship of glutamine to citric acid is not at all obvious but has assumed theoretical significance in recent years (38). But tartaric acid and the much rarer malonic acid are not closely related to either of the amides nor to any of the better known protein amino acids and their occurrence in plant extracts presents a difficult problem. On the other hand, oxalic acid is a well known end-product of carbohydrate oxidation *in vitro* and may well occupy a similar position in the cell.

It would seem likely that a fairly strong case can be made out for a theoretical metabolic relationship between certain of the members of these groups of substances; but this must not blind us to the fact that many other, possibly equally important, substances are being overlooked. For example, many plants contain nitrogenous basic substances, frequently of very complex chemical constitution, the function of which in the plant is quite unknown. Of these, the group known as alkaloids is of enormous practical importance because of the physiological effects of many of these substances upon the animal organism. Quaternary bases, such as stachydrin, the betaine of proline, in alfalfa, and choline are also very widely distributed. One may assume with some degree of confidence that choline is in some way allied with the phosphatide metabolism, but no relationship for the other quaternary bases has yet been suggested save that they appear to be derived by methylation from ordinary protein amino acids and thus may share in some as yet obscure way in the protein metabolism.

From these considerations, it is clear that a discussion of the details of the chemical aspects of metabolism is an exceedingly speculative and uncertain undertaking. In any specific case it is necessary to introduce analogies in chemical behavior from other plants and even from animal tissues. A few facts are definitely known; certain substances are present in large proportions and discussion of these is accordingly invited. But the presence of many substances of unknown nature must not be disregarded and it is also probable that substances, traces of which only can be detected, have a significance as great as that of the major known constituents. Their very paucity is an evidence of their activity in the metabolic scheme.

THE COMPOSITION OF RHUBARB LEAVES

The analytical results given in detail in Parts I and II permit calculation of the composition of rhubarb leaves at different stages in the culture period. To obtain a measure of the soluble substances, the previously dried tissue was treated in a continuous extraction apparatus with hot 75 percent alcohol; the soluble carbohydrates, the amino acids and amides, most of the organic acids, the pigments and probably certain lipoid components, together with a relatively small group of unknown substances including certain ash constituents, were thereby brought into solution. In view of the precision with which this extraction process can be carried out, the weight of the residual material was taken to represent the total quantity of insoluble substances present for the purposes of the calculation, and the loss in weight was taken as the total quantity of soluble substances. The insoluble residue contains the protein, the fiber, a group of complex carbohydrates such as hemicelluloses and pectins, a certain amount of inorganic

material including calcium oxalate as one of the more important known components and, in addition, a small amount of water-soluble nitrogenous material. This was, of course, removed by extraction with boiling water previous to the determination of the insoluble nitrogen which was reckoned as protein.

The division of the tissue constituents into soluble and insoluble fractions—the solvent being hot diluted alcohol—is purely a matter of convenience, and attention has already been directed to the vagueness of the concept, "soluble nitrogen". The chief advantage is that the complex carbohydrates and glucuronides remain insoluble and thus do not interfere with the determination of the sugars. Actually the only determinations made on extracts secured in this way were those of the sugars, it being more convenient to determine the soluble nitrogenous components in water extracts and the organic acids in extracts prepared by means of ether from acidified tissue samples.

1936 DW Experiment

Table 25 shows the calculations of the composition of the rhubarb leaves collected in 1936. The data for soluble nitrogenous components have been calculated from analyses of the cold water extracts of ether-cytolyzed fresh tissues (E samples, Part I), these being probably the most reliable. The other values are derived from analyses of dried tissue. The data for soluble reducing carbohydrate and for ammonia and glutamine require no comment, save that the glutamine is calculated from the glutamine amide nitrogen rather than from the acid hydrolyzable amide nitrogen. Thus any asparagine that may have been present towards the end of the culture period is relegated to the amino acid fraction. Reasons have been given in Part I for the belief that very little asparagine was present at any stage.

The group of amino acids was estimated from the soluble amino nitrogen (fresh leaf extract, E samples, see Part I). The total nitrogen of the glutamine was subtracted, since nearly all (90 percent) of this reacts in the amino nitrogen apparatus, and the difference was multiplied by 10. This involves the assumption that the average nitrogen content of the mixture of amino acids other than glutamine in the tissues is 10 percent. Actual monoamino acids vary in nitrogen content from 7.7 (tyrosine) to 18.7 percent (glycine) while the basic amino acids contain a higher proportion of nitrogen; accordingly this factor is possibly somewhat too large but is close enough for the present purpose.

The "other soluble nitrogenous components" are calculated from the water-soluble nitrogen by deducting the ammonia and the amino nitrogen (including therefore the glutamine) and multiplying by 5. Substances included in this group would be proline and methylated bases (choline and betaines if present), the purines, and also the nitrogen of bases other than the α-amino nitrogen. Many substances in this group are rich in nitrogen and the factor 5 involves the assumption that the average nitrogen content is 20 percent. Although this is a very rough estimate, its use is justified by the smallness of the quantity of nitrogen in this form. The organic acids include the soluble oxalic acid, as determined in the cold water extracts from the cytolyzed E samples, and the acidity due to unknown acids is arbitrarily calculated as malic acid.

TABLE 25. COMPOSITION OF RHUBARB LEAVES AT DIFFERENT STAGES DURING CULTURE IN DARKNESS IN WATER

Figures are grams per kilo of fresh weight of whole leaves.

Hours	0	24	72	165	261
			Blade		
Soluble reducing carbohydrate (as glucose)	2.28	1.02	1.13	0.70	1.14
Ammonia (ammonia N × 1.214)	0.107	0.103	0.110	0.313	0.324
Glutamine (glutamine amide N × 10.4)	0.094	0.136	0.521	2.16	2.11
Amino acids (amino N corrected for glutamine × 10)	0.64	0.87	1.88	2.33	1.60
Other soluble N compounds (soluble N corrected for ammonia and amino acids × 5)	0.145	0.34	0.75	1.18	0.845
Malic acid	2.04	1.56	1.67	1.46	1.39
Citric acid	1.16	1.14	0.89	0.78	0.70
Soluble oxalic acid	0.76	0.73	0.96	1.04	0.88
Unknown acid (as malic acid)	2.44	2.02	1.89	1.64	1.89
Undetermined substances soluble in 75% alcohol	5.04	5.50	4.62	4.06	4.83
Protein (protein N × 6.25)	9.13	9.13	6.81	2.38	2.62
Non-protein insoluble substances	20.06	18.21	18.23	19.48	19.59
Total solids	43.89	40.76	39.46	37.52	37.91
Water	280.0	295.0	295.0	115.0	18.0
			Petiole		
Soluble reducing carbohydrate	5.87	4.68	3.20	1.42	1.00
Ammonia	0.387	0.283	0.249	0.350	0.482
Glutamine	0.050	0.061	0.239	0.571	0.489
Amino acids	0.480	0.400	1.32	0.860	0.920
Other soluble N compounds	0.00	0.315	0.485	0.937	0.665
Malic acid	12.20	11.72	11.06	9.18	5.69
Citric acid	0.832	0.812	0.858	0.799	0.572
Soluble oxalic acid	2.22	1.37	1.84	2.22	1.40
Unknown acid	0.536	2.13	1.24	1.03	0.985
Undetermined substances soluble in 75% alcohol	3.29	3.19	2.13	1.73	2.21
Protein	2.18	2.04	2.15	2.33	1.88
Non-protein insoluble substances	24.2	24.8	23.9	26.2	24.5
Total solids	52.23	51.80	48.67	47.62	40.79
Water	624.0	674.0	654.0	644.0	558.0

The sum of these soluble components is subtracted from the total 75 percent alcohol-soluble material, as directly determined, to give the "undetermined soluble" fraction. This group probably includes a part, at least, of the "lipoid" fraction, i.e. true fats and sterols, as well as the chlorophyll and yellow pigments. It also contains a little of the inorganic components. Together these doubtless make up half of this fraction and the residuum of substances of totally unknown chemical nature is thus small, being probably within the limits of 10 to 20 percent of the soluble fraction.

The protein is estimated from the alcohol- and water-insoluble nitrogen by the use of the conventional factor 6.25, and the quantity so found is deducted from the alcohol-insoluble material directly determined to give the "non-protein insoluble" fraction. This group of substances is large in

proportion to the total solids, but much of it belongs to substances the nature of which can be assumed. The cellulose and other components of the "crude fiber" belong here together with pectins and other glucuronides and hemicelluloses. Furthermore, much of the inorganic material, including that part of the oxalic acid in combination with calcium, is present. Thus only a small part of even this large residue belongs to totally unknown substances.

No estimate is introduced for the inorganic constituents, these being included in the "undetermined" fractions in spite of the fact that ash determinations were made. The reason for this omission is one that is frequently overlooked in the consideration of plant tissue analyses. The weight of the ash does not necessarily represent the weight of the actual inorganic constituents at all; what is weighed, for example, as calcium oxide to be sure represents the calcium present in the tissue, but the oxygen may have been derived from the tissue or from the air in the muffle furnace. Thus the total weight of a complex ash may be appreciably greater than the weight of the inorganic ions from which it is derived. We do not wish to appear to deprecate the value of ash determinations, but, failing a detailed analysis of the ash constituents from which a measure of the inorganic ions may be calculated, and also a knowledge of the form in which many of these substances are combined in the living cells, we hesitate to present an interpretation of the ash data in terms of tissue components.

The calculations in Table 25 have been made for the initial fresh leaf, and also at certain definite points during the culture period which correspond roughly to the completion of recognizable physiological events. The changes in the first period of 24 hours are those which are immediately noticeable and to some extent may illustrate the continuation of chemical changes going on in the normal leaf. The 72-hour stage represents the point at which the chlorophyll was still largely undecomposed but was beginning to disappear; shortly after this time the blades began to lose their turgidity and became yellow. The 165-hour point represents the end of the period of rapid protein decomposition in the blade; chlorophyll had nearly all disappeared and death of some of the cells, as evidenced by browning of the margins of the blades, had occurred; the petioles were, however, still turgid and apparently healthy. At the end of 261 hours, the blade tissue was brown and shrivelled and the petioles were becoming flaccid; the changes in the blade in this last interval were therefore mostly *post mortem* but those in the petiole represent only the beginning of obvious decomposition.

The chemical changes in the first 24 hours have clearly to do chiefly with the carbohydrates and organic acids. There was a sharp loss of soluble reducing sugar from both blade and petiole, and the malic acid also diminished. There was a slight increase in glutamine in the blade, a possibly significant increase in soluble nitrogenous substances and a probably significant decrease in unknown organic acids in the blade, and an increase in the petiole. There was also an apparent loss of 2 gm. from the non-protein insoluble group (pectin, etc.) of the blade. The protein was apparently unchanged.

In the next period, the loss of carbohydrate continued in the petiole but not in the blade, the glutamine increased rapidly in both blade and petiole and the protein diminished in the blade but not in the petiole. The

increase in amino acids and other soluble nitrogenous substances is doubtless correlated with the protein changes, but the parallel increase in these factors in the petiole is obviously due to transport of soluble nitrogen from the blade. The organic acids evidently shared to a considerable extent in the chemical transformations, but the non-protein insoluble material remained unaffected and there was evidence for only a small change in the group of undetermined alcohol-soluble substances.

Between 72 and 165 hours, the soluble carbohydrate changes continued though on a reduced scale; the great change is in the protein of the blade, three-quarters of which had disappeared at the end. There was a rapid increase in all forms of soluble nitrogen in the blade, especially of glutamine, but in the petiole the amino acids other than glutamine diminished while the other soluble nitrogenous substances increased. The changes in organic acids in the blade were not great but the malic acid in the petiole continued to diminish. There was little, if any, significant change in quantity of either the soluble or the insoluble groups of unknown substances.

In the last period, the carbohydrate changes were relatively small and there was no significant change in the blade protein. The ammonia increased, especially in the petiole; the glutamine decreased slightly, and the amino acids decreased in the blade and increased in the petiole. This is probably only in part, if at all, due to translocation, since in this period the protein of the petiole began to decompose and this would lead to increase in the soluble nitrogen. Furthermore, the loss of glutamine was about equivalent to the increase in amino acids. There was little change in the organic acids of the blades but there was a relatively large loss of malic acid from the petiole.

1937 DW Experiment

The results of the experiments carried out in 1937 (Part II) are similarly calculated in Table 26. Comparison of the columns at zero time in Tables 25 and 26 shows the marked difference in the composition of these two lots of leaves to which attention has already been drawn in Part II. The 1937 leaves were larger and heavier than the 1936 leaves but contained considerably less solids, both in the blades and in the petioles, in terms of one kilo of whole leaves. This difference was due to the greater hydration of the 1937 leaves, which, in turn, may have been a result of the heavy rain of the night before collection.

Probably the most important difference is in the carbohydrate content: The 1937 leaves contained less than half as much soluble reducing substance calculated as glucose as the 1936 leaves. On the other hand, the 1937 leaves were far richer initially in glutamine than the 1936 leaves. In the discussion in Part II, attention has been drawn to a number of points of difference in behavior of these two lots of leaves during culture in water in darkness. For example, the initiation of protein metabolism in the 1936 leaves was definitely delayed, whereas in the 1937 leaves the ammonia began to increase and the protein of the blades began to decrease from the start. Glutamine was itself apparently drawn into the metabolism early in the 1937 experiment whereas, in the 1936 leaves, it promptly began to increase at the expense of ammonia and probably also of the citric acid already present (see below). The same remarks apply to the petioles, with the exception that

TABLE 26. COMPOSITION OF RHUBARB LEAVES AT DIFFERENT STAGES OF CULTURE
Figures are grams per kilo of fresh weight of whole leaves.

Culture conditions		Darkness						Light		
		Water (DW)			Glucose (DG)			Water (LW)		
Hours	0	25	93	165	25	93	165	25	93	165
					Blade					
Soluble reducing carbohydrate	0.81	0.46	0.34	0.49	0.78	0.50	0.50	0.56	0.49	0.50
Ammonia	0.051	0.094	0.171	0.281	0.080	0.144	0.238	0.075	0.131	0.189
Glutamine	0.223	0.161	0.520	1.38	0.247	0.605	1.28	0.262	0.588	0.783
Amino acids	0.134	0.581	1.59	1.71	0.484	1.03	1.83	0.520	0.860	1.27
Other soluble N compounds	0.585	0.760	1.05	1.24	0.785	0.97	1.10	0.61	0.825	1.39
Malic acid	1.08	0.757	0.548	0.630	0.901	0.682	0.462	0.857	0.837	0.684
Citric acid	0.558	0.442	0.312	0.279	0.430	0.423	0.292	0.403	0.356	0.279
Oxalic acid	1.43	1.77	1.40	1.55	1.38	1.54	1.41	1.35	1.21	1.26
Unknown acid	0.99	1.12	1.14	1.60	1.21	1.03	1.16	1.27	1.24	1.17
Undetermined soluble substances	3.32	2.22	1.20	0.21	2.21	1.85	0 88	3.73	1.98	1.34
Protein	6.81	6.31	3.61	2.06	5.22	4.04	2.22	5.27	3.38	1.95
Non-protein insoluble substances	12.06	11.72	10.74	12.44	10.57	11.33	11.44	8.53	9.88	10.21
Total solids	28.04	26.40	22.61	23.92	24.30	24.14	22.81	23.44	21.78	21.03
					Petiole					
Soluble reducing carbohydrate	2.96	1.90	1.32	0.898	4.06	3.71	3.64	1.88	1.44	1.12
Ammonia	0.378	0.38	0.363	0.593	0.376	0.365	0.558	0.352	0.636	0.604
Glutamine	0.484	0.385	0.495	0.434	0.536	0.520	0.825	0.282	0.520	0.798
Amino acids	0.0	0.0	0.19	0.37	0.0	0.0	0.0	0.245	0.32	0.19
Other soluble N compounds	1.29	1.63	1.85	1.28	1.83	1.76	1.60	1.47	2.10	2.22
Malic acid	9.38	9.38	8.98	7.77	10.23	10.72	9.51	10.98	8.17	6.89
Citric acid	0.442	0.434	0.441	0.448	0.510	0.579	0.638	0.457	0.529	0.509
Oxalic acid	2.85	2.89	2.90	3.06	2.72	2.97	2.95	2.91	3.02	2.89
Unknown acid	0.824	0.522	0.897	1.53	0.509	1.52	2.51	0.449	2.83	3.29
Undetermined soluble substances	−0.03	−0.04	0.61	−0.73	−0.28	1.66	−0.26	−0.91	1.04	0.16
Protein	1.85	1.66	1.70	1.73	1.55	1.70	1.89	1.80	2.15	2.42
Non-protein insoluble substances	19.12	18.35	17.95	20.01	17.83	19.69	21.41	19.36	21.33	21.18
Total solids	39.55	37.49	37.70	37.39	39.87	45.19	45.28	39.28	44.10	42.27

the protein was not affected in either set of leaves until very late in the culture period. Thus the 1937 DW experiment furnishes confirmation of the behavior of the leaves in the 1936 experiment only in certain respects; the two lots of leaves were definitely different from the start, and the two experiments in some ways show the differences in behavior that might be expected. It would seem that the poverty of the 1937 leaves in carbohydrates threw the load of metabolic change upon other components of the tissues and the response to the conditions of culture was therefore different.

This is particularly striking in the case of the glutamine. Whereas the 1936 leaves ultimately became extraordinarily enriched in this substance, the 1937 leaves elaborated only moderate quantities.

The organic acids furnish a somewhat similar picture in both cases with the exception that, late in the culture period in the 1937 experiment, a little malic acid was regenerated, whereas there was a continuous loss in 1936. No conclusion should be drawn from the apparently different behavior of the oxalic acid in the two cases. The figures in Table 25 represent *soluble* oxalic acid, i.e. oxalic acid extracted from the tissue by cold water. Those in Table 26 represent *total* oxalic acid since separate determinations of the oxalic acid brought into solution by cold water were not made.

Attention should be directed to the values for "amino acids" in the petioles of the 1937 leaves. Small negative values were obtained in many cases and these are recorded as zero. Evidently practically the whole of the soluble amino nitrogen in this tissue consisted of glutamine save at the end of the DW culture and in the LW culture.

1937 DG Experiment

Examination of the data for the DG culture as compared with the DW culture shows certain definite effects of the soluble carbohydrate made available. In spite of the influx of many grams of glucose, the loss of solids from the blades was not influenced but there was a marked increase of solids in the petiole. The soluble reducing carbohydrate changed very little in the blade and increased in the petiole, though not in a manner at all commensurate with the quantity of glucose known to have been absorbed (some 13 gm., see Part II). Neither the protein nor the ammonia metabolism were noticeably affected, and the demand made upon the organic acids, particularly of the petiole, was diminished. There was a slight stimulation of glutamine formation at the end of the culture period but, when the data for the whole leaves are considered in order to eliminate the effect of possible transport of glutamine from blade to petiole, this is obviously too small to warrant any definite conclusion that glucose may behave directly as a precursor of glutamine.

1937 LW Experiment

The effect upon the carbohydrate of culture in light, as compared with culture in darkness, was not particularly striking and many of the changes in the nitrogenous components are obscured by the extensive transport of nitrogen from blade to petiole that took place. Production of ammonia and of glutamine in the whole leaf was actually smaller in light, although the redistribution that occurred indicates that the petioles became relatively enriched in glutamine. The apparent smaller production may have been due to utilization of glutamine in the petiole for protein regeneration. The protein of the blade behaved in essentially the same way in both LW and DW experiments, but the protein of the petiole increased strikingly in the LW experiment. As is shown in Part II, this was the result of the formation from transported nitrogen of a water-soluble, protein-like substance, and it is evident that amino acids, ammonia, and probably also glutamine were drawn upon. But the most striking difference is in the behavior of non-protein insoluble material in the two cases. It seems clear that this group—

the pectins, hemicelluloses, etc.—was actively concerned in the metabolism during the early phases of the LW experiment, being heavily drawn upon at first and then in part replaced by the formation of new insoluble material. The data upon which this conclusion rests were obtained by difference and are therefore affected by a considerable experimental error, but the consistency of the values in both DW and DG experiments suggests that this error is materially less than the order of magnitude of the changes noted in the LW experiment.

There are a number of negative values for undetermined soluble material in the petioles. These arise from the fact that the whole of the oxalic acid is reckoned as soluble, whereas it is unlikely that more than half of it was actually present in the alcohol-soluble fraction.

Tables 25 and 26 thus give a general picture of the metabolic changes that took place in this series of samples of rhubarb leaves during culture. It is necessary to see to what extent these can be interpreted in terms of physiological reactions.

The information available permits inferences with respect to the behavior of three main groups of components, the proteins together with the simpler nitrogenous substances, the simple carbohydrates, and the organic acids. The detailed changes in the proteins, the amino acids and amides are obviously closely correlated with each other, and evidence has been discussed in Parts I and II which shows that the nitrogen of the glutamine arises almost exclusively from the α-amino nitrogen of the amino acids produced from the protein by proteolytic enzymes. The intermediate substance in the conversion is ammonia, and the problem of the origin of the amide reduces to the investigation of the mechanism whereby the carbon compound necessary for the production of glutamine from ammonia is provided. As will become clear, this, in turn, involves the organic acids.

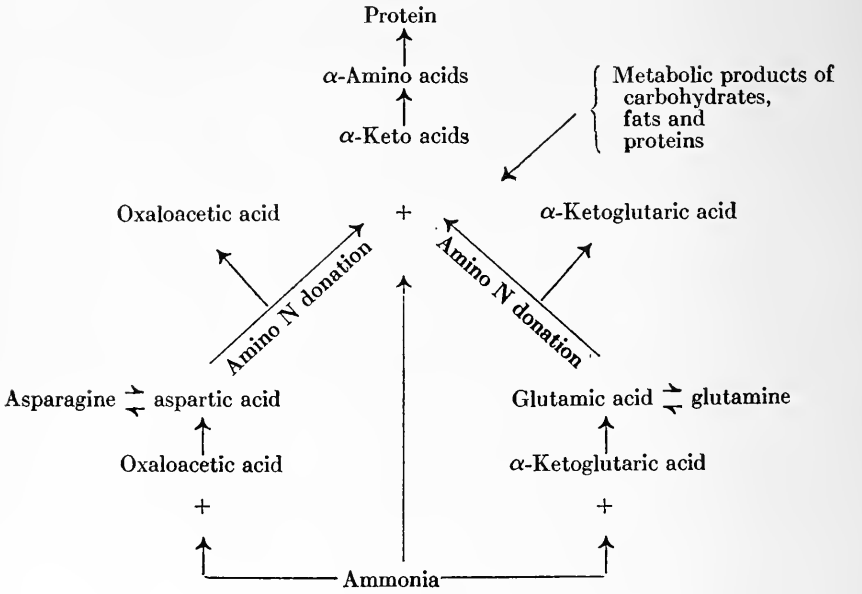
The other important change is that which involves loss of organic substances from the tissues. It seems clear that a part, at least, of this loss is provided by the carbohydrate and examination of the tables also suggests that malic acid may be involved. Furthermore, the striking loss of protein during a large part of the period is not compensated by any parallel increase in substances in other groups, and the inference is clear that some, at least, of the digested protein was ultimately lost from the tissues. These are the changes involved in the reactions of respiration and it is of fundamental importance to discover to what extent the carbohydrates, the organic acids, and especially the proteins may contribute to the oxidation reactions whereby carbon dioxide and water are liberated and energy is made available.

THE PROTEIN METABOLISM

According to the views advanced by Schulze in finally summarized form in 1906 (72), the metabolism of proteins in seedlings as well as in leaves may be summed up in the statement that proteins are hydrolyzed by the intracellular enzymes, the amino acids produced are oxidized with the production of ammonia, and this substance is in large part recombined with the aid of metabolic products derived probably from the carbohydrates into one or both of the amides asparagine and glutamine. These, in turn, act as storehouses for nitrogen which can be transported to any part of the

plant where they are needed for the resynthesis of protein. For this purpose, according to Schulze, they are preëminently suited, although amino acids may also share in the reaction. Prianischnikow (64) has extended these views and has pointed out that the nitrogenous substance involved in all of the reactions is ammonia. Ammonia is thus both the starting-point and the end-point or, as he put it, the *alpha* and *omega* of nitrogen metabolism.

Chibnall (20), in his Silliman Lectures at Yale, 1938, has presented these relationships in a diagramatic form and has added to them ideas that express present-day views of the chemical mechanisms that are involved in the synthesis of proteins.



This scheme includes certain conversions, many of which are reversible, that are today well recognized enzymatic reactions, others that are only probabilities and still others that are pure assumption. It is therefore worth while to discuss the different phases in detail, to see to what extent the suggested relationships of ammonia to the metabolism of proteins, on the one hand, and of the amides, on the other hand, are substantiated.

That proteins are normally decomposed to amino acids by certain intracellular enzyme systems is thoroughly established. But that proteins are formed in the cells by the condensation of amino acids under the action of enzymes is still an assumption, and alternative views have been advanced

and discussed by Alcock (4). No *in vitro* experiment has yet resulted in the synthesis from amino acids of a protein, or even of a recognizable normal protein decomposition product, with the possible exception of the recent enzymatic synthesis from amino acid derivatives by Bergmann and Fraenkel-Conrat (13) of several compounds that contain the peptide bond. It is customary to quote the extensive work of Wasteneys and Borsook (89) as evidence of the possibility that condensation of amino acids to protein-like products can be brought about by enzymes. To a certain extent this is so; they have shown that a concentrated digest of proteins, under the action of a relatively high concentration of pepsin, yields moderately high proportions of a material designated as plastein. The product has a low ratio of amino to total nitrogen, gives a positive biuret test, and possesses solubility relationships to be expected of a complex substance allied to the proteins. They concluded that a substance of high molecular weight which had many properties analogous to those of native proteins had been produced. Subsequent research (90) has confirmed the idea that a substance more complex than the amino acids of which it is composed is indeed formed and plastein is frequently referred to as a protein; but ultracentrifuge study has shown that the molecular weight is not excessively high (c. 1000) (29), and recent investigation of the alleged antigenic properties of plastein (28) has shown that the preparations, when due allowance is made for possible protein impurities derived from the enzyme used in their preparation, possess no antigenic properties.

However this may be, there is ample evidence that proteins are synthesized both in plants and in animals from simple substances, and Schulze's conclusion, drawn from a vast experience, that asparagine can supply a large part of the necessary nitrogen for this synthesis is today fully substantiated. That the actual intermediates are the α-amino acids, that is, that synthesis is the reverse of hydrolysis, is a conclusion that is widely held but is still by no means a certainty.

Since the work of Knoop and Oesterlin (39) in 1935, it has been generally realized that α-keto acids must occupy an exceedingly important position in the metabolism of nitrogen, and the obvious significance of aspartic and glutamic acids, or rather their amides, in plant metabolism at once focuses attention on oxaloacetic and α-ketoglutaric acids as the two α-keto acids the behavior of which in the organism must be understood.

Krebs, in 1933 (41), demonstrated that kidney tissue contains an enzyme which, in the presence of arsenite to inhibit further steps in the oxidation, oxidizes aspartic acid to oxaloacetic acid, and glutamic acid to α-ketoglutaric acid. At about the same time, Andersson (7), working in v. Euler's laboratory, showed that wheat and cucumber seeds likewise contain an enzyme which oxidizes aspartic acid or glutamic acid in the presence of a hydrogen acceptor (methylene blue) and of cozymase obtained from yeast. The actual preparation of cozymase used had not been purified, and the further development of the details of the reaction required considerable time. The results were described in five recent papers by v. Euler and his coworkers (1, 2, 3, 26, 27), of which the one by v. Euler, Adler, Günther and Das (27) contains the most comprehensive summary of the present position.

Meanwhile, and quite independently, Damodaran and Nair (24), working in India, established the presence of a *l* (+)-glutamic acid dehydro-

genase in two-day old seedlings of three species of legumes (*Phaseolus mungo*, *Phaseolus radiatus*, and *Pisum sativum*). They concentrated the enzyme by precipitation of water extracts of the seedlings with ammonium sulfate but found that enzyme activity was lost on dialysis. Accordingly, buffered solutions of the ammonium sulfate precipitate were used in their studies which resulted in the demonstration that the enzyme is most active in the region pH 7.8 to 8.0, that the reaction involves the absorption of one atom of oxygen per molecule of glutamic acid oxidized, that α-ketoglutaric acid is the product of the oxidation, that such inhibitors as fluoride, cyanide and arsenite have little or no effect on the oxidation, and that this is not stimulated by such hydrogen transporters as methylene blue or ascorbic acid. Furthermore, the enzyme was found to oxidize only *l* (+)-glutamic acid out of eight amino acids tried. It is perhaps to be inferred, from the failure of their enzyme preparation to react after dialysis, that other essential components of the system were removed by this process—a possibility to which they did not refer.

The work of v. Euler and his collaborators established the existence of an enzyme which brings about the dehydrogenation of glutamic acid to α-imino glutaric acid. This substance is unstable and is automatically hydrolyzed to α-ketoglutaric acid. The reactions may be represented as follows:

$$\begin{array}{l} CH_2\text{-}CH_2\text{-}CH\text{-}COOH \\ \quad\quad\quad\quad\quad\quad | \\ COOH \quad\quad NH_2 \end{array} + Co \longrightarrow \begin{array}{l} CH_2\text{-}CH_2\text{-}C\ COOH \\ \quad\quad\quad\quad\quad\quad\ \| \\ COOH \quad\quad\ NH \end{array} + CoH_2$$

$$\xrightarrow{H_2O} \begin{array}{l} CH_2\text{-}CH_2\text{-}C\text{-}COOH \\ \quad\quad\quad\quad\quad\ \| \\ COOH \quad\quad\ O \end{array} + NH_3$$

where Co denotes the cozymase, CoH_2 the reduced cozymase.

The first reaction is catalyzed by the apodehydrase (the prefix *apo* signifies separate, distinct, and hence specific) for the dehydrogenation of glutamic acid and requires for its action the presence of a coenzyme. In this case cozymase preparations from yeast were effective, but it was found that there were differences in the behavior of the apodehydrase preparations from various sources with respect to the particular coenzyme required. Glutamic acid apodehydrase from yeast and *Bacillus coli* required codehydrase II (Warburg's co-ferment), whereas the apodehydrase from higher plants (beets, celery, wheat seeds, cucumber seeds) required codehydrase I. The apodehydrase preparations from liver and other animal tissues reacted satisfactorily in the presence of either codehydrase, and may possibly, therefore, contain more than one specific enzyme.

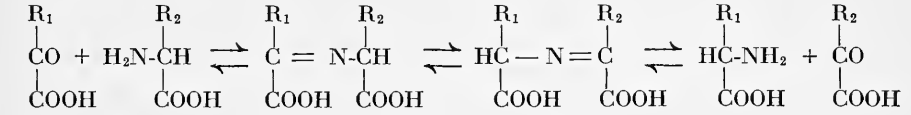
The by-product of the first reaction is the reduced form of the coenzyme (CoH_2), which is a very powerful reducing reagent that can also be prepared from coenzyme solutions by treatment with hydrosulfite. Owing to the color change that takes place during its formation, it is possible to follow the rate of reduction of the coenzyme spectrophotometrically and, accordingly, three methods are available to define the course of the enzymatic reaction, namely the more or less qualitative methylene blue decolorization technic of Thunberg, the quantitative rate of oxygen consumption as meas-

ured in the Warburg manometric apparatus, and the spectrophotometric method mentioned. In addition, isolation methods may be applied in large scale experiments to demonstrate the actual products of the reactions.

The enzyme reaction was shown to be an equilibrium since ammonia and α-ketoglutaric acid repress the rate of oxygen absorption and also, if α-ketoglutaric acid and ammonia are allowed to react with the reduced form of the coenzyme (CoH_2) in the presence of the apodehydrase, glutamic acid is formed. The maximum rate was found to be at or near pH 7.5, and no amino acid other than *l*(+)-glutamic acid (15 tried), nor any peptide, even glutathione, would react.

v. Euler and his coworkers pointed out the great significance of this enzyme reaction not only in animal physiology but also in the plant. It provides a mechanism for the synthesis of a specific amino acid from ammonia and a specific α-keto acid. Once this amino acid is formed, the production of other amino acids becomes possible by means of the amino nitrogen donation reaction of Braunstein and Kritzmann (17) and, accordingly, the metabolism of the proteins and the carbohydrates is linked through that of the organic acids.

The amino nitrogen donation reaction, which plays an important part in these speculations upon protein metabolism, has also only recently been demonstrated. Braunstein and Kritzmann studied the effect of muscle tissue upon amino acid oxidation. It had already been noted by Needham (55, 56) that muscle tissue oxidizes glutamic and aspartic acid to succinic acid, but that there was no increase in ammonia nor any change in the amino nitrogen. The latter observation was confirmed, but it was found that glutamic acid is converted to α-ketoglutaric acid and that alanine is formed. The reaction is therefore essentially a transfer or donation of amino nitrogen to an α-keto acid, in this case pyruvic acid. Detailed study showed that the reaction is enzymatic, is reversible, and may probably be generalized in such a way that glutamic acid may be converted into any other amino acid provided the proper α-keto acid is available. A mechanism was suggested which is based upon a transformation worked out by Herbst and Engel (37).



In muscle, the reaction was found to result in approximately an equimolecular mixture of the reaction products. The main investigation was confined to the glutamic acid-α-ketoglutaric acid equilibrium, but the analogous aspartic acid-oxaloacetic acid relationship was also observed. The enzyme that brings about this reaction has been definitely demonstrated as yet only in muscle and in a few other animal tissues. Virtanen and Laine (88), however, believe that they have obtained evidence of a similar reaction catalyzed by an enzyme in pea seedlings and there is every reason to expect that it will soon be recognized in many plant tissues.

Returning now to the metabolic scheme suggested by Chibnall, shown on page 116, it is clear that the interrelationship of ammonia to the two

α-keto acids, of these respectively to aspartic and glutamic acid and hence, by formation of the ammonium salts and dehydration by means of the respective amidases, the relationship of these in turn to asparagine and glutamine are all well-defined enzymatic reactions known to occur in plant or animal tissues. The possibility of the donation of the amino nitrogen of aspartic and glutamic acid to suitable α-keto acids connects these two important amino acids to the complete set of amino acids required for the formation of proteins, and only two steps remain which must still be regarded as wholly hypothetical. The one is the assumption that, given the proper amino acids and the proper conditions, i.e. concentration, hydrogen ion activity, and other purely physical conditions together with the correct enzyme system, protein will be produced. There is at present no alternative to the acceptance of this as a matter of faith; it is, however, supported by a considerable degree of intrinsic probability as well as by some experimental evidence, alternative views being exceedingly vague. The other assumption is that the α-keto acids required for the production of α-amino acids by amino nitrogen exchange are products of the metabolism of the carbohydrates and perhaps also of the fats. This assumption is so important that it will require careful examination and, as will become clear, it is still highly speculative.

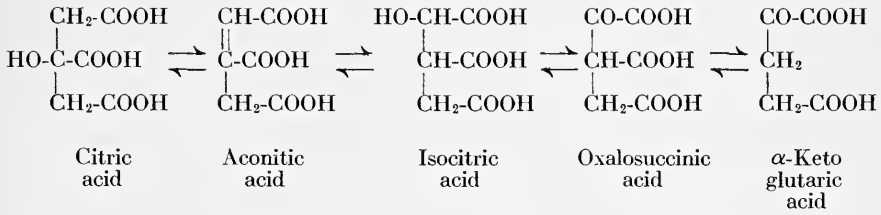
THE CARBOHYDRATE-ORGANIC ACID METABOLISM

Progress in the unraveling of the details of intermediary metabolism in tissues has been so rapid during the past few years that views that were recently entirely speculative are now well established, while others of apparently equal intrinsic probability have proved unsound. It is mostly in the field of animal physiology that these advances have been made, but so many analogies have been observed in plants that the temptation to transfer ideas of metabolism from the discussion of one organism to that of another is scarcely to be resisted. The recent detailed review of protein metabolism in plants by Chibnall (20) has shown that a considerable degree of success is to be anticipated from this type of argument, and the following discussion is largely based upon his views.

The primary function of living tissue by means of which energy is obtained for the various chemical transformations that take place is respiration. This is generally assumed to consist, for the most part, in the conversion of carbohydrates to carbon dioxide and water, but the steps whereby the overall change is effected are certainly numerous and many of the details are still only dimly understood. However, the facts that oxygen is absorbed and carbon dioxide is evolved furnish powerful methods for the detection of many of these steps. If it can be shown that a given substance added to a tissue or tissue extract, still provided with a part at least of its equipment of enzymes in an active form, exerts a measurable effect upon either the oxygen consumption or the carbon dioxide evolution, that substance can be quite confidently regarded as being in some direct or indirect way concerned in the respiration of the tissue.

The work of Szent-Györgyi and his collaborators on animal tissue respiration (10) has thrown much light upon the share taken by the four-carbon atom dicarboxylic acids. Malic, succinic, fumaric, and oxaloacetic acids are definitely involved in the transformations together with the specif-

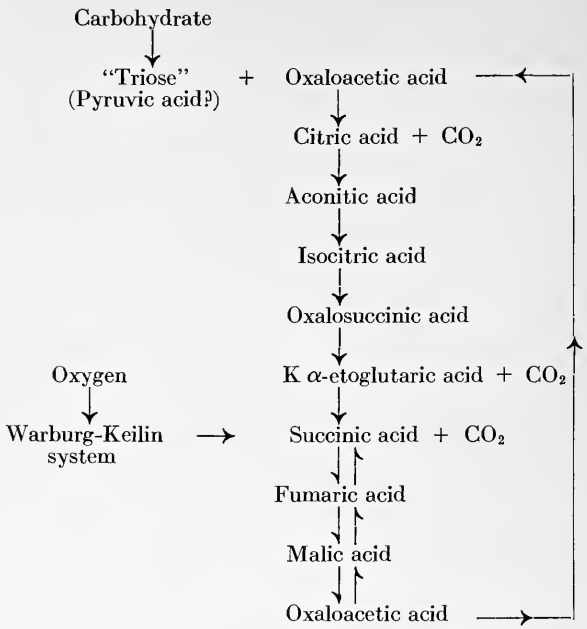
ic enzymes and coenzymes that form an essential part of the system, since each of these is oxidized. But the share taken by the five-carbon atom dicarboxylic acids, and the more complex acids such as citric acid, has only recently been appreciated. Knoop and Martius (38, 48) and Martius (46) have shown that citric acid may arise through the condensation of pyruvic with oxaloacetic acid and subsequent oxidation, and that citric acid can be converted into α-ketoglutaric acid by an enzyme system in liver tissue, the intermediates probably being the well-known constituents of certain plants, aconitic and isocitric acids. Krebs (42) has confirmed this observation, as has also Breusch (18). The respiration of liver tissue was found to be stimulated to almost exactly the same extent by citric acid, cisaconitic acid, isocitric acid, and α-ketoglutaric acid. Accordingly, there is evidence for the existence of the equilibrium relationships shown by the following formulae:



Citric acid | Aconitic acid | Isocitric acid | Oxalosuccinic acid | α-Keto glutaric acid

Martius in a more recent paper (47) has furnished evidence that bean and cucumber seeds, as well as muscle and liver tissue, contain enzyme systems that can convert half of the optically inactive synthetic isocitric acid employed into citric acid. Equilibrium appears to be attained at about the point where the organic acid mixture consists of 10 percent isocitric acid and 90 percent citric acid. Cisaconitic acid is also nearly completely converted into citric acid and it is evident that only one of the isomers of the isocitric acid enters into the enzymatic reaction. The significance of this scheme for citric acid metabolism both in plant and animal organisms thus appears to be thoroughly established.

Krebs (42) has suggested that the mechanism of the oxidation of carbohydrates in animal tissues is really a closed system or cycle of reactions: sugar enters the cycle as an hypothetical "triose" which is very likely pyruvic acid, and this substance, in combination with oxaloacetic acid, yields citric acid. The citric acid proceeds through the steps outlined above, carbon dioxide being eliminated at the last, the α-ketoglutaric acid being then oxidized with further elimination of carbon dioxide, by means of the Warburg-Keilin system, to succinic acid. This subsequently passes through the successive reversible stages fumaric and malic acid back to oxaloacetic acid. The hypothetical cycle may be illustrated as follows:



The net result of the operation of this cycle is that carbohydrate enters through the reactions of glycolysis; oxygen enters through the Warburg-Keilin system of oxidizing ferments; carbon dioxide is eliminated and energy is made available. The evidence in its favor is that each component has been demonstrated to promote cell respiration, the citric acid to α-ketoglutaric group inclusive by Knoop and Martius, the succinic to oxaloacetic acid group by Szent-Györgyi and his collaborators and by others. The unique position of oxaloacetic acid is evident from the fact, established by Krebs, that citric acid is regenerated if oxaloacetic acid is added to muscle tissue anaerobically but by no other intermediate. Citric acid is thus a catalyst in the oxidation of carbohydrate in animal tissues The steps from succinic to oxaloacetic acid are reversible and it would appear from the work of Breusch and also of Martius that certain of the steps from citric to α-ketoglutaric are also, at least in part, reversible; but oxalosuccinic acid could not be shown to be converted to aconitic nor to isocitric acid and the position of this substance in the scheme is therefore less well established than the others.

This view of the mechanism of respiration has been criticized by Breusch in Szent-Györgyi's laboratory and is still a matter of debate. It provides, however, a convenient working hypothesis and many of the detailed steps are definite enzyme reactions that occur in some animal, as well as in certain plant, tissues. Furthermore Krebs, Salvin and Johnson (43) have produced evidence in its favor from experiments on the intact animal based on the feeding experiments of Orten and Smith (59) and the great importance of citric acid in animal metabolism is emphasized by the recent work of Hallman and his colleagues in Finland (35, 36, 73). But the transfer

of so elaborate a system *in toto* from the animal to the plant is not a step to to be lightly undertaken. It is this transfer that Chibnall has made in his Silliman Lectures and it is necessary to examine the evidence that may be advanced for its justification.

In the first place, every substance mentioned in the scheme has been found in plant tissues with the exception of oxalosuccinic and oxaloacetic[1] acids. The two key substances, oxaloacetic and α-ketoglutaric acid, are essential products of aspartic and glutamic acid oxidation and the latter has been actually isolated in the form of a derivative from plant tissue. Citric and malic acids are infinitely more important from the quantitative point of view in the plant than in the animal, and it is only recently, indeed, that citric acid has been shown to play any intermediary role at all in the animal. The probable significance of oxaloacetic and α-ketoglutaric acids in the plant is based upon their obvious chemical relationship to the important plant amides asparagine and glutamine; direct evidence that α-ketoglutaric acid must have some function has been mentioned already in connection with the demonstration in v. Euler's and also in Damodaran's laboratory of a dehydrase system in plants specific for glutamic acid, and further evidence will be given below. With respect to oxalosuccinic acid, it may be pointed out that its conversion to α-ketoglutaric acid is merely a decarboxylation; it is a reaction that can be carried out *in vitro* by heating with acid, and to assume that it may proceed in tissue either spontaneously or under the influence of a decarboxylating enzyme introduces no difficulty.

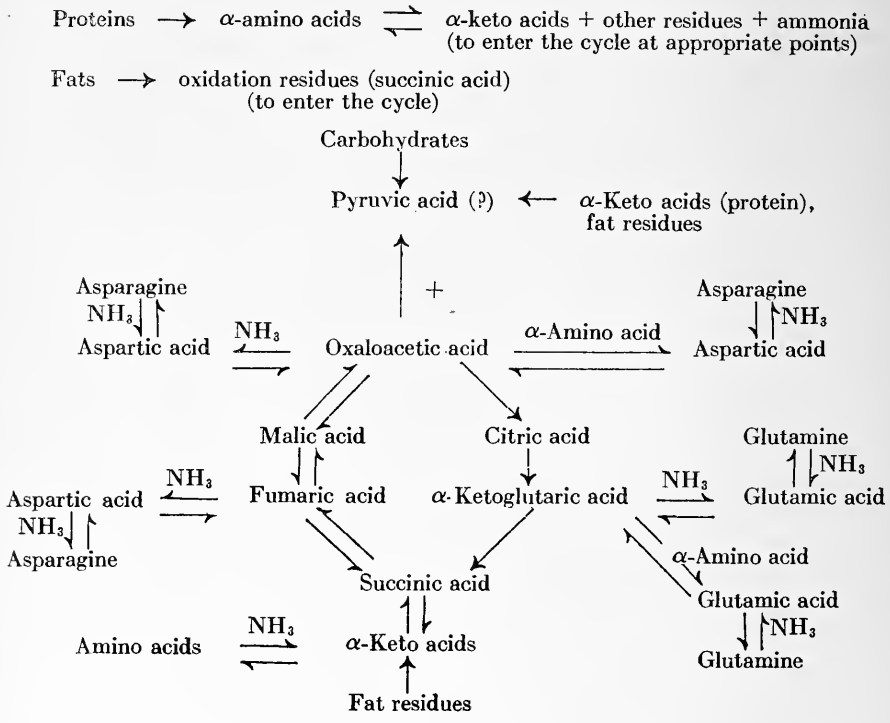
Perhaps the strongest argument in favor of the existence of the citric acid respiration cycle in plants is a purely pragmatic one. If this cycle is postulated as a mechanism for respiration, many relationships between a number of well-known plant constituents become clear and certain aspects of the behavior of the components of the tissues receive a rational explanation.

Chibnall, in his application of the Krebs cycle to the explanation of respiration in plants, has added to it formulations to represent the share taken by the proteins and the fats in the scheme of reactions. The diagram on page 124 includes these modifications together with a few additions intended to clarify some of the relationships. In order to simplify the presentation as much as possible it is assumed that, in plants, proteins undergo digestion to amino acids and that these, in turn, are oxidized to ammonia and a mixture of α-keto acids. It is further assumed that fats, which are exceptionally important constitutents of many seeds, although they play a smaller role in leaves, undergo oxidation to what are denoted as "fat residues". Of these, succinic acid and acetic acid are doubtless very important and α-keto acids may also play a part. The chief product of carbohydrate decomposition is assumed, for the purposes of representation in the present scheme, to be pyruvic acid. Krebs, in his original article, employed the non-committal term "triose" and in a later paper wrote pyruvic acid with a query. It is to be understood that the conversion of citric to α-ketoglutaric acid takes place through the intermediate well-known plant acids already mentioned. The right-hand half of the cycle represents reactions

[1] Evidence of the presence of oxaloacetic acid in plants has been obtained by Virtanen and his associates since this was written (Virtanen, A. I., Laine, T., and Roine, P., Suomen Kemistilehti, 11B: 25. 1938).

that proceed as a whole only in one direction, although certain steps are reversible; the left-hand half represents the reversible equilibria of Szent-Györgyi and others.

The positions of pyruvic and oxaloacetic acid are unique; the symbols are intended to convey the idea that these two substances can condense to form citric acid, a reaction that has been demonstrated by Knoop and Martius. On the other hand, oxaloacetic acid can be decarboxylated to yield pyruvic acid. Thus fat residues entering the cycle at succinic acid can contribute to the pyruvic acid and hence, if the glycolytic reactions are reversible, can be converted to carbohydrates.



The position of the protein decomposition products is also unique. Ammonia, the chief nitrogenous product, can combine with oxaloacetic or with α-ketoglutaric acid to yield respectively, aspartic and glutamic acid; these, with further ammonia, are then converted to the amides asparagine and glutamine by dehydration, a reaction known to occur in animal tissues in the case of glutamine. Another way in which asparagine may arise is by the addition of ammonia to the double bond of fumaric acid. The amino acids themselves may also react with oxaloacetic and α-ketoglutaric acid to yield aspartic and glutamic acids and the corresponding α-keto acids. This is the reversible reaction of amino nitrogen donation and, as has been pointed out above, it furnishes the possibility that any amino acid may be derived from its corresponding α-keto acid in the presence of aspartic or glutamic acid.

The scheme therefore provides an explanation of the mechanism of the respiration of the carbohydrates. It shows that respiration involves a series of interconversions of the organic acids and, by assumptions with respect to the speed of the different reactions, provides an explanation of the enrichment of plant tissues in one or more of these substances. Furthermore the proteins are linked into the scheme via amino acids and ammonia and especially through aspartic and glutamic acids, substances that obviously have definite functions in animal protein metabolism and which are represented in plants by the exceedingly important and universally distributed respective amides. The fats likewise enter the scheme, and in many seedlings doubtless play an almost dominating role.

Thus respiration, the property by which life is most surely recognized, becomes in plants a function of all three of the main groups of cell constituents each bearing its appropriate share of the load, although details may be expected to differ in different species and under various conditions. This is perhaps the chief advantage of the present hypothesis; it specifies definite links in metabolism between the carbohydrates, the fats, the proteins, and the organic acids, but provides an explanation for the widest differences in actual composition in any particular case. It further suggests the great complexity of these reactions.

There are, however, certain deficiencies in the present formulation that should be mentioned. No place is provided for the two very important substances, tartaric and oxalic acids. Tartaric acid occurs plentifully in many species (30) but by no means universally; it may therefore be a product of a specialized metabolism. It is known as a product of relatively mild oxidation of sugars *in vitro* and may therefore be linked with the carbohydrate metabolism. Oxalic acid is almost invariably present in plant tissues and in many species is the dominant acid, notably so in highly acid plants. It is a common end-product of oxidation of carbohydrates by energetic reagents *in vitro* and may also result from carbohydrate oxidation in plants. This seems definitely to be so in fungi (6). But oxalic acid undoubtedly can enter to some extent into the general metabolism of the intact higher plant. It is, for example, withdrawn from the leaves and laid down in the fruit during seed development in the tobacco plant (83). Furthermore, as one of the strongest of the common organic acids, it plays an important part in the buffer systems of acid plants.

The relationship of malonic acid is not considered in the cycle. In the animal, malonic acid acts as a specific inhibitor of succinic dehydrase (67) and, accordingly, promotes the excretion of succinic acid when fed to rabbits or rats (43). It has been identified in appreciable quantities in the leaves of alfalfa (77) and of wheat (57) as well as in a few other species. One possible metabolic relationship has been indicated by Butterworth and Walker (19) who detected it in appreciable amounts at one stage of the decomposition of citric acid by certain bacteria. It was supposed to arise from the oxidation or hydrolysis of acetone dicarboxylic acid, an intermediate in the transformation of the citric acid to acetic acid by the organism.

Lactic acid, one of the most important of the organic acids in animal physiology, is also found in plants, for example in raspberry leaves (31). Like tartaric acid, it is probably a product of the carbohydrate metabolism,

and its close relationship to pyruvic acid suggests that it may be involved in some part of the metabolic scheme not far separated from the relationships already indicated.

Another point upon which the scheme is necessarily vague is the matter of the production of the α-keto acids required for the synthesis of all save three of the amino acids. Once these are provided the mechanisms for the synthesis of proteins are reasonably clear up to a point, but, except for the suggestion that the necessary substances may arise from the carbohydrates in some as yet undefined way, no information has been secured. In this connection the ingenious speculations of Hall (34) are of the utmost importance.

Finally, no formal account is taken of the capacity of the plant to assimilate nitrogen from without in the form of nitrate. There is much reason to suppose, however, that nitrate obtained from the soil enters the metabolism of the plant in the form of ammonia, which would enter the cycle at the appropriate points. And Virtanen and Laine (87) have presented evidence that nitrogen assimilated from the air by the symbiotic root bacteria of certain legumes may be absorbed by the host plant in the form of aspartic acid, the mechanism being the production of hydroxylamine by the reduction of nitrate through nitrite, the condensation of this with oxaloacetic acid to form the oxime of aspartic acid, and the further reduction of this to aspartic acid. If this is indeed the case, the relation of soil or of atmospheric nitrogen to the plant metabolism can be quite simply represented. That hydroxylamine is one of the intermediates in the reduction of nitrate to ammonia in the metabolism of certain bacteria seems to have been established by Woods (91).

If the course of respiration in plant tissues actually follows a scheme in which a series of organic acids figures in such a way that the final product of the interconversions is identical with one of the initial products, it is clear that the entire sequence of reactions is controlled by the slowest individual transformation. Accordingly, the conditions with respect to the relative concentrations of the various components may change from species to species, as well as from time to time in the same species, as one or another of the transformations is stimulated or repressed. This is the concept of bars or inhibitions in the cycle which enables one to account for enrichment in one or more of the components under various circumstances.

The accumulation of any single component does not necessarily involve the depletion of the tissue in the component that next precedes it in the cycle but this may occur. Consequently the search for evidence of the nature of the individual precursor, for example of glutamine, may become futile if the cycle is operating normally. Each of the members of the equilibria may furnish its quota of material and the failure of an attempt to set up a balance sheet between, for example, citric acid and glutamine does not necessarily mean that glutamine does not, in fact, arise from α-ketoglutaric acid. Metabolism, if it follows a cyclic path, must therefore be considered not from a static but from a dynamic point of view.

THE SYNTHESIS OF GLUTAMINE IN RHUBARB LEAVES

1936 DW Experiment

It was pointed out in Part I that the increase in glutamine amide nitrogen in the blades of the rhubarb leaves subjected to culture in 1936

amounted to 0.218 gm. per kilo of original fresh weight of whole leaves during 261 hours of culture in water in darkness. A duplicate experiment with leaves of the same lot confirmed the order of magnitude of the change.

This conversion represents the formation of 2.27 gm. of glutamine per kilo of whole leaves and it is necessary to inquire into its origin. Two sources are obvious; some of the glutamine may have arisen directly from the protein as a primary product of enzyme hydrolysis, while the balance may have been formed from the combination of ammonia with a suitable carbon compound or precursor. The quota from the protein can be readily estimated if it be assumed that the rhubarb leaf proteins, in common with the many leaf proteins analyzed in Chibnall's laboratory, yield glutamic acid to the extent of about 8 percent of their nitrogen. The protein nitrogen loss from the blade was 1.06 gm.; 8 percent of this is 0.085 gm. and this is accordingly equal to the maximum amount of glutamine amide nitrogen that may have been derived from the protein. There remains 0.133 gm. of glutamine amide nitrogen to be accounted for. This is equivalent to 1.39 gm. of glutamine that must be regarded as newly synthesized from ammonia and a suitable precursor.

On purely chemical grounds the most likely precursor of glutamine is α-ketoglutaric acid, and biochemical evidence that this substance is involved in a reversible enzymatic relationship with glutamic acid both in animals and in plants has already been discussed. The critical experiment of administering α-ketoglutaric acid to leaf tissue and noting the effect on the glutamine content has recently been performed by Chibnall and his associates (20). The infiltration technic was employed and the loss of the ammonium salt of α-ketoglutaric acid from the infiltered solution as well as the increase in glutamine were determined. The quantities involved corresponded closely to the requirements of theory and the experiment would be completely conclusive if there had not been a considerable loss of carbohydrates from the tissue during the operations. Chibnall points out, however, that this is an essential part of the interconversion since the demands for energy of the living tissue must be satisfied and, although it is conceivable that the carbon chain of the newly formed glutamine may have arisen from the carbohydrate rather than from the α-ketoglutaric acid, the chance of this seems remote. Thus we may assume with a degree of probability that amounts almost to certainty that α-ketoglutaric acid is the immediate precursor of glutamine in plant tissues. Accordingly, the search for an explanation of the reaction in the case of the present series of rhubarb leaves becomes a search for possible precursors of α-ketoglutaric acid.

In Bulletin 399 (85, p. 808), a speculation according to which glutamine may have arisen in tobacco leaves from citric acid was discussed. If it be assumed that this mechanism applies in the present case, 1.83 gm. of citric acid would be required to form 1.39 gm. of glutamine in the blade; this is 28.1 milliequivalents, or very close to the citric acid content of the whole leaf at the beginning (30.7 milliequivalents) and nearly four times as much as the loss of citric acid from the blade (7.1 milliequivalents). Evidently, therefore, if citric acid contributes directly to the synthesis of glutamine in the rhubarb leaf during culture in darkness, it must have been regenerated during the process.

Another hypothetical origin of the carbon chain that is ultimately converted to glutamine is the fermentable carbohydrate. Without regard

to the nature of the intermediate reactions that must occur, it is worth while to point out the possibilities involved if it be assumed that one mole of glucose is so converted as to give rise to one mole of glutamine. In the present case 1.71 gm. of glucose would be required to produce the 1.39 gm. of glutamine synthesized in the blade tissue. The loss of fermentable carbohydrate from the blade was, at the outside, of the order of 1 gm. (Table 13) and the loss of total reducing carbohydrate was of the order of 1.5 gm. If one is prepared to assume that a part of the carbohydrate of the petiole migrated to the blade, there to be converted into glutamine, the production of this substance from carbohydrate can be, to some extent, explained. Failing this, it is obvious that not nearly as much fermentable carbohydrate disappeared from the blade tissue as could account for the glutamine produced.

Accordingly, one is forced to the conclusion that the glutamine synthesized in the blade of the present series of samples of rhubarb leaves must have arisen from several sources. The citric acid cycle provides an explanation of how this may have come about and, in the following discussion, the possibilities that are presented by a rigid stoichiometric application of the cycle are given.

The origin of the 1.39 gm. of glutamine which must have been synthesized from ammonia and α-ketoglutaric acid is required. Since the molecular weights of the two substances are identical, 1.39 gm. of α-ketoglutaric acid would be needed, and this, in turn, would require the transformation of 1.83 gm. of citric acid.

The actual loss of citric acid from the blade was 0.46 gm. (7.1 milliequivalents); hence 1.37 gm. of citric acid must have been newly formed through the operation of the cycle. The blades initially contained 1.28 gm. (17.9 milliequivalents) of citric acid, and the assumptions therefore lead to the conclusion that slightly more than this amount must have been regenerated in the course of the metabolism. This is by no means unreasonable. Tobacco leaves, for example, have been found to synthesize no less than 6.3 gm. of citric acid per kilo of fresh weight under similar conditions of culture (85). The ratio of blade weight to total weight of the present series of rhubarb leaves was 0.323; hence the hypothesis requires a synthesis of only 4.3 gm. of citric acid per kilo of blade tissue, which is much less than tobacco leaves have been demonstrated to be able to form.

In order to produce this quantity of citric acid, 0.94 gm. of oxaloacetic and 0.63 gm. of pyruvic acid would be required, and in the course of the synthesis 0.31 gm. of carbon dioxide would be liberated. According to the cycle, oxaloacetic is derived from malic acid and, to yield this quantity, 0.95 gm. would be necessary on the assumption of mole for mole conversion. The actual loss of malic acid from the blades was 0.64 gm., leaving a deficit of 0.31 gm. to be accounted for.

If the pyruvic acid arose from the fermentable carbohydrate, assuming mole for mole conversion, 1.29 gm. of glucose would be required. The actual loss of fermentable carbohydrate from the blades was approximately 1.0 gm. and there is again a small deficit.

The cycle provides another source for oxaloacetic and pyruvic acids. The α-keto acids derived from the decomposition of the protein are sup-

posed to enter at two points; alanine would yield pyruvic acid directly on deamination and so may supplement the supply from the carbohydrates. Other amino acids may be presumed to undergo transformation along the general lines of fatty acid oxidation to succinic acid and thus contribute to the oxaloacetic through the operation of the equilibria via fumaric and malic acid. Furthermore, oxaloacetic acid itself may be directly derived from the aspartic acid of the protein. In the present case it is necessary to find, if possible, the origin of about 0.31 gm. of oxaloacetic acid and about 0.21 gm. of pyruvic acid, or roughly 0.5 gm. of organic substance.

At this point in the argument it is necessary to abandon the stoichiometric reasoning that has been hitherto applied, and resort to the device of inquiring merely whether a sufficient quantity of protein disappeared to provide the necessary amounts of these two acids. It has already been pointed out (Part I, p. 50) that the losses of organic solids due to respiration can be accounted for if it is assumed that a large part of the protein that disappeared was ultimately completely oxidized to carbon dioxide and water. The actual loss of protein from the blades in the 1936 experiment was 7.63 gm. and, if half of this were completely oxidized to volatile end-products (disregarding the nitrogen), an approximate accounting for the actual organic solids loss can be made. As will be shown later, this estimate is not unreasonable; an approximate maximum limit of about 65 percent can be set, but there is no way to establish a lower limit in view of the possibility that the fermentable carbohydrate and malic acid losses may have contributed to the synthesis of glutamine and that other unknown components of the leaf may have contributed to the respiration. For the present, however, we have the problem of accounting for the production of about 0.5 gm. of oxaloacetic and pyruvic acid from 7.63 gm. of protein. This means that approximately 7 percent of the protein may have had this fate, no regard being paid to the fate of the nitrogen nor to the fact that oxygen entered into the reactions.

With respect to the oxaloacetic acid needed, it is only necessary to suppose that this may have been derived from the aspartic acid of the protein. Although we have no information with respect to the aspartic acid yielded by rhubarb leaf proteins, most of the leaf proteins analyzed in Chibnall's laboratory yielded in excess of 4 percent of this acid; 4 percent of the actual protein loss from the rhubarb blades is 0.31 gm., or exactly sufficient to account for the deficit of oxaloacetic acid. Incidentally, this result accounts for the fact that no asparagine could be demonstrated to be present in rhubarb leaves even after prolonged culture in darkness. The small amount of pyruvic acid needed may be assumed to arise directly from the alanine of the protein and there is thus no difficulty in accounting for the deficit of 0.21 gm. of this substance.

There is one additional detail of the data for the 1936 leaves that merits attention from the point of view of the cycle. It will be recalled that the ammonia nitrogen of the blades diminished sharply during the first 48 hours and that glutamine was synthesized during this period. With respect to the whole leaves, the amount of ammonia that had disappeared accounted fairly accurately for the whole of the nitrogen of the glutamine that was formed. It is of interest to see if the carbon compound that shared in this synthesis may have been citric acid. At the 48-hour point, 0.012 gm. of

glutamine amide nitrogen had been formed in the blades. This is equivalent to 0.125 gm. of glutamine. The citric acid loss from the blades at this point was 2.7 milliequivalents, or 0.175 gm. This could provide the carbon skeleton for the synthesis of 0.133 gm. of glutamine and, accordingly, the whole of the newly formed glutamine may have arisen from citric acid present originally in the blade tissue. At the 72-hour point, 0.041 gm. of glutamine amide nitrogen, or 0.428 gm. of glutamine, had been formed. The citric acid loss was 4.1 milliequivalents, or 0.267 gm., which could supply the carbon skeleton for only 0.203 gm. of glutamine. Thus other carbon compounds must have been called upon to furnish the balance. The apparent change in the metabolism of the glutamine of the blades in the interval between 48 and 72 hours is probably significant. It was in this period that ammonia began to increase in the blades, an evidence that protein decomposition had begun, with the possibility that glutamine of primary protein origin may have been liberated. It is, of course, recognized that the close agreement between fact and theory at the 48-hour point is fortuitous; the data are not as reliable as this agreement might suggest. The important point is that the direction and order of magnitude of the changes conform to what might be expected.

1937 Experiment

In Table 27 are shown the quantities of glutamine formed in the blades of the leaves studied in 1937. If it be assumed that the glutamic acid of that part of the protein that was digested was liberated as glutamine, and Chibnall's average value of 8 percent of leaf protein nitrogen as glutamic acid nitrogen be taken, the third line of the table shows how much glutamine amide nitrogen may have been derived from the protein. The quantities agree moderately well with the actual glutamine amide nitrogen increases save at the 165-hour points of the DW and DG experiments. Thus it is necessary to assume that synthesis of glutamine from ammonia and a nonnitrogenous precursor took place only in the interval from 93 to 165 hours in these two experiments.

TABLE 27. APPLICATION OF THE KREBS CITRIC ACID CYCLE HYPOTHESIS TO THE CALCULATION OF THE ORIGIN OF THE CARBON CHAIN OF GLUTAMINE IN RHUBARB LEAF BLADES

Figures are grams per kilo of fresh weight of whole leaves.

Culture conditions	Darkness						Light		
	Water (DW)			Glucose (DG)			Water (LW)		
Hours	25	93	165	25	93	165	25	93	165
Glutamine amide N increase	−0.006	0.030	0.111	0.002	0.037	0.087	0.004	0.036	0.054
Protein N loss	0.08	0.512	0.761	0.254	0.443	0.734	0.246	0.549	0.778
Glutamine amide N from protein	0.006	0.041	0.061	0.020	0.035	0.059	0.020	0.044	0.062
Synthesized glutamine amide N			0.050			0.028			−0.008
Synthesized glutamine			0.520			0.291			
Citric acid required			0.683			0.383			

Similar calculations are shown in Table 28 for the whole leaves. In this case the data for the LW culture likewise show that a small amount of glutamine must have been newly synthesized in the same interval. The difference between the figures in the two tables for the quantity of glutamine synthesized in the DG and LW experiments is clearly due to the transport of some of the glutamine, or possibly of the substances from which it was formed, from the blade to the petiole in each case. Transport was especially well marked in the LW leaves.

An attempt to account for the precise origin of the citric acid that would be needed, according to the cycle hypothesis for the synthesis of glutamine, does not lead to conclusive results. If the changes in the composition of the whole leaves with respect to citric acid, malic acid, and fermentable carbohydrate during the entire period of culture are taken, there is no difficulty whatever. The losses in these components were more than ample in each case to cover the requirements of the hypothesis. On the other hand, if it is assumed that glutamine synthesis took place exclusively in the blades during the interval from 93 to 165 hours, and that none of the necessary material was obtained by transport from the petiole, there is a considerable deficit both of malic acid and of fermentable carbohydrate in the DW experiment, but only a small deficit of carbohydrate in the DG experiment. In this latter, however, a large excess of glucose was doubtless available from the culture solution. The calculation is meaningless in the LW experiment where an unknown quantity of the products of photosynthesis were present.

Table 28. Application of the Krebs Citric Acid Cycle Hypothesis to the Calculation of the Origin of the Carbon Chain of Glutamine in Whole Rhubarb Leaves

Figures are grams per kilo of fresh weight of whole leaves.

Culture conditions	Darkness						Light		
	Water (DW)			Glucose (DG)			Water (LW)		
Hours	25	93	165	25	93	165	25	93	165
Glutamine amide N increase	−0.015	0.031	0.106	0.007	0.040	0.119	−0.015	0.039	0.084
Protein N loss	0.110	0.540	0.783	0.310	0.465	0.731	0.260	0.504	0.691
Glutamine amide N from protein	0.009	0.043	0.063	0.025	0.037	0.058	0.021	0.040	0.055
Synthesized glutamine amide N			0.043			0.061			0.029
Synthesized glutamine			0.447			0.630			0.302
Citric acid required			0.588			0.828			0.397

With respect to the deficit of malic acid and of fermentable carbohydrate in the DW experiment one further item of information has been obtained that may have significance. In the discussion of the changes in the forms of carbon in these leaves in a later section, it is shown that the loss

of carbon belonging to soluble substances other than organic acids, glutamine, and carbohydrates (Table 31, Column 3), in the interval between 93 and 165 hours of culture in this experiment, was very large indeed, there being far more than ample to account for the formation of the glutamine. Thus, although the details of the cycle do not suffice to show how the necessary citric acid may have arisen, there is every reason to suppose that the necessary carbon chains were available in the form of soluble substances which might enter the cycle and contribute to the formation of the glutamine, and in any case considerable quantities of protein decomposition products were present at this period of the culture.

A GENERAL VIEW OF AMIDE METABOLISM

The assumption that the citric acid cycle of Krebs may be the mechanism whereby α-ketoglutaric acid is supplied for the synthesis of glutamine in rhubarb leaves during culture is thus in accordance with the observations that have been made upon the parallel changes in composition that took place. Adequate amounts of the necessary substances were present or could have been formed. This is, however, in no way a demonstration that the Krebs cycle is, in fact, the mechanism whereby the synthesis was effected, and whether or not rigid proof that such a complex series of reactions may be in operation can ever be obtained is problematical. It is at once the merit and the deficiency of such schemes that they are so flexible that an *experimentum crucis* is almost impossible to devise. The point of the present discussion is to show that a rational explanation of the behavior of the tissue can be assigned. It remains to be seen whether this explanation possesses unique advantages over any other that may be later suggested.

Nevertheless, the present conception of the mechanism of amide synthesis throws much light upon the widely different behavior not only of the two series of rhubarb leaves discussed herein but also upon the differences between the behavior of rhubarb and of tobacco leaves. The provision of the actual precursor of the amide — in the case of rhubarb α-ketoglutaric acid—seems clearly to be a function of the respiratory activity of the leaves. Under different circumstances and with different species or even with samples of the same species collected at times when the initial composition was different, the exact course of the chemical reactions that lie at the basis of respiration may be expected to differ. Thus the provision of the necessary α-keto acid in adequate quantities for amide synthesis may not at all correspond with the other phases of the general metabolism which lead to the production of ammonia. Amide synthesis is thus a result of a complex of reactions of which ammonia liberation is only one of the component members.

The almost exclusive formation of asparagine in darkness and the formation of both asparagine and glutamine in light in the case of tobacco leaves cultured in water (85) are thus to be regarded as results of the differences in the details of the chemical mechanism of the respiration under the two conditions. It would appear that the conditions that lead to the liberation of ammonia in tobacco leaves in darkness at the same time direct the respiration along a course in which oxaloacetic acid is readily formed. The synthesis of asparagine in considerable amounts is the necessary outcome and, during the time the α-keto acid is being provided in excess, the

net result would be a maintenance of the ammonia at a very low level. Thus the amide synthesis may be described as being highly efficient as a mechanism for the control of the ammonia concentration. If and when the supply of α-keto acid is restricted, the ammonia concentration may rise although asparagine formation would continue. These are exactly the conditions that were observed in tobacco leaves cultured in darkness (85). The ammonia level was maintained very low for nearly 100 hours but then increased rapidly although asparagine formation continued at an unabated rate. It seems clear that partial exhaustion of the substances consumed in respiration restricted the rate of oxaloacetic acid formation with the result that the ammonia liberating mechanisms attained predominance.

In light, on the other hand, the respiratory activity may follow a different course since the products of a very efficient photosynthesis became available, and it may be supposed that both oxaloacetic acid and α-ketoglutaric acid arose. Both asparagine and glutamine would, therefore, be synthesized, and again the apparent efficiency of the reactions as a means of restricting the rise in the ammonia level would be a function of the rate of supply of these two substances. In the tobacco leaves cultured in light (85) only a minimal increase in free ammonia was observed and thus, in this case, it may be supposed that the situation was completely under the control of the respiratory activity. This corresponds with the fact that these leaves increased remarkably in organic solids as a result of photosynthesis in spite of the demands of respiration.

In the case of the present samples of rhubarb leaves, attention has already been directed to the fact that the 1936 leaves contained considerably more carbohydrate initially than the 1937 leaves. The results of this difference in composition, as they affected the details of the respiration, will be discussed in the next section, and it will be shown that there were wide differences in the two lots of leaves with respect to the chemical nature of the substances drawn upon. If this be assumed for the moment, the difference in the general picture of amide metabolism in the two series of samples can be accounted for. The discussion is, of course, restricted to that part of the glutamine that arose by synthesis from ammonia and a nonnitrogenous precursor.

In the 1936 leaves, during the initial phase of the culture, it has been shown that a small amount of glutamine was synthesized and this has been explained as arising from the transformation of citric acid already present into α-ketoglutaric acid. In the later phases of the culture it may be supposed that a more or less continuous supply of this substance was made available particularly in the interval between 72 and 165 hours with the result that glutamine accumulated in considerable amounts.

In the 1937 leaves, however, there was very little, if any, synthesis of glutamine until after the expiration of 93 hours of culture under any of the three conditions studied. This means that the respiratory activity of this lot of leaves followed a path different in some details from that of the 1936 leaves. Insufficient α-ketoglutaric acid was present at any time up to 93 hours to permit of the formation of glutamine in spite of the presence of large quantities of ammonia. Later, however, as the drain of respiration upon the components of the leaves affected a wider variety of substances or, perhaps, as the drain upon the α-ketoglutaric acid, formed in the normal

operation of the cycle for purposes other than amide synthesis relaxed, it became possible for glutamine to be formed.

It is clear that this conception of the mechanism of amide synthesis places little emphasis upon the concentration of ammonia present. The efficiency of the process with respect to the restriction of the increase in ammonia was of a totally different order of magnitude from that in tobacco leaves. The present account places the responsibility for this difference upon the respiration rather than upon the mechanisms that liberate ammonia and throws some light, therefore, upon the inadequacy of Prianischnikow's detoxication hypothesis as an explanation of amide formation in rhubarb leaves.

Furthermore, the distinction between neutral plants and acid plants, emphasized by Ruhland and Wetzel as an explanation of what were conceived to be differences in ammonia and amide metabolism, loses much of its force. Although a discrimination may perhaps be justified on the grounds of ammonia content, or even of acidity, it is clear that the difference, if any, is more deep-seated than this. It is a difference in the details of the chemical mechanisms of respiration, and there is reason to suppose that, when these details are more fully understood, the classification may be placed upon a more rational basis.

The view that the amide metabolism in plants is a function of the respiration of the tissues is by no means novel. It was suggested probably for the first time by Boussingault in 1864 (16) as a possible explanation of the carbon losses he observed from seedlings and has been occasionally considered by others since. Prianischnikow (63), in particular, pointed out that the ammonia that arises in seedlings from amino acids does so as the result of an oxidation reaction that would likewise liberate carbon dioxide, and one, at least, of the nitrogen atoms of the asparagine formed must have this origin. Thus he connected amide synthesis with the series of reactions through which carbon dioxide is produced but was very careful to state that knowledge was limited to the beginning and end of such reactions, that is, to the facts that oxygen is absorbed and carbon dioxide is set free but that nothing was known of the nature of the intermediate reactions that must occur. His later work with seedlings grown upon ammonia-containing culture solutions led him to place greater emphasis on the process by which ammonia was disposed of than upon the oxidation reactions that must accompany this process.

RESPIRATION OF RHUBARB LEAVES

The mechanism proposed by Krebs to account for the share taken by organic acids in the respiration of carbohydrates has been discussed in a previous section. Chibnall has pointed out that, in plants, certain relationships exist between the organic acids and other components that make it possible for contributions to the energy supply to arise not only from the sugars and organic acids but from the proteins and fats as well. The papers of Yemm (92) have made it strikingly clear that the respiration of starved leaves must draw upon components other than carbohydrates and have established a high degree of probability that the proteins may be extensively involved. Similar conclusions have also been reached from our own previous work with tobacco leaves (85).

In the present experiments several groups of data have been assembled from which conclusions may be drawn with respect to the nature of the components of the tissue that contribute to the respiration. In the first place, the loss of organic solids provides an estimate of the order of magnitude although it cannot be assumed to be a precise measure. Secondly, although it was not possible to analyze the gas phase in contact with the leaves, determinations of total carbon were made at each stage, by a modification[1] of the wet combustion method of Friedemann and Kendall (32). From these the actual carbon loss may be calculated. Furthermore, carbon determinations were made on the dried residues of the tissues after extraction with diluted alcohol to remove soluble carbohydrates, organic acids[2], pigments, and other alcohol-soluble components. The total soluble carbon was calculated by difference, and from the insoluble carbon it was possible to calculate the changes in the carbon of the group of insoluble non-protein components which may be assumed to include such substances as the pectins and other glucuronides and the complex polysaccharides.

1936 DW Experiment

The total carbon of the blade tissue of the 1936 series of leaves (Table 13, Part I) was initially 19.6 gm. per kilo; of this 3.86 gm. had disappeared at the end of 165 hours of culture. The petioles initially contained 20.5 gm. of carbon and lost 1.87 gm. in the same period; the entire loss of carbon during 165 hours was thus 5.73 gm. The loss of organic solids, as determined by subtracting the ash from the total solids, was 11.0 gm. and the average carbon content of the substances removed by respiration was, therefore, of the order of 50 percent. This is undoubtedly an overestimate since the weight of the ash, from which the loss of organic solids is calculated, includes a certain amount of extraneous oxygen and consequently leads to an underestimate of the true organic solids. Nevertheless the result strongly suggests that components of higher carbon content than sugars must have entered into the reactions of respiration.

If the respiration of the carbohydrates is assumed to take place through the operation of the citric acid cycle, it has already been pointed out, in the discussion of the application of this hypothesis to the synthesis of glutamine, that the quantity of carbon dioxide formed during one of the essential transformations would amount to 0.31 gm., or 0.085 gm., of carbon. This is obviously only a small part of the total loss. Furthermore, in the course of the reactions of glutamine synthesis, the whole of the citric acid and malic acid that disappeared from the blade may have been utilized together with about 1 gm. of soluble carbohydrate. Thus, in the blades, the tissues in which most of the glutamine synthesis probably occurred, it is necessary to set aside the citric and malic acid losses, the loss of about 1 gm. of carbohydrate and even of a little of the protein to account for the newly formed glutamine alone. These substances, or their equivalent, may have contrib-

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[1]The combustion was carried out as described by the authors but the carbon dioxide was absorbed in 4 N alkali and was determined gasometrically by the method of Van Slyke. In this way difficulties from the absorption of acid fumes were avoided. The precision was approximately ± 2 percent.

[2]It is unlikely that all of the organic acids are extracted by diluted alcohol under these conditions but tests on rhubarb tissue have shown that most of the malic and citric acid does pass into solution. The error in calculating the results falls upon the "other alcohol-soluble" carbon (see below), which would be a little too low, and upon the "alcohol-insol uble non-protein" carbon, which would be a little too high. For purposes of interpretation as used here, these errors have little effect upon the conclusions.

uted very little to the loss of carbon from the leaves, the greater part being involved in transformations within the tissue.

The simplest method to present the results, and one which also avoids any assumptions with respect to the intermediary mechanisms, is to calculate the data for the changes in the quantity of carbon of the individual components during each successive interval of the culture period. The results of such calculations are given in Table 29. The assumptions made are as follows: The protein carbon is calculated from the protein nitrogen by the use of the conventional factor 6.25 to convert to weight of protein, and the carbon content is taken as 50 percent. The carbon content of the soluble carbohydrate calculated as glucose is taken as 40 percent. The glutamine carbon of protein origin is calculated on the assumption that 8 percent of the nitrogen of the protein that was digested belonged to glutamic acid, it being assumed that this was liberated in the plant as glutamine. The glutamine carbon of other origin is calculated from the increase of glutamine amide nitrogen corrected for the glutamine of protein origin. The "other alcohol-soluble" carbon is calculated from the soluble carbon by subtracting the sum of the carbon of the carbohydrate, the organic acids and the glutamine. The "alcohol-insoluble non-protein" carbon is calculated by subtracting the protein carbon from the total carbon of the alcohol-extracted residues.

From the quantities of carbon attributed to each component at each stage of the culture (Table 29, Columns 1 to 5) the differences which represent the change in each successive interval of culture time were calculated. These are shown in Columns 6 to 9.

During the first 24 hours there was a loss of 1.37 gm. of carbon from the blades (Table 29, Column 6). The soluble carbohydrate and the "other alcohol-soluble carbon" diminished respectively by 0.5 and 0.7 gm. and the malic acid by 0.17 gm. There was a slight increase in glutamine carbon, but it is clear that the greater part of the change was due to loss of carbon from the carbohydrate and the "other alcohol-soluble" fraction. Loss of carbon by the respiration of the carbohydrates is to be anticipated, but the nature of the substances in the other fraction that were also oxidized is a most interesting and difficult question. A glance at the subsequent behavior of this group of substances shows that no additional significant quantity of carbon was lost from it until the final period when the blades were undergoing post-mortem decomposition. Accordingly, the fraction must contain a portion, amounting to about 10 percent of its carbon, which is far more readily oxidized than the rest. The analogous fraction in the petiole showed only a small parallel loss, hence this easily oxidized part is chiefly a blade constituent.

The carbon loss from the petiole fell largely on the soluble carbohydrate, smaller quantities being contributed by the "other alcohol-soluble" fraction and the malic acid. The malic acid data show a continuous and accelerating loss throughout the period of culture. The demands upon this substance for glutamine synthesis in the petiole, according to the Krebs cycle, were probably nil; it seems likely, therefore, that the loss of malic acid may have been largely due to respiration.

In the period between 24 and 72 hours of culture, the carbon loss from the blades was 1.03 gm. and from the petioles 0.25 gm. (Column 7). There

TABLE 29. CARBON CONTENT AND CARBON CHANGES OF THE COMPONENTS OF RHUBARB LEAVES DURING CULTURE IN WATER IN DARKNESS

Figures are grams of carbon in each component per kilo of fresh weight of whole leaves.

Hours	Carbon present 0	24	72	165	261	Carbon changes 0–24	24–72	72–165	165–261
	1	2	3	4	5	6	7	8	9
Blade									
Protein C	4.56	4.56	3.41	1.19	1.31	0.000	−1.15	−2.22	+0.12
Soluble carbohydrate C	0.912	0.408	0.452	0.280	0.456	−0.504	+0.044	−0.172	+0.176
Malic acid C	0.730	0.559	0.597	0.523	0.499	−0.171	+0.038	−0.074	−0.024
Citric acid C	0.435	0.427	0.336	0.292	0.262	−0.008	−0.091	−0.044	−0.030
Glutamine C of protein origin	0.000	0.000	0.127	0.369	0.355	0.000	+0.127	+0.242	−0.014
Glutamine C of other origin	0.038	0.056	0.087	0.518	0.510	+0.018	+0.031	+0.431	−0.008
Other alcohol-soluble C	6.51	5.81	5.96	5.79	5.44	−0.70	+0.15	−0.17	−0.35
Alcohol-insoluble non-protein C	6.44	6.44	6.26	6.81	6.96	0.00	−0.18	+0.55	+0.15
Total C	19.63	18.26	17.23	15.77	15.79	−1.37	−1.03	−1.46	+0.02
Petiole									
Protein C	1.09	1.02	1.08	1.17	0.94	−0.07	+0.06	+0.09	−0.23
Soluble carbohydrate C	2.35	1.87	1.28	0.57	0.40	−0.48	−0.59	−0.71	−0.17
Malic acid C	4.37	4.20	3.96	3.29	2.04	−0.17	−0.24	−0.67	−1.25
Citric acid C	0.311	0.305	0.322	0.300	0.214	−0.006	+0.017	−0.022	−0.086
Glutamine C	0.021	0.026	0.098	0.235	0.201	+0 005	+0.072	+0.137	−0.034
Other alcohol-soluble C	3.81	3.62	4.39	4.14	3.35	−0.19	+0.77	−0.25	−0.79
Alcohol-insoluble non-protein C	8.57	8.64	8.31	8.95	8.14	+0.07	−0.33	+0.64	−0.81
Total C	20.52	19.68	19.43	18.65	15.28	−0.84	−0.25	−0.78	−3.37
Whole leaf									
Total C	40.15	37.94	36.66	34.42	31.03	−2.21	−1.27	−2.24	−3.35

was a loss of 1.15 gm. of protein carbon from the blades but the gain in alcohol-soluble carbon that would be assumed to represent the products of protein digestion was only 0.15 gm. The changes in the organic acids were small and suggest that interconversions within this group, such as glutamine synthesis, were the most important changes that occurred. Two possibilities are open to explain the loss of somewhat more than 1 gm. of carbon of protein origin from the blade; the products of protein digestion may have been transported to the petiole or they may have entered into the respiration. These possibilities are not mutually exclusive—both phenomena may have occurred; but it seems best first to examine the evidence with respect to translocation.

The data for total nitrogen in Table 13 (Part I) show that the blades lost about 0.2 gm. of nitrogen and the petioles gained a nearly equivalent amount. Thus transport of nitrogenous substances seems to have occurred and the question remains to what extent carbon accompanied the nitrogen. An estimate of a possible order of magnitude can be formed if it be assumed that the substances involved were chiefly the products of protein hydrolysis. The weight of protein associated with 0.2 gm. of protein nitrogen would be

roughly 1.25 gm., and of this one-half would be carbon. Accordingly, one might expect to find an increase of alcohol-soluble carbon in the petiole of the order of 0.6 gm.; reference to Table 29 shows that the increase in alcohol-soluble carbon was 0.77 gm.

More detailed examination of the nitrogen data suggests, however, that this estimate of the quantity of carbon that may have accompanied the nitrogen in the form of protein decomposition products is excessive. There was a slight loss of ammonia from the petiole and the amino nitrogen increased by probably less than 0.1 gm. If, on the average, five carbon atoms accompanied one amino nitrogen atom, the increase in soluble carbon should thus have been of the order of 0.4 gm. On the other hand, if transport of nitrogen occurred, it is possible that soluble, non-nitrogenous organic substances may have been carried along with the nitrogenous material so that the observed increase of "other alcohol-soluble" carbon in the petiole may be largely due to transport.

The problem remains, however, of accounting for the loss of carbon from the leaves. Examination of the figures in Column 7 of Table 29 shows that the carbohydrate carbon loss from the petioles was 0.59 gm., the malic acid loss was 0.24 gm., and the combined loss of "alcohol-insoluble non-protein" carbon from blades and petioles was 0.51 gm. The actual carbon loss of 1.27 gm. from the whole leaf may therefore be reasonably accounted for if the whole of the soluble carbohydrate and considerable part of the alcohol-insoluble fraction are assumed to have been respired. Whether or not the malic acid entered into the respiration reactions cannot be decided, but its subsequent behavior in the petiole suggests that a part of it, at least, had this fate.

The changes that occurred in this period from 24 to 72 hours may, however, be accounted for somewhat differently. This explanation depends upon the assumption that the products of protein hydrolysis migrated to the petiole to a considerable extent. But a reasonable estimate of the quantity of carbon of protein origin that could have been transported is only 0.6 gm., or about half the loss of protein carbon that took place; the fate of the remaining 0.5 gm. is not evident from any of the figures for blade components in Column 7 of the table, and the conclusion may be drawn that this quantity of protein carbon may have contributed to the respiration loss from the leaves. If this be granted, the loss of 1.27 gm. of carbon from the whole leaves may clearly be accounted for in terms of respiration of 0.59 gm. of carbohydrate carbon from the petiole and an equal amount of protein carbon from the blade. The changes in the other components may then be explained as interconversions.

In the interval from 72 to 165 hours (Table 29, Column 8), the carbon loss from the blade was 1.46 gm. and that from the petiole was 0.78 gm., in all 2.24 gm. The soluble carbohydrates of the blade could have contributed only 0.17 gm., those of the petiole 0.71 gm.; thus, if it be assumed that this carbohydrate was respired completely, 1.36 gm. of carbon remain to be accounted for. The protein of the blade lost 2.22 gm. of carbon and the alcohol-soluble carbon of both blade and petiole diminished; hence any transport of carbon from blade to petiole in the form of products of protein hydrolysis is entirely masked by the subsequent changes that took place, and the ultimate fate of this carbon remains open. Glutamine synthesis in

the blade required 0.43 gm. of carbon, in the petiole 0.14 gm. The increase in glutamine of protein origin in the blade was 0.24 gm. Thus, if the hypothesis of glutamine synthesis advanced in an earlier section has any validity, the whole of the malic acid, citric acid, and carbohydrate losses from the blade, together with the relatively small loss of "other alcohol-soluble" carbon, would be needed to account for the newly formed glutamine in the blade alone. Furthermore, if the increased amount of glutamine in the petiole was transported as such from the blade, rather than having been synthesized in the petiole, still other components of the blade must have been drawn upon. The only component that remains is the protein and we are faced with the alternatives of supposing that, if the sugars and organic acids were used in glutamine synthesis, about 2 gm. of protein carbon were respired, or that protein decomposition products contributed more extensively to glutamine synthesis than from purely chemical considerations would seem likely. The further alternative that the necessary glutamine precursors migrated out of the petiole into the blade, perhaps as malic acid and carbohydrate, is most improbable in view of the physical condition of the tissues in this period. The blades were yellowing rapidly and had become completely flaccid.

It is thus extremely difficult to escape the conclusion that protein decomposition products entered into the respiration. On the other hand, it seems unlikely that so much as 2 gm. of protein carbon could have had this fate. The increase of alcohol-insoluble, non-protein carbon in both blade and petiole remains unaccounted for. Possibly aldehydes produced by oxidative deamination of amino acids may have undergone polymerization with the formation of insoluble products.

In the last period, during which most of the cells in the blade tissue were probably dead, there were only minor interchanges in the forms of carbon; evidently alcohol-soluble carbon compounds were drawn upon to some extent and there was an increase in reducing sugar, possibly due to hydrolysis of complex carbohydrates, but no carbon was lost from the tissue. In the petiole, on the other hand, respiration increased enormously—no less than 3.37 gm. of carbon were lost and it is obvious that a considerable part of this came from malic acid, the constituent that showed the greatest change. Proteolytic enzymes began to affect the protein to a small extent, but the other major changes were in the alcohol-soluble and the alcohol-insoluble fractions and it seems likely that the complex carbohydrates of the alcohol-insoluble fraction were attacked.

To recapitulate, in general, respiration in the blade tissue involved prompt participation by the carbohydrates together with a part of the alcohol-soluble components. As soon as protein digestion began and amino acids were produced, there appears to have been a little migration of these to the petiole, but most of them ultimately underwent oxidative deamination with the production of ammonia. The fate of the residues is not clearly evident in the earlier stages of this process but, towards the end of the period in which protein was being digested, some of the residues almost certainly contributed to the carbon lost from the tissue. Other components of the blade were also involved. The carbon data provide little evidence with respect to the respiration of organic acids in the blade, but the mechanism that has been suggested in an earlier section for the synthesis of glu-

tamine involves the liberation of a relatively small part of the carbon of these substances as carbon dioxide. In the petiole, there is little doubt that respiration of the carbohydrates dominated the situation for many hours, but it seems clear that an increasing share of the load was gradually assumed by malic acid. In the final period, about half of the carbon lost from the petiole must have been derived from malic acid, the balance being largely provided by the groups of unknown soluble and insoluble substances.

The respiration of rhubarb leaves during culture in water is thus a very complex process. Carbohydrates, in the present series of samples, could have provided only about half of the carbon lost and it would seem that many other components, including the protein of the blade, contributed their respective shares. Definite phases of respiration, in which the major part of the load was borne by a single type of compound, are difficult to recognize although the carbohydrate and "other alcohol-soluble" substances were earliest involved. In the petiole, however, the load appears to have been borne at first largely by the carbohydrate and then to an increasing extent by the malic acid, although here also other components in the unknown groups of soluble and insoluble substances played a part.

A summary of the distribution of carbon in the leaves studied in 1937 is given in Table 30, the same assumptions having been made in the calculations as in the first four columns of Table 29. The successive changes in the carbon of each component are shown in Table 31. It will be necessary to consider each series separately.

1937 DW Experiment

The DW series of 1937, as has already been pointed out, behaved somewhat differently from the DW series of 1936, and the carbon data furnish further details of these differences. The 1937 leaves contained much less soluble carbohydrate than the 1936 leaves and, although they were initially larger and heavier, they contained considerably less organic solids. The demands of respiration which, in the 1936 leaves, were to a considerable extent satisfied by the carbohydrates, were extended in the 1937 leaves to other groups of substances. In the first 25 hours the blades lost 0.8, and the petioles 1.1 gm. of carbon (Table 31, Column 1) and it is quite clear that the carbohydrates could have provided only slightly more than 0.5 gm. of this. Accordingly the source of about 1.3 gm. of carbon must be sought among the other components of the leaf. There was little or no migration of nitrogen from the blade to the petiole in this period and there is no clear evidence of a migration of carbon. The changes in the blade and in the petiole can therefore be considered independently.

In the blade the carbon losses were distributed more or less equally over several groups of substances. The carbohydrates supplied less than one-fifth of the carbon loss and the "other alcohol-soluble" and the alcohol-insoluble groups each provided a similar amount. In view of the loss of 0.25 gm. of carbon from the protein, one might have expected the "other-alcohol soluble" carbon to increase but, on the contrary, it diminished. Thus it is quite possible that, even at this early stage of the culture, substances of protein origin may have been completely oxidized.

In the petiole, less than half of the loss was due to carbohydrate oxidation and the balance was made up chiefly from the "other alcohol-soluble"

TABLE 30. CARBON CONTENT OF THE COMPONENTS OF RHUBARB LEAVES DURING CULTURE

Figures are grams of carbon in each component per kilo of fresh weight of whole leaves.

Culture conditions	Darkness							Light		
	Water (DW)				Glucose (DG)			Water (LW)		
Hours	0	25	93	165	25	93	165	25	93	165
	1	2	3	4	5	6	7	8	9	10
Blade										
Protein C	3.41	3.16	1.81	1.03	2.61	2.04	1.11	2.64	1.69	0.98
Soluble carbohydrate C	0.324	0.184	0.136	0.196	0.312	0.200	0.200	0.224	0.196	0.200
Malic acid C	0.386	0.271	0.196	0.225	0.323	0.244	0.166	0.307	0.296	0.244
Citric acid C	0.209	0.166	0.117	0.105	0.161	0.159	0.109	0.151	0.133	0.105
Oxalic acid C	0.378	0.468	0.370	0.410	0.365	0.407	0.373	0.357	0.320	0.332
Glutamine C of protein origin	0.000	0.027	0.175	0.260	0.087	0.160	0.260	0.084	0.188	0.266
Glutamine C of other origin	0.092	0.039	0.043	0.305	0.014	0.088	0.202	0.024	0.068	0.056
Other alcohol-soluble C	3.37	3.24	3.17	2.25	3.25	3.51	3.35	2.99	2.97	2.82
Alcohol-insoluble non-protein C	4.25	4.06	3.69	4.27	3.62	3.89	3.99	3.57	3.57	3.74
Total C	12.42	11.62	9.71	9.05	10.74	10.70	9.76	10.35	9.43	8.74
Petiole										
Protein C	0.925	0.830	0.850	0.865	0.775	0.850	0.945	0.900	1.07	1.21
Soluble carbohydrate C	1.18	0.760	0.528	0.359	1.62	1.48	1.46	0.752	0.576	0.448
Malic acid C	3.36	3.36	3.21	2.78	3.66	3.84	3.40	3.93	2.92	2.47
Citric acid C	0.166	0.163	0.165	0.168	0.191	0.217	0.239	0.171	0.198	0.191
Oxalic acid C	0.753	0.764	0.767	0.809	0.719	0.785	0.780	0.769	0.798	0.764
Glutamine C	0.199	0.158	0.204	0.179	0.221	0.214	0.340	0.116	0.214	0.329
Other alcohol-soluble C	2.17	1.81	2.59	2.09	2.33	3.04	3.30	1.99	3.90	3.64
Alcohol-insoluble non-protein C	6.82	6.65	6.69	7.17	6.46	7.45	7.95	6.89	7.83	7.61
Total C	15.57	14.50	15.00	14.42	15.98	17.88	18.41	15.52	17.51	16.66
Whole leaf										
Total C	27.99	26.12	24.71	23.47	26.72	28.58	28.17	25.87	26.94	25.40
Glucose C absorbed					1.91	4.56	5.22			

TABLE 31. CHANGES IN CARBON CONTENT OF THE COMPONENTS OF RHUBARB LEAVES IN SUCCESSIVE INTERVALS OF CULTURE

Figures are grams of carbon in each component per kilo of fresh weight of whole leaves.

Culture conditions	Darkness						Light		
	Water (DW)			Glucose (DG)			Water (LW)		
Culture interval (hrs.)	0–25	25–93	93–165	0–25	25–93	93–165	0–25	25–93	93–165
	1	2	3	4	5	6	7	8	9
Blade									
Protein C	−0.250	−1.350	−0.780	−0.800	−0.570	−0.930	−0.770	−0.950	−0.710
Soluble carbohydrate C	−0.140	−0.048	+0.060	−0.012	−0.112	0.000	−0.100	−0.028	+0.004
Malic acid C	−0.115	−0.075	+0.029	−0.063	−0.079	−0.078	−0.079	−0.011	−0.052
Citric acid C	−0.033	−0.049	−0.012	−0.048	−0.002	−0.050	−0.058	−0.018	−0.028
Oxalic acid C	+0.090	−0.098	+0.040	−0.013	+0.042	−0.034	−0.021	−0.037	+0.012
Glutamine C of protein origin	+0.027	+0.148	+0.085	+0.087	+0.073	+0.100	+0.084	+0.104	+0.078
Glutamine C of other origin	−0.053	+0.004	+0.262	−0.078	+0.074	+0.114	−0.068	+0.044	−0.012
Other alcohol-soluble C	−0.130	−0.070	−0.920	−0.120	+0.260	−0.160	−0.380	−0.020	−0.150
Alcohol-insoluble non-protein C	−0.190	−0.370	+0.580	−0.630	+0.270	+0.100	−0.680	0.000	+0.170
Total C	−0.794	−1.908	−0.656	−1.677	−0.044	−0.938	−2.072	−0.916	−0.688
Petiole									
Protein C	−0.095	+0.020	+0.015	−0.150	+0.075	+0.095	−0.025	+0.170	+0.140
Soluble carbohydrate C	−0.420	−0.232	−0.169	+0.440	−0.140	−0.020	−0.428	−0.176	−0.128
Malic acid C	0.000	−0.150	−0.430	+0.300	+0.180	−0.440	+0.570	−1.010	−0.450
Citric acid C	−0.003	+0.002	+0.003	+0.025	+0.026	+0.022	+0.005	+0.027	−0.007
Oxalic acid C	+0.011	+0.003	+0.042	−0.034	+0.066	−0.005	+0.016	+0.029	−0.034
Glutamine C	−0.041	+0.046	−0.025	+0.022	−0.007	+0.126	−0.083	+0.108	+0.115
Other alcohol-soluble C	−0.360	+0.780	−0.500	+0.160	+0.710	+0.260	−0.180	+1.910	−0.260
Alcohol-insoluble non-protein C	−0.170	+0.040	+0.480	−0.360	+0.990	+0.500	+0.070	+0.940	−0.220
Total C	−1.078	+0.509	−0.584	+0.403	+1.900	+0.538	−0.055	+1.998	−0.844
Whole leaf									
Total C	−1.87	−1.40	−1.24	−1.27	+1.86	−0.40	−2.13	+1.08	−1.53
Glucose C absorbed				+1.91	+2.65	+0.66			

and the alcohol-insoluble fractions. The malic acid did not change, unlike that of the petioles of the 1936 leaves in the same period, although there was a definite utilization of malic acid in the blades in both cases.

In the interval from 25 to 93 hours (Table 31, Column 2) the blades lost 1.9 gm. and the petioles gained 0.5 gm. of carbon. The data for total nitrogen in Table 24 (Part II) show that the petioles acquired nearly 0.2 gm. of nitrogen from the blades and we therefore must consider the possibility that a substantial quantity of carbon was transported along with this nitrogen. The "other alcohol-soluble" carbon of the petiole did, in fact, increase by a quantity commensurate with the nitrogen on the assumption that protein hydrolysis products were the substances involved in the migration, but the soluble amino nitrogen increased very little and the ammonia

nitrogen diminished (Table 24). Thus the nitrogen must have been transported in some form other than amino acids or ammonia, or else underwent a prompt change on reaching the petiole. Whatever the form may have been, however, it seems clear that carbon accompanied it and the net gain by the petioles of only 0.5 gm. of carbon indicates that a part of this newly acquired carbon or its equivalent must have been utilized in respiration. The carbohydrate loss from the whole leaves was only 0.28 gm., or one-fifth of the net loss of carbon. Thus slightly more than 1 gm. of carbon lost from the leaves must have been derived from other components. The protein of the blade lost 1.35 gm. of carbon but the "other alcohol-soluble" carbon of the blade scarcely changed at all. Thus most of this carbon must have been either transported to the petiole or oxidized. An approximate upper limit of about half of this quantity for the amount transported is set by the nitrogen transport figure, and about 0.15 gm. of carbon remained in the blades as glutamine. It would thus appear that approximately 0.7 gm. of protein carbon was completely oxidized. The balance may well have been provided by the alcohol-insoluble, non-protein carbon of the blade.

In the interval from 93 to 165 hours (Column 3) the loss of carbon from the blade was only 0.66 gm., and that from the petiole was 0.58. There was little or no evidence of transport of nitrogen in this period and there was a considerable loss of "other alcohol-soluble" carbon from the petiole. The changes in the blade and petiole can therefore be considered independently. It was in this period in the present samples of leaves that glutamine was synthesized from carbon compounds already present in the blade, but the changes in the organic acids are far too small to account for it. The major change was in the "other alcohol-soluble" fraction and attention has already been drawn to the probability that this fraction must have provided much of the carbon ultimately converted into glutamine. The alcohol-insoluble fraction increased by nearly 0.6 gm. and if this, together with the glutamine, were derived from the alcohol-soluble fraction, less than 0.1 gm. of carbon from this group of substances was available for respiration. On this assumption most of the respiration loss must have been derived from the protein. On the other hand, if the protein contributed to the increase of the alcohol-insoluble group, soluble substances already present would have been available for respiration. Even so, however, the loss of protein carbon exceeded the increase in alcohol-insoluble material so that some of the protein carbon must have been respired.

In the petiole it is clear that the loss of 0.6 gm. of carbon may have been provided almost wholly by the carbohydrate and malic acid if it is assumed that the formation of the alcohol-insoluble substances drew upon the "other alcohol-soluble" fraction.

1937 DG Experiment

The consideration of the carbon changes in the DG experiment is complicated by the advent of glucose from the culture solution. Data given at the bottom of Table 31 (Columns 4 to 6) show the quantity of carbon supplied in this way and it is clear that 1.9 gm. of glucose carbon entered in the first 25 hours but that the leaves nevertheless lost about 1.3 gm. of carbon. There was an appreciable transport of nitrogen from the blade to the petiole (Table 24, Part II) and the changes must be considered in the light of the

possibility that carbon not only migrated from the blade to the petiole in the form of nitrogenous substances, but that a little glucose may have been transported in the reverse direction into the blade from the culture solution. There were increases in many components of the petiole and the fact that the loss of soluble carbohydrate from the blade was scarcely appreciable suggests that sufficient glucose reached it to compensate for the small amount of soluble carbohydrate that would otherwise have been respired. The protein in the blade was digested considerably more extensively during the first 25 hours than that in the DW experiment, but there was no corresponding increase in alcohol-soluble carbon; on the contrary this decreased substantially as did also the alcohol-insoluble, non-protein carbon. If the behavior of the blade is compared with that of the parallel DW experiment and, for the moment, the possibility that a little glucose entered it is disregarded, it is clear that the loss of 1.7 gm. of carbon was mostly contributed by the protein and the alcohol-insoluble material. The "other alcohol-soluble" substances furnish a small share which, however, was of the same order of magnitude as was observed in the DW series. What proportion of this loss represents respiration and what proportion represents transport is not certain. These leaves were in definitely better condition than the DW leaves at later stages of the culture so that it is not possible to assert that their detailed behavior with respect to either of these functions was the same.

The petioles show striking evidence of the effects of the supply of glucose. The carbohydrate and organic acids increased and there was an increase in "other alcohol-soluble carbon". The alcohol-insoluble fraction, however, decreased.

The total loss of carbon from these leaves in the first 25 hours must have been of the order of 3.2 gm. since there was a net loss of 1.3 gm. in spite of the acquisition of 1.9 gm. of glucose carbon. If it be assumed that the increases in organic acids in the petiole represent transformations of glucose, and the behavior of the DW series indicates that this is probable, then approximately 0.32 gm. of glucose carbon had this fate. In addition, 0.44 gm. remained in the petiole as an increase in soluble carbohydrate so that the fate of 0.76 gm. of glucose carbon seems clear. Thus 1.15 gm. of the glucose carbon from the culture solution remains to be accounted for. Even if this were all respired, there remains a balance of approximately 2 gm. of carbon derived from original components of the tissues that must also have met this fate. The sum of the losses of protein carbon and of the alcohol-insoluble fractions from both blade and petiole is 1.9 gm., and it seems highly probable that these components supplied much of this carbon. In this estimate no account is taken of the small changes in the other components. The possibility that protein shared in the respiration even in the earliest phase and in spite of the presence of glucose derived from the culture solution is especially noteworthy.

In the period from 25 to 93 hours (Table 31, Column 5), the leaves acquired 2.65 gm. of glucose carbon and increased in carbon content by 1.86 gm. Thus the total respiration loss was of the order of only 0.8 gm. and it is difficult to allocate this relatively small quantity. Both blade and petiole carbohydrate diminished, in all by 0.25 gm. The protein of the blade lost 0.57 gm. while the "other alcohol-soluble" carbon of the blade increased by

0.26; hence 0.3 gm. seems a reasonable estimate of the amount of protein carbon that may have been involved in subsequent oxidation since there was very little transport of nitrogen from the blade in this period to complicate the situation. The increases in almost every other form of carbon clearly show that the glucose that entered from the culture solution brought about extensive alterations in the composition of the tissues, especially in the alcohol-insoluble, non-protein fraction.

In the final period (Column 6) the leaves absorbed 0.66 gm. of glucose carbon but lost 0.4 gm. of total carbon. The respiration loss was therefore about 1 gm. of carbon. A part of this was probably contributed by the malic acid of the petiole but most of the newly acquired glucose must have been used in the petiole for the formation of soluble and insoluble products. The blades lost 0.95 gm. of carbon, mostly derived from the petiole. There was little or no evidence of transport of nitrogen from the blade to the petiole and it thus appears that a considerable part of the protein carbon was employed in respiration, the balance contributing to the increase in glutamine and possibly of the alcohol-insoluble solids.

1937 LW Experiment

Interpretation of the carbon losses from the leaves cultured in light is difficult because there is no measure available of the quantity of carbon acquired by photosynthesis. The organic solids (Table 24, Part II) at first diminished and then increased, indicating that there was a considerable respiration loss in the first 25 hours followed by a period in which photosynthesis outstripped respiration. The net loss of carbon from the whole leaves in the first period, and the gain in the second (Table 31, Columns 7 and 8) confirm this conclusion. The respiration loss in the first 25 hours was at least 2.1 gm. of carbon and how much greater it may have been does not appear. However, of this 2.1 gm., only 0.53 gm. can be assigned to soluble carbohydrate originally present. Transport of nitrogen and, accordingly, also of carbon played an unusually large part in this experiment and attention has been drawn in Part II to the evidence for protein synthesis in the petiole in the later phases of the culture. Thus the data for the whole leaves only can be dealt with in an attempt to assign the respiration loss.

In the first 25 hours the migration of nitrogen was small (Table 24, Part II) and the loss of 2.1 gm. of carbon from the blade fell mostly upon the protein and on the two groups of unknown substances. The petiole gained a large amount (0.57 gm.) of malic acid, possibly through the metabolism of products of photosynthesis transported probably in large part from the blade. The combined losses of carbon from the unknown alcohol-soluble and insoluble components were 1.2 gm. The loss of 0.53 gm. of carbohydrate carbon is approximately offset by the gain in malic acid of 0.57 gm. Thus about 0.8 gm. of carbon lost from the leaves must have come from some other source. The protein carbon loss from the blade was 0.77 gm. and it seems difficult to escape the conclusion that this contributed to the respiration. The alternative that it was mostly converted into malic acid in the petiole seems far from probable.

In the second period (Column 8) photosynthesis predominated over respiration and there was a large increase in the total carbon of the petiole, the most striking individual increase being that of 1.9 gm. of "other alcohol-

soluble" carbon, much of which may have been due to the synthesis of unknown organic acids. A part is also doubtless to be attributed to soluble substances transported from the blade. The soluble carbohydrate loss was small, but the protein was rapidly hydrolyzed in the blade and regeneration took place to some extent in the petiole. Malic acid carbon in the petiole decreased by no less than 1 gm.

There is no clue as to the actual amount of carbon lost through respiration but there are striking differences in the behavior of several of the groups of components as compared with the first period. The very rapid initial loss of alcohol-soluble and insoluble components in the blade ceased but there were large increases in these factors in the petiole. Thus the general course of events must have changed considerably. The rapid hydrolysis of protein in the blade continued and, as has been shown in Part II, nitrogen in the form chiefly of ammonia and glutamine was transported to the petiole, there to be resynthesized in part into protein.

In the last period (Column 9) there was a loss of 1.5 gm. of carbon from the leaves. Transport of nitrogen from the blade continued, although to a smaller extent, and the digestion of protein in the blade and regeneration in the petiole was again evident. Of the carbon lost, only 0.13 gm. was derived from soluble carbohydrate present in the petiole at the beginning of the period, and the losses can be accounted for to the extent of only 0.86 gm. by assuming that the malic acid of the petiole and the "other alcohol-soluble" components of both blade and petiole were involved. An additional 0.2 gm. of carbon from the alcohol-insoluble fraction in the petiole may be included but, to account for the balance of about 0.5 gm., it is again necessary to suppose that products derived from the blade protein were involved in the respiration.

Discussion

The description of the carbon changes in these experiments has been given in considerable detail since it was felt that the present data furnish an exceptionally favorable opportunity to discuss respiration in terms of the changes in the individual substances in the tissues. Under the conditions that obtained in these sets of samples of rhubarb leaves, it seems clear that the demands of respiration were satisfied by the oxidation of a wide assortment of the components, and it is particularly striking that, even when carbohydrate was supplied in the form of glucose, or as a product of photosynthesis, the respiratory process still made demands upon the products of protein hydrolysis. It would seem that, once having been deaminized, the amino acid residues were of little further value for subsequent synthetic reactions and were employed as a source of energy. Even in the case of the protein regeneration in the petioles late in the LW experiment, there is evidence that much of the nitrogen required was transported in the form of ammonia and glutamine (see Part II) so that the amino acids needed for the synthesis may have been largely synthesized *de novo* from other substances already present or from the incoming stream of products of photosynthesis.

Probably the earliest suggestion that leaf proteins may be in any way concerned in respiration was made by Borodin sixty years ago (14, 15) although no cogent evidence could be advanced at that time, and the matter

has subsequently been discussed by many students of the problem. With the advent of better technical equipment and methods in the present century, respiration losses from plant tissues have been found in general to run parallel with carbohydrate losses and it has become a matter of conviction that these substances, and, in certain cases also the fats, form the chief source of the carbon dioxide, and therefore of the energy. This conclusion has been supported by experiments in which the respiratory quotient of leaves was ascertained. The oxidation of carbohydrates should give a quotient of unity and quotients of this magnitude are, in fact, usually observed in tests made soon after leaves are excised.

Nevertheless such experiments as those of Deleano (25) and of Meyer (51) indicated that, after the carbohydrate reserves were exhausted, the protein residues, that is, the carbon chain compounds that remain after deamination of the amino acids, may be drawn upon for respiration, and much data has been accumulated in recent years that shows that respiration losses from leaves must involve substances other than carbohydrates. For example, in our own studies of the curing of tobacco leaves (84) in 1933, we found that the loss of organic solids far exceeded the loss of soluble carbohydrates, and pointed out that it was difficult to account for the loss unless it could be assumed that a considerable part of the protein were involved. The work of Yemm (92) on the respiration of barley leaves has recently added more precise information on this point. After an initial period of about 24 hours, in which respiration was undoubtedly concerned mainly with the carbohydrates, the respiratory quotient fell and an increasingly large share of the load was borne by other components of the leaf. The behavior of the nitrogenous substances left little doubt that the amino acid residues were the components that became involved.

Gregory and Sen (33) have also reached the conclusion, from experiments on the respiration of leaves of barley plants grown with the most careful control of the inorganic nutriment supplied, that the protein is in some way involved in the respiration. Statistical analysis of their very comprehensive data showed that there was a highly significant positive correlation between the respiration rate and the protein content, and an even higher correlation with the amino nitrogen. In order to account for this they postulated the existence of a cyclic relationship between protein synthesis and protein digestion which were assumed to follow separate paths.

The products of glycolysis are drawn into this cycle either directly or through intermediary organic acids which combine with ammonia to form amides and amino acids. The products of protein hydrolysis are oxidized more or less completely to ammonia and residues which may then be further oxidized with the production of carbon dioxide. The cycle of protein synthesis and decomposition is traversed rapidly and its demands upon the organic acids and carbohydrate decomposition products regulate the rate of glycolysis. The output of carbon dioxide is thus in turn related to the rate at which protein is decomposed and reformed. The carbon dioxide eliminated may or may not originate mainly from the amino acids and it would seem that, in general, quotas would be provided from several sources. In all cases, however, the several processes are interrelated and are connected by some general regulatory process which controls the whole mechanism. Gregory and Sen's own experiments were carried out with leaves from plants grown

at various levels of potassium and nitrogen deficiency with phosphorus held constant and optimal. Richards (68) extended the series to plants with various levels of potassium and phosphorus deficiency with nitrogen constant and optimal, and likewise found that the respiration rate was closely correlated with the protein content. He pointed out that the simple view that protein content is merely a measure of the mass of protoplasm respiring, or even of the enzyme systems concerned, is not adequate to account for the observations. The relationship between protein and respiration is reciprocal, each being dependent on the other; and other components of the leaves, which might be assumed to influence the rate of respiration, varied between wide limits under the different nutritive conditions without effect upon this relationship.

These views of Gregory and his co-workers are of great interest in the light of the present data. Although the actual chemical mechanisms involved can at present be expressed only in the vaguest and most general terms, they serve admirably to show how such interrelations between the various groups of substances in the tissues as have been observed in the present experiments may be brought about. The great difficulty with all experimental attack upon such systems is the necessity of working with detached leaves. This necessity arises from the purely technical considerations that require the isolation of the system before measurements upon it can be interpreted. Yet excision of a leaf at once introduces a factor that ultimately proves lethal and may well produce such fundamental changes with respect to hormone supply as to go far to defeat the very purpose for which it is done.

Nevertheless, under conditions such as those that exist in barley plants at about the time of ear formation where, as both Yemm and Gregory have pointed out, the whole of the nitrogen required for subsequent development of the plant may be derived from the decomposition of the proteins of the basal leaves, it would seem very likely that the chemical behavior of the lower leaves, while still *in situ*, would have many analogies with the behavior of excised leaves in water culture.

The present results are thus in general agreement with conclusions that have been drawn from experiments with other species, and it remains to be seen to what extent suggested detailed mechanisms for respiration can be applied to account for them.

The Krebs cycle, with the addenda provided by Chibnall to show the relationships of respiration to amide metabolism, has already been found adequate to explain in general outline the synthesis of glutamine in the rhubarb leaf. But the maximal amount of carbon dioxide that would be eliminated in the course of amide synthesis falls far short of the actual carbon lost by the samples. Clearly, therefore, the synthesis of the glutamine can be regarded as at most a by-product of the respiratory activity, important as it may have been in connection with the nitrogen relationships.

It is desirable to gain an inkling of the actual magnitude of the contribution made by the protein to the carbon dioxide loss. In the calculations given in Table 29, it is merely assumed that all the carbon of the protein that disappeared entered into some subsequent reaction, but the figures do not show how much of it may have remained in the tissue in the form of

resultant products. It has been assumed throughout the discussion of the amide metabolism of these leaves that the glutamic acid of that part of the protein that was digested survived the subsequent oxidation reactions and appeared in the soluble fraction as glutamine. Although the actual glutamic acid content of the proteins is not known, the evidence from Chibnall's analyses of a long series of leaf proteins indicates that 8 percent of the nitrogen is usually present as glutamic acid nitrogen after acid hydrolysis. Since this is almost certainly combined in the protein molecule largely as glutamine radicals, it is reasonable to suppose that 16 percent of the protein nitrogen was originally present in this form. This is certainly a maximal estimate since the amide nitrogen of leaf proteins seldom exceeds 6 percent.

The loss of protein nitrogen from the blades of the 1936 samples was 1.08 gm.; 16 percent of this is 0.173 gm. The amino acids other than glutamic acid that resulted from the digestion of this protein were in large part deaminized, the ammonia being subsequently, in part, metabolized to glutamine. Some amino nitrogen, however, survived, and the quantity can be calculated from the increase in soluble amino nitrogen corrected for the glutamine (Part I, Table 13). The increase in the blades of the 1936 leaves was 0.208 gm. Accordingly, of the 1.08 gm. of protein nitrogen, 0.38 gm. probably survived chemical change subsequent to the digestion leaving amino acid residues equivalent to approximately 0.7 gm. of protein nitrogen for possible respiratory changes. This result sets a roughly estimated upper limit upon the proportion of the protein that may have been completely oxidized of the order of 65 percent.

The products of oxidative deamination are α-keto acids, which on decarboxylation would yield aldehydes. The mechanism of the subsequent steps in the complete oxidation in the plant is unknown, but the animal is able to bring about complete oxidation of most of the amino acids (23), the fatty acids usually being thought to be largely converted to succinic acid which may be assumed to enter the cycle. Whether or not the aromatic amino acids are completely oxidized in the plant is unknown; in the animal they frequently are not.

It is also clear that the whole of the nitrogen of that part of the protein which is completely oxidized must be made available for further metabolism, probably mostly in the form of ammonia. The excess of the available ammonia over that calculated from the potential amino nitrogen, to which attention was directed in the discussion of amide metabolism in Parts I and II (Tables 7 and 22), thus receives an explanation; and Chibnall's assumption (20) that most of the nitrogen of the digested protein becomes available in metabolism is, to some extent, justified.

THE REGULATION OF PROTEIN METABOLISM IN LEAVES

It is a striking fact that when a leaf is detached from the plant and cultured in water, digestion of the protein with an attendant increase in the soluble nitrogen soon becomes manifest. The best known exception to this rule in the literature is provided by the experiments of Deleano (25) with vine leaves. Deleano employed the half-leaf method and found that leaves collected in the fall, which appear to have been provided with large stores of starch and other carbohydrates, showed no evidence of loss of protein until they had been cultured in darkness for 100 hours. The failure of the

protein to diminish and the close relationship that is evident, in his data, between the losses of carbohydrates and the quantity of carbon dioxide evolved, have been widely quoted as evidence that carbohydrates are alone concerned with the respiration of leaves under normal conditions. The problem of the behavior of the protein of vine leaves is in need of further investigation, since the extensive studies of Mothes, of Paech, and of Yemm, as well as the results obtained in this laboratory, have shown that protein hydrolysis is detectable usually very soon after the leaf is removed from the plant, being frequently quite extensive within 24 hours. The leaves described in Part I of this Bulletin were, in our experience, unusual in that protein decomposition was initiated so slowly as to be clearly recognizable only after 48 hours of culture in darkness.

Mothes (54) has considered the relationship of the age of the leaf, as well as of other factors, to the activity of the proteolytic enzymes and has suggested that the oxygen tension is most important as a controlling factor, in that at low oxygen tensions protein decomposition is favored, while at high concentrations it is restricted, or synthesis occurs. Paech (60), however, has shown that Mothes' experiments in the absence of oxygen are vitiated by the fact that leaves, in general, survive the absence of oxygen for a very short time only so that the intense proteolytic activity observed under these conditions is almost wholly a post-mortem phenomenon. Furthermore, Paech observed that the proteolytic activity of leaf-cell enzymes is much reduced by destruction of the cell organization and exposure of the enzymes to the action of oxygen. From an extensive review of the literature, as well as from his own experiments, Paech concluded that the disposition of the intact cell towards synthesis or decomposition of protein is dependent upon the relative quantities present in the cell of chemically active carbohydrate and of an active form of nitrogen which he considered to be ammonia. The component of the system present in minimal amount determines whether decomposition or synthesis shall take place. The different components of the equilibria are balanced against each other and, within the whole organism, are regulated in amount by the translocation stream, the direction and velocity of which is in turn determined by forces exerted by the centers of growth. Paech supported this view by many experiments which indeed showed that protein decomposition was depressed in the presence of an unusually large supply of carbohydrate—he employed glucose introduced by infiltration—but, as Chibnall has pointed out (20), Paech's experiments are unconvincing because of his failure to determine carbohydrates in the tissues he examined. He regarded changes in the protein content as small as .2 percent as significant, but provided only qualitative evidence, or even merely a presumption deduced from the choice of material, that such changes were, in fact, brought about by changes in the carbohydrate concentration.

A striking example of the fact that protein decomposition in the leaves of plants may have nothing to do with the carbohydrate content is furnished by data obtained with young tobacco leaves collected in 1935 and cultured in water in continuous light (Table 32). The figures show that the hydrolysis of the protein proceeded rapidly after the first 24 hours in spite of the accumulation in the leaves of glucose to the extent of more than 6 percent of the organic solids in 120 hours and of 10 percent in 169 hours. Sucrose, unfermentable carbohydrate, and even starch, likewise increased markedly.

Other examples with tobacco leaves cultured in light that lead to the same conclusion have been described in Bulletin 399 (85).

Table 32. The Effects of Culture in Water in Light upon the Protein and Carbohydrates of Tobacco Leaves

Figures are grams per kilo of fresh weight.

Hours	0	24	74	120	169
Protein N	1.69	1.71	1.32	1.11	0.98
Organic solids	64.9	71.0	79.1	78.0	82.5
Sucrose	0.29	1.07	2.09	1.83	2.09
Glucose	0.76	2.10	6.15	7.19	8.71
Unfermentable sugar	0.43	1.13	2.97	3.46	3.72
Starch	0.18	1.94	1.26	1.58	2.68

The results with rhubarb leaves described in Part II likewise illustrate the failure of a supply of carbohydrate acquired from without to influence the rate at which the blade protein is hydrolyzed. In both the DG experiment and the LW experiment the hydrolysis of the blade protein was greater in the first 25 hours than in the control DW experiment (Table 24). The final level of protein content attained at 165 hours was practically the same in all three and the most that can be said for the effect of glucose supplied to the leaves cultured in darkness is that protein hydrolysis was slightly delayed at the 93-hour point.

On the other hand, the initial carbohydrate content of these leaves was low and culture in glucose solution did not increase the sugar content of the blades; it served merely to maintain the reducing sugar level unchanged for 25 hours. Furthermore the rate of formation of products of photosynthesis in light was not sufficient to maintain the carbohydrate content against the demands of respiration. Thus these results are perhaps less striking than those with tobacco leaves already mentioned, but it is clear that in both species the rate of protein hydrolysis in the blades is not in any obvious way controlled by the quantity of carbohydrates present.

These experiments also furnish an illustration of the fallacy in Paech's implied assumption that the ammonia concentration may serve as a control upon the rate of protein hydrolysis. As has been pointed out in Part II, the ammonia in the blades of the rhubarb leaves amounted to 3 percent of the total nitrogen, and nearly 20 percent of the soluble nitrogen. The leaves of neutral species such as tobacco and tomato contain in the vicinity of only 1 percent of the soluble nitrogen as ammonia.

Paech's view that the disposition of the cells towards hydrolysis or synthesis of proteins is controlled by some factor according to a mass action law is not without value, however. Chibnall (20) has pointed out that amino acid synthesis, upon which protein synthesis is generally assumed to depend, is from the chemical standpoint a matter of the provision of the necessary α-keto acids which may react either with ammonia or with glutamic or aspartic acid to produce the corresponding amino acid. There

is as yet no information as to the source from which these α-keto acids may be derived save in the case of the three α-keto acids which yield alanine, aspartic, and glutamic acids. These are known products of carbohydrate and organic acid metabolism in plants. We can, at present, only assume a capacity on the part of the plant cell to synthesize the necessary eighteen or so other α-keto acids from which the respective amino acids may then be in turn derived, and it is only natural to invoke the carbohydrate metabolism to account for this. Mechanisms whereby the plant may be supposed to bring about such syntheses have been suggested by Hall (34), although his speculations upon amino acid synthesis deal chiefly with possible interconversions of amino sugars.

Whatever the exact mechanisms may be, however, the plant clearly has this capacity and the increase in protein nitrogen in the petioles of the LW experiment provides an example. On Chibnall's modification of Paech's view, this could be explained in terms of an excess supply of α-keto acids derived from the products of photosynthesis which combined with ammonia or reacted with glutamine in the petiole to form the necessary amino acids. These then condensed to form the new protein.

Alternatively, on Gregory and Sen's hypothesis, the behavior may be the result of the influence of the extra supply of carbohydrate and organic acid derivatives upon the cycle of protein decomposition and synthesis. Synthesis predominated under these special conditions.

It is of interest to note that the relative order of magnitude of the conversion was considerable; the total nitrogen of the petiole increased by 57 percent, and the protein nitrogen by 31 percent, of the quantities originally present. Parallel with this increase in apparent protein there was a striking increase in organic solids and of total carbon, although the various forms of carbohydrates did not increase (Table 24, Part II). Clearly, therefore, organic substances either newly synthesized in the blades or original components displaced by the products of synthesis must have migrated to the petiole. If we may assume that among these substances there was a supply of the proper material, the synthesis of protein becomes intelligible. Paech's hypothesis in its original form is inadequate to account for this since the actual carbohydrate components decreased owing to the demands of respiration.

This discussion of the regulation of the changes in protein has left untouched the fundamental problem of why the protein is digested at all when leaves are cultured. It would seem from the data for carbon losses that protein decomposition products sooner or later become involved in the respiration. Gregory and Sen's concept of a continuous and rapidly traversed cycle of hydrolysis and regeneration of protein throws some light on this matter, and their further suggestion that this is the fundamental mechanism which in turn controls the rate of decomposition of sugars is helpful in discerning the relations between these main groups of components. It seems clear that their conclusion that some regulatory influence is at work is justified though the precise nature of this influence remains obscure. Whatever it may be, it seems to be correlated with the respiratory activity and thus with the demands of the cell for energy.

SUMMARY

The results of the analyses described in Parts I and II of samples of rhubarb leaves cultured in darkness and in light have been discussed in terms of present-day views of the chemical mechanisms that have been proposed to explain the reactions that may take place in leaf tissues. The composition of the leaves at each stage in the culture period has been calculated in terms of known components, so far as this was possible, and the unknown balance has been classified on the basis of solubility so that certain inferences concerning its chemical composition may be drawn.

In order to account for the formation of glutamine it is necessary to assume the presence of an equivalent amount of α-ketoglutaric acid which, by reaction with two moles of ammonia, forms glutamic acid and glutamine in two successive steps. The Krebs citric acid cycle, proposed recently as a mechanism for the respiration of carbohydrates in animal tissues, provides a means whereby α-ketoglutaric acid may arise through enzymatic reactions and, following the suggestion of Chibnall, this scheme of reactions has been invoked as a speculative possibility in the present case. It is shown that the requirements of the scheme are satisfied and that the amount of glutamine newly synthesized in the leaves may have arisen from transformations to which the citric acid, the malic acid, the soluble carbohydrate, and possibly even a little of the protein, each furnished quotas. Amide formation in these circumstances is not a reaction in which a single specific precursor reacts with ammonia but is a complex process that may involve many components of the tissue. The actual immediate precursor of glutamine, α-ketoglutaric acid, need have only a transitory existence and may never be present in notable amounts.

From the point of view of the Krebs cycle, the synthesis of amides in plants is a by-product of carbohydrate respiration, and the velocity of the reaction and its efficiency in maintaining the concentration of ammonia at a low level may depend on considerations quite different from those envisaged by Prianischnikow's ammonia detoxication hypothesis. The presence of an appreciable concentration of ammonia is only one of the conditions that must be satisfied before amide synthesis can occur, and the new views therefore throw considerable light upon the problem presented by the wide difference between the concentrations of ammonia that occur in different species of plants. It would appear that the conditions that tend to bring about increases in the ammonia content of the leaves of such a plant as tobacco likewise stimulate the formation of the precursors of the amides, with the result that the ammonia concentration increases significantly only when the more readily respired components of the leaves approach exhaustion. The amide synthesizing mechanism thus appears to be highly efficient with respect to the removal of ammonia. In the rhubarb leaf on the other hand, the whole complex of conditions is not satisfied so readily nor is it dependent to the same extent upon the ammonia concentration. Thus amide synthesis may be delayed materially, as it was in one of the series of samples examined, until the necessary precursor of the glutamine was provided in adequate amounts by the respiratory process. Under such circumstances, the apparent efficiency of the amide synthesizing mechanism as a means to maintain a low level of ammonia in the tissues may suffer. These considerations thus lead to what is really a new and more generalized concept of the problem of amide metabolism in plants.

The respiration of rhubarb leaves in culture has been discussed in terms of the data for losses of carbon from the leaves. Calculations of the changes in the amount of carbon present in certain known components and in the groups of unknown components that can be classified in terms of solubility show the general nature of the substances that were drawn upon to provide energy by oxidative reactions. Although the interpretation assigned to the various changes contains much that is speculative, there is no doubt that respiration in the leaf blades involved substances other than carbohydrates very early in the culture period and the conclusion is drawn that a part of the protein of the blade was included in the reactions. It is suggested that the residues of the amino acids, after these had been oxidatively deaminized, were subsequently to an appreciable extent completely oxidized, presumably by mechanisms allied to those provided for the oxidation of fatty acids. In the petiole, respiration appears to have drawn mainly upon the carbohydrates and the malic acid, although other components to some extent shared the same fate.

Respiration in the leaves cultured in darkness in glucose solution was definitely more complex than would be supposed on the assumption that the glucose supplied to the leaves furnished all of the energy required. The digestion of the blade protein was if anything stimulated, and the evidence points strongly to the possibility that the amino acid residues contributed to the respiration in this case also. During a part of the culture period, however, synthetic reactions predominated over the carbon losses due to respiration, so that allocation to definite components is difficult.

The leaves cultured in light acquired carbon by photosynthesis to an extent that cannot be demonstrated from the data obtained. Here also, however, the possibility that the blade protein contributed to the respiration loss was clearly evident.

It is shown that Paech's hypothesis that the carbohydrate supply behaves as a regulator of protein decomposition is inadequate to account for the observations, particularly in the case of the leaves cultured in glucose solution in darkness. On the other hand, the highly generalized views of Gregory and Sen, which include an observed close correlation between respiration and protein content and provide for a chemical relationship between carbohydrates, organic acids, and proteins, and of these in turn with the respiration, throw considerable light upon the behavior of these groups of substances in rhubarb leaves under the various conditions of culture that have been studied.

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