Bulletin 399 August, 1937

Chemical Investigations of the Tobacco Plant
VI. Chemical Changes That Occur in Leaves During Culture in Light and in Darkness

HUBERT BRADFORD VICKERY, GEORGE W. PUCHER, ALFRED J. WAKEMAN and CHARLES S. LEAVENWORTH

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CONTENTS

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INTRODUCTION

In a previous study (93) of the chemical changes that occur in tobacco leaves during culture in water in the dark, evidence was obtained which rendered it highly probable that digestion of the leaf protein gave rise to amino acids. These were, in turn, deaminized, and the ammonia produced was employed for the synthesis of amides. Other sources of ammonia were, however, also drawn upon for this purpose, and it seemed probable that renewed study might give a clearer picture of the chemical changes that take place under these conditions. Furthermore no attempt was made in the previous investigation to study the effect of light upon the reactions that occur.

The experiments have accordingly been repeated and attention has been given, not only to the effect of light, but also to the possibility that on the one hand glucose, and on the other hand inorganic salts, including an ammonium salt as a source of nitrogen, might have an effect upon the chemical changes.

The results of these attempts to influence the behavior of the components of tobacco leaf tissue during culture are described in the following pages. As will become apparent, we were not successful in demonstrating specific effects of glucose as a component of the culture solution owing, probably, to the use of too low a concentration. The study of the effect of ammonia was successful, however, inasmuch as the amide metabolism was increased. Since the amide metabolism is closely associated with the protein metabolism, these experiments have been of some value.

In view of the significance of amide metabolism in this relationship, it may be useful to review the early literature of asparagine and glutamine, the two amides that have been found most widely distributed in plants, and to show how our present knowledge of these two substances and their relationship to the other nitrogenous constituents of plants has been ascertained.

ASPARAGINE

Asparagine, the mono-amide of aspartic acid, $CONH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$, crystallizes with unusual ease in the form of a mono-hydrate even from very complex mixtures of amino acids, and it is therefore not

Note: The chemical investigations of tobacco 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, New Haven, Conn. The Department has enjoyed the benefit of close coöperation from the Tobacco Substation at Windsor. The expenses were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

surprising that asparagine was the first substance belonging to the class of α-amino acids to be discovered in nature. It was first isolated in 1806 by Vauquelin and Robiquet (85) from juice expressed from asparagus shoots. The juice, after being evaporated, had been left for a few days and, during this period, deposited crystals. Some among these had, as they said, "a shape, transparency and taste" which were unfamiliar to the investigators and that enabled them readily to separate the new substance from the other constituents of the mixture. The crystals were shown to be those of a neutral nitrogenous substance which was neither an acid nor a salt in spite of its capacity to crystallize, and its exact chemical nature remained a puzzle for many years. It was again prepared by Dulong (19) in 1826, and in his paper is referred to as the substance Vauquelin and Robiquet had named "asparagine", although no name is mentioned in their earlier paper. Bacon (3), in 1826, prepared a substance from the roots of the marsh mallow which he called althèine, but the following year Plisson (50) repeated this work and identified althèine with asparagine. He likewise succeeded in isolating asparagine from the roots of the comfrey and licorice plants (6, 51). Plisson made the first attempt to ascertain the chemical nature of asparagine. He found that, after it had been boiled with lead hydroxide, it was converted into an acid which crystallized from hot water in plates, and named this new substance aspartic acid.

The first analyses were made by Plisson and Henry(52) in 1830 and by Pelouze (46) in 1833. Liebig (29) also analyzed it at this time, but his results did not agree with those of the other investigators and, in fact, correct values were not obtained by him until 1838 (30). These early workers were somewhat confused by the difference in chemical behavior of the two nitrogen atoms of asparagine, but Pelouze clearly recognized it as the amide of aspartic acid. Liebig questioned this at first, but later, as a result of his new and correct analysis, became convinced.

The significance of asparagine in plant physiology was scarcely discussed at this time. In 1844, however, a chance observation by Menici, a pharmacist of Pisa, led to a study of asparagine which at one stroke made many of the chemical and physiological relationships of this substance clear. Menici had had occasion to sprout vetch seeds in the dark and, from an extract of the etiolated seedlings, had prepared a small quantity of a crystalline compound that he took to Piria at the University of Pisa with a request for identification. Piria (48) suspected its nature at once and repeated Menici's experiment on a large scale. He showed that asparagine could be obtained in equally large quantities from vetch seedlings whether these were sprouted in light or in the dark; that none of the substance was present in the unsprouted seeds; and that it disappeared from the tissues of the plants as these approached flowering, none at all being found as the fruit ripened. Accordingly, he inferred that light has no influence on the formation of the substance, that it is produced from the protein of the seed, and is utilized by the plant in the later stages of growth.

Piria recognized that asparagine has acid properties inasmuch as a well-crystallized and insoluble copper salt could be prepared, and asparagine could be recovered unchanged from this compound. When a solution of asparagine was allowed to stand in a warm place, he noted

what he designated as a fermentation (today, we should say putrefaction), as a result of which asparagine was converted into the ammonium salt of succinic acid. When treated with nitrous acid, nitrogen and malic acid are produced from asparagine and this acid could be isolated as the crystalline lead salt. Piria, therefore, regarded asparagine as the diamide of malic acid analogous to oxamide, and pointed out that its properties have much in common with succinimide and butyramide. According to this view, aspartic acid would be the mono-amide of malic acid.

Although this conclusion was incorrect, Piria's demonstration of the chemical relationship of asparagine to succinic acid and to malic acid, and his inferences that it is produced from the seed protein and can later be utilized by the plant were destined to have a great influence on subsequent studies of this substance. Furthermore, the ease with which it could be prepared from vetch seedlings, and the relatively enormous quantities of it in these tissues not only stimulated investigation but presented physiological problems of the greatest importance.

The chemical relationship of asparagine to malic acid revealed by Piria, and the later synthesis of aspartic acid by Dessaignes (18), who obtained it by heating ammonium malate or fumarate, led Pasteur (45) to a study of the optical properties of these substances. He showed that asparagine is configurationally related to the natural *l*-malic acid of plants; that Dessaignes' synthetic substance was optically inactive; and that the diamide of malic acid is isomeric, but not identical with asparagine, inasmuch as malamide gives off all of its nitrogen as ammonia when hydrolyzed with alkali, whereas asparagine gives only one-half in this form. Kolbe (27), somewhat later, showed that aspartic acid is an amino acid derived from succinic acid, and that asparagine is its monoamide. The final step of synthesis of aspartic acid as a proof of chemical structure was made by Piutti (49) in 1887.

Meanwhile aspartic acid had been obtained from another natural source which served to increase its significance in plant physiology. Ritthausen (66) had prepared the proteins of various leguminous seeds and, in the course of a study of conglutin and legumin in 1868, had isolated a new amino acid from mother liquors from which glutamic acid had previously been crystallized. He did not recognize the new substance at first, but the following year (67), showed that it was aspartic acid, identical with that obtained by Plisson from asparagine, and homologous with his recently discovered glutamic acid (65).

$COOH\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$	$COOH\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$
Aspartic acid	Glutamic acid

The one yielded malic acid on being treated with nitrous acid; the other gave what he called glutanic acid, today usually referred to as α-hydroxyglutaric acid. Both the new amino acids were found among the products of hydrolysis of many proteins, whether of plant or of animal origin, and it was at once clear that they must play an important rôle in physiology.

A clearer explanation of the relationship of the asparagine of plant tissues to the aspartic acid of proteins was first suggested a few years later. Nasse (41) in 1872 observed that proteins, when subjected to hydrolysis, yield a considerable part of their nitrogen as ammonia. The quantity formed during alkaline hydrolysis invariably exceeds that re-

sulting from acid hydrolysis, but the rate at which it is given off is rapid at first and then slow. He inferred that there are two different ammonia-yielding groups in proteins both of which are decomposed by hot alkali, and he pointed out that the stability of the more easily decomposed group had analogies with the ammonia-yielding group of the acid amides. He referred to the ammonia that can be set free from proteins as "loosely bound nitrogen", and suggested that among other possibilities it may be present in the protein in the form of acid amide groups. In his discussion, he pointed out that it behaves like the nitrogen of asparagine.

This brilliant suggestion was followed up a year later by Hlasiwetz and Habermann (24) who prepared glutamic and aspartic acids from casein. They emphasized the probability that these two acids and the ammonia, which is always formed, have a common origin in the asparagine and glutamine which they felt must be the form in which the two acids are present in the protein. Although this was pure speculation, it has been supported by all subsequent investigations (44), and recently (16, 17) has been demonstrated to be the case by the isolation of asparagine from edestin and of glutamine from gliadin after enzymatic digestion of these proteins.

GLUTAMINE

The discovery of glutamic acid among the products of hydrolysis of proteins by Ritthausen in 1866, and the demonstration that, when treated with nitrous acid, it yields α-hydroxyglutaric acid, which was recognized as being allied to malic acid, suggested that a higher homologue of asparagine must exist in nature. Hlasiwetz and Habermann in 1873 referred specifically to a hypothetical glutamine which they thought must be the form in which glutamic acid is combined in the protein. So far as we have been able to discover, this is the earliest reference to the substance by name in the literature.

Schulze began his investigations of the chemical composition of plant tissues at Zürich in 1872 with the study of a formation of asparagine in lupine seedlings along the lines already suggested by the early work of Piria. In 1875 he turned his attention to the composition of the roots of the beet (78). Extracts of this tissue were found to contain considerable nitrogen in a form that would dialyze through parchment, but very little protein was present. A considerable part of the dialyzable nitrogen could be readily liberated as ammonia by hydrolysis with dilute acid. This was an indication of the presence of asparagine, but, on concentration and long standing, no asparagine could be induced to crystallize. If asparagine were added to the solution, there was no difficulty in recovering it almost quantitatively by crystallization, and Schulze concluded that some amide must be present which did not possess the remarkable capacity for crystallization of asparagine.

In order to see if the free acid had properties that would make it easier to isolate, Schulze subjected an extract of beets to hydrolysis with dilute hydrochloric acid and removed the reagent with lead acetate. An excess of lead acetate was then added and, since no precipitation occurred, the solution was treated with alcohol in considerable volume. A flocculent precipitate of a lead salt separated, and this was collected, decomposed with hydrogen sulfide and the free acid was crystallized. Schulze's great technical ability is well shown by the way in which he handled the crystal

fractions obtained. Glutamic acid is dimorphic, separating sometimes in rhombic prisms and sometimes in thin, lustrous plates. Furthermore it yields a copper salt which under certain conditions separates as a blue, insoluble powder, under others as deep blue, transparent prisms. To make the situation even more difficult, there was a small amount of aspartic acid present. In spite of the confusing properties of the main constituent, Schulze showed that the different crops of crystals, whether prisms or plates, consisted of glutamic acid from which he prepared the copper and barium salts and the hydrochloride, and identified his material with Ritthausen's glutamic acid, notwithstanding the fact that the melting point given by Ritthausen was much lower than that he himself observed.

To make certain that he was not dealing with an isomeric substance, he converted his specimen into α-hydroxyglutaric acid with nitrous acid, and reduced this to glutaric acid which was found to be identical with the substance prepared from Ritthausen's glutamic acid by Markownikoff (32).

Schulze concluded from these results that the original beet extract must have contained glutamine, the amide of glutamic acid, homologous with asparagine. No glutamic acid could be obtained from such an extract unless it were hydrolyzed, and hydrolysis produced both ammonia and glutamic acid.

Schulze and his collaborators continued their investigations of the amides of plant extracts for many years, and in 1882 (73) announced the discovery that asparagine can be precipitated by means of mercuric nitrate, and also that he intended to attempt the isolation of glutamine with the aid of this reagent. The following year Schulze and Bosshard (76) described their experiments. The method they developed is extremely simple and, save in matters of improvement in technical detail and the use of modern apparatus (89), is still the only method by which this extremely rare substance has been obtained from plants. Although a synthesis has been described recently (4), the substance is far more readily prepared in quantity from natural sources.

Schulze and Bosshard first treated the juice of beet roots with basic lead acetate and added neutralized mercuric nitrate solution to the filtrate. The white precipitate was collected, decomposed with hydrogen sulfide, the solution was neutralized with ammonia, and concentrated on a water bath at 40 to 50° until crystallization occurred. The substance, after being purified by recrystallization, was shown to be the mono-amide of glutamic acid. Schulze's genius as an experimenter is again revealed by this apparently simple procedure. Glutamine is an unstable substance, being rapidly hydrolyzed in acid or alkaline solution in the cold, or in neutral aqueous solution at boiling temperature. None would have been obtained had Schulze not recognized this property and had the patience to wait for the extremely slow concentration. The yields obtained were low, from 20 to 25 percent of the amount actually present as determined by the amide nitrogen; but even with modern technic, such as vacuum distillation at low temperature to evaporate the solution, yields of only 80 percent are ordinarily obtained.

Morever, the selection of ammonia to neutralize the solution previous to evaporation is essential. Crystals of glutamine will not separate if this neutralization is effected with sodium, lithium or magnesium hydroxide. Glutamine does not form a hydrate and is entirely unlike asparagine in its capacity to crystallize from complex mixtures.

During the following years Schulze and his students detected glutamine in the tissues of many plants (74) and were soon able to generalize with the statement that this substance replaces asparagine in the metabolism of many species. Throughout the literature even to the present, these two amides are regarded as equivalent to each other in metabolism, and it is one of the purposes of this bulletin to show that this equivalence has certain limitations.

AMIDE METABOLISM IN PLANTS

The physiological significance of the amides was the theme which motivated much of Schulze's work. In 1872, the distinguished botanist Pfeffer (47) had published an enormous work in which he recorded the examination of the tissues of many species under the microscope after the sections had been treated with alcohol. Under these conditions, if asparagine is present in notable amounts, the characteristic crystals can be seen. Study of the distribution of asparagine in seedlings and of the relationship to the protein convinced Pfeffer that, during germination, the protein is converted into asparagine, and that this substance serves as the chief means whereby nitrogen is translocated out of the cotyledon through cell membranes to the growing tissue in the axial organs. Here the amide combines with carbohydrate for the construction of new protein in the tissue.

It must be remembered that this view was propounded by a botanist only a few years after aspartic acid had been demonstrated to be a component of proteins, and long before any clear conception of the structure and composition of proteins had been reached. It was this hypothesis, published the same year that Schulze took up his duties at Zürich, that stimulated him to begin the labors devoted to the explanation of the function of the amides. But even as early as 1878, Schulze (72) had begun to have doubts that the transformation of protein to asparagine was as simple as Pfeffer had supposed.

As nearly as he could tell, the reactions that take place during germination of the seed consist chiefly of protein decomposition to yield products similar to those produced by acid hydrolysis. These are then used for protein regeneration in the growing tissue but in unequal proportions so that unused parts accumulate. Hence asparagine is found in large amounts in lupine seedlings. In this particular case, however, the storage of asparagine is so phenominal—up to 80 percent of the converted protein nitrogen—that he could only assume that a part of the protein decomposition products undergo further chemical changes whereby asparagine is produced. Thus, within a few years, Schulze had already recognized that asparagine is by no means the only translocatory substance for nitrogen.

It is hardly worth while to discuss in detail the vast amount of careful investigation that Schulze carried out during the following years. By 1898 (75), however, he was in a position to state clearly his views of the series of events that takes place during germination of seeds. The protein undergoes rapid decomposition, the path followed being not unlike that brought about by the action of acids, and the final products are amino acids. He felt that it is highly probable that a certain amount of glutamine and of asparagine arises directly from the protein—and in this he was probably correct. The greater part of the amino acids, however, undergo further decomposition with the production of ammonia, which is employed for

the formation of asparagine and glutamine, the purpose being to convert substances which are not suitable for protein regeneration into substances that are available for this.

The amides are therefore secondary products of protein decomposition, and are formed from ammonia and nitrogen-free organic substances. As evidence of the conversions, Schulze pointed out that considerably more protein decomposition products of the amino acid type could be isolated from seedlings during the early stages of germination than during the later, whereas asparagine steadily accumulated in the later phases.

Schulze obtained a vast store of information on the changes that occur during the germination of seeds, but he was necessarily somewhat vague with respect to the chemical mechanisms whereby these changes are brought about. This aspect of the problem was dealt with more fully by the Russian investigator Prianischnikow. In 1895, Prianischnikow (53) carried out a careful series of analyses of vetch seedlings of different ages in Schulze's laboratory. He showed that the protein decomposition products are chiefly of the amino acid type, but that asparagine is a by-product that accumulates in the axial organs. Later (54), he showed that the time at which maximal protein decomposition occurs in etiolated seedlings corresponds with that at which maximal asparagine synthesis takes place, and pointed out the analogy to urea formation in the animal—an idea suggested many years before by Boussingault (9). He was very clear, however, on the point that asparagine is not an excretory substance like urea, but serves as a storehouse for nitrogen which can later be used by the plant.

The evidence secured in part by Schulze, in part by himself, indicated that the quantity of asparagine formed in plants is far in excess of the amount that could possibly arise directly by protein hydrolysis. In the later stages of etiolation, the chief final product of the protein that disappears is asparagine and, furthermore, the distribution of the asparagine in the seedling—mainly in the growing parts, very little in the cotyledon—is not what would be expected if the protein of the cotyledon were directly decomposed to asparagine. Some data of Prianischnikow (55) will illustrate the kind of phenomena observed, the figures given being expressed in percent of the total nitrogen.

	ASPARAGINE NITROGEN (percent of total nitrogen)		
	Cotyledons	Stems	Roots
Vicia sativa seedlings	14.5	38.2	
" " "	13.0	39.2	
Pisum sativum "	10.2	33.7	
Vicia faba "	11.7	39.3	43.5

That asparagine arises by a definite synthetic reaction was made strikingly clear by some experiments of Butkewitsch (12), another former pupil of Schulze. When seedlings are anesthetized by means of toluene vapor, synthetic reactions are suspended, but decomposition reactions continue. Under these circumstances, amides do not accumulate; instead there is a rapid accumulation of ammonia. The accumulation of ammonia was largely prevented, however, if the experiment was conducted in the absence of oxygen.

	LUPINE SEEDLINGS (percent of total nitrogen)	
	Untreated	3 days in toluene vapor
Soluble N	32.1	34.5
Amide N	6.1	2.6
Ammonia N	0.9	8.3

These observations provided proof that asparagine synthesis is dependent upon the presence of ammonia which is produced by an oxidative process, and verified the inferences drawn by Schulze from his early work. The next step, therefore, was to see if plants can take up ammonia from the culture solution and convert it into asparagine. Suzuki (83), in 1897, had demonstrated that asparagine accumulates in plants when these are removed from the soil and placed in nutrient solutions that contain ammonium salts. There is very little accumulation of ammonia but, especially if sugar is added to the culture solution, enrichment in asparagine occurs.

Prianischnikow (58) repeated Suzuki's experiments under more easily controlled conditions. Barley was sprouted for 14 days in water and in 0.1 percent ammonium chloride, and the results of the analysis of the tissues were calculated in terms of milligrams per 100 plants.

	BARLEY (mg. in 100 plants)			
	Total N	Protein N	Asparagine N	Ammonia N
Water	145.8	61.8	36.8	0.55
NH_4Cl	161.5	61.5	56.4	0.89

The preliminary experiment with peas failed, but when calcium carbonate or sulfate was added to the ammonium chloride culture solution, the following results were obtained. The figures are the increases in nitrogen over the control plants cultured in water and expressed in milligrams per 100 plants.

	PEAS (mg. in 100 plants)		
	NH_4Cl	NH_4Cl + $CaCO_3$	NH_4Cl + $CaSO_4$
Increase N absorbed	44.8	93.9	195.7
Increase asparagine N	25.0	116.	182.

The important point to note is that the increase in total nitrogen is **closely paralleled by the increase in total asparagine nitrogen.** This means that the ammonia from the culture solution provided the source of both the amino and the amide groups, and consequently we are not dealing with a synthesis of asparagine from aspartic acid and ammonia, but from some non-nitrogenous substance, for example, as he suggests, malic acid. Disregarding his suggested origin of the asparagine, this statement is of the greatest importance. For the first time, asparagine synthesis was experimentally clearly dissociated from a direct relationship to protein metabolism. Aspartic acid plays no essential part in the picture—the amide arises *de novo* from ammonia and a non-nitrogenous precursor.

Precisely similar results have been obtained in this laboratory (90) with respect to glutamine synthesis in the beet. In order to obtain samples of this tissue of high glutamine content for purposes of preparation on the large scale, a plot of beets was heavily watered with a molar solution of ammonium sulfate applied at the rate of a ton of nitrogen per acre—four or five times as much as would be used in ordinary agricultural practice. The effect upon glutamine synthesis was striking.

	BEET ROOTS (gm. per kilo of fresh tissue)		
	Control	3 days	9 days
Soluble N	1.96	2.10	2.16
Glutamine N	0.37	0.54	0.58
Increase soluble N		0.15	0.21
Increase glutamine N		0.16	0.21

The increase in soluble nitrogen and the increase in total glutamine nitrogen agree quantitatively, and it is clear that, under these conditions, the synthesis of glutamine consists in the combination of ammonia with a non-nitrogenous precursor. Two types of reaction must occur—the synthesis of an α-amino group, and the dehydration of an ammonium salt to form the amide. The metabolism of glutamine in the beet, when extraneous ammonia is supplied, is therefore strictly analogous to that of asparagine in pea seedlings—the amide metabolism under certain definite conditions can be entirely dissociated from the protein metabolism.

Prianischnikow was intensely interested in accounting for this phenomenon. Butkewitsch had shown that plant enzymes decompose proteins to amino acids in the same way as animal enzymes, and also that, when synthetic reactions are suspended by means of anesthetics, ammonia accumulates. As early as 1910, Prianischnikow inferred that both animals and plants avoid the harmful effects of ammonia in the cells by a dehydration process—namely the synthesis of urea on the one hand and of an amide on the other. Twelve years later, in 1922 (56), he was in a position to generalize more fully. The facts were as follows:

Asparagine accumulates in certain seedlings when these are cultured on ammonium salts in the dark, and ammonia does not. The seedlings assimilate nitrogen from the solution as the amide. With seedlings of other species, this assimilation does not occur; instead ammonia accumulates. Now the seedlings which do assimilate nitrogen as amides are those which possess a considerable store of carbohydrate. Those which do not synthesize asparagine are characterized by very small stores of carbohydrate. The issue is a little confused by the fact that certain plants, in particular legumes, also seem to require calcium, or at any rate the presence of a reagent ($CaCO_3$) in the culture solution that will preserve neutrality. The lupines, however, are organisms which, even when provided with calcium, fail entirely to synthesize asparagine but become enriched in ammonia. These seeds are very low in carbohydrates.

LUPINES IN DARK
(mg. in 100 plants)

	H_2O	$(NH_4)_2SO_4$	$(NH_4)_2SO_4 + CaCO_3$
Total N	567	575	535
Protein N	152	160	170
Asparagine N	258	175	158
Ammonia N	26	57	68

Three types of experiment can readily be suggested to demonstrate the essential nature of the carbohydrate. In the first place, will a seed which contains carbohydrate behave like the lupines if the cotyledons which contain the carbohydrate stores are cut off and removed? This was tried on barley seedlings, and the data showed clearly that asparagine synthesis was prevented while ammonia accumulated in unusual amounts.

BARLEY WITH COTYLEDONS REMOVED
(mg. in 100 plants)

	H_2O	NH_4Cl	$NH_4Cl + CaCO_3$
Total N	163	202	242
Protein N	81	95	87
Asparagine N	45	57	37
Ammonia N	4	41	73

Secondly, how will lupines behave if given an opportunity to supply themselves with carbohydrate by means of photosynthesis? This was test-

ed by allowing lupine seeds to sprout in light on ammonium salts solutions; the data show that assimilation of asparagine was pronounced and that little ammonia accumulated.

	LUPINES IN LIGHT (mg. in 100 plants)		
	H_2O	NH_4Cl	$NH_4Cl + CaCO_3$
Total N	885	968	978
Protein N	617	521	617
Asparagine N	151	291	227
Ammonia N	20	35	24

Finally, how will lupines behave in dark culture on ammonium salts if glucose is also added to the nutrient solution? The experimental data for the solution of this problem were much more difficult to obtain. Smirnow (80), working in Prianischnikow's laboratory, developed the technic—a matter of conducting the cultures under sterile conditions—from previous work of Petrow. The answer was perfectly clear; the quantity of asparagine was much increased over the controls when glucose was added, and at the same time the ammonia was diminished. Furthermore, protein synthesis was stimulated.

	LUPINES IN DARK (mg. in 100 plants)	
	$(NH_4)_2SO_4 + CaSO_4$	$(NH_4)_2SO_4 + CaSO_4$ + glucose
Total N	1002	1249
Protein N	216	380
Asparagine N	490	619
Ammonia N	123	82

The results of these experiments were summarized in a table which shows the effects of the presence of carbohydrate on the synthesis of amides in seedling plants in the presence of extraneous ammonia.

CONDITIONS		RESULTS	
Carbohydrate	Light	Amide synthesis	Ammonia enrichment
+	−	+	−
−	−	−	+
+	+	+	−
−	+	−	+

This generalization appears to be of far reaching application in plant physiology, and Prianischnikow pointed out that the analogy with urea synthesis in the animal is very close. Asparagine and urea alike are to be regarded as detoxified forms of ammonia. The great difference is that the animal, which does not need to be economical of nitrogen, excretes the urea, whereas the plant, which must carefully conserve all of its nitrogen, converts it into a product from which it can be again called into action should need arise. Another difference is that an animal merely requires carbon dioxide to detoxify ammonia, and consequently can continue to eliminate urea even under conditions of starvation. But the plant requires a precursor with at least two carbon atoms still in the reduced form, and so, in starvation, may make use of its stores of asparagine for respiration with the result that ammonia accumulates and a vicious circle is established.

To sum up the facts hitherto presented, it is clear that amide metabolism is usually closely associated with protein metabolism, but that the relation is indirect, the bridge between the two being ammonia. The other

factor involved is the carbohydrate, this being, so far as can be established at present, the source of the carbon chain which ultimately is converted into asparagine or glutamine. It is upon this point that the experiments described in the present bulletin bear.

The great Russian plant physiologist and musician Borodin (8) was the first to demonstrate that mature leaves placed with their petioles in water become enriched in asparagine. This aspect of amide metabolism has not been studied very extensively until recent years. Chibnall (15), in 1924, reviewed the early work of Schulze and others, and demonstrated that runner bean leaves, on being "starved" in water culture for several days, become greatly enriched in asparagine at the expense of the protein. Mothes (37) has also studied the amide metabolism of mature leaves. He found in 1925 that there is normally little or no amide synthesis at the cost of protein save when there is a deficiency of carbohydrate; that is, protein is ordinarily "spared" in the presence of excess of carbohydrate. On complete starvation of carbohydrate, ammonia may accumulate. The digestion of protein is a reaction which takes place independently of the carbohydrate content of the tissue, but amide formation is a secondary reaction connected with oxidation of the protein decomposition products, and with the detoxication of the ammonia produced. Protein regeneration may occur in young leaves in the presence of carbohydrate at the expense of ammonia or asparagine, but this capacity is gradually lost with increasing age. Older leaves show protein breakdown and amide synthesis up to the time of yellowing which is a condition that corresponds to a minimal protein content.

Mothes (39) has also studied the metabolism of the leaves of conifers in order to obtain a check by modern methods on Suzuki's contention that arginine is synthesized in these plants in response to enrichment in ammonia. He found that the soluble basic nitrogen of conifer seedlings is indeed unusually high, but obtained support for the view that it originates entirely from the protein by normal hydrolytic processes. Conifer seedlings possess an amide metabolism of the normal type inasmuch as amide nitrogen is promptly formed when ammonia of extraneous origin becomes available.

Our own earlier studies of the amide metabolism of tobacco leaves have been briefly mentioned in the introduction. The following pages describe experiments in which the leaves of this plant have been cultured in water, in dilute glucose, and in dilute nutrient salt solution both in light and in darkness. The data are given in considerable detail, not only with respect to the problem of amide metabolism, but also with respect to other chemical changes that occurred in the tissues under the experimental conditions. Sufficient discussion is introduced to illustrate the bearing of these experiments on the general physiological problems raised.

PREPARATION OF MATERIAL

The leaves used in the water culture experiment consisted of the lowest three picked from plants grown under shade at the Tobacco Substation in Windsor, Conn., on July 17, 1934. Picking was completed by 9:30 A. M. and the leaves, carefully stacked in piles, were transported to the New Haven laboratory. Samples of 60 leaves each were selected at random, with care to exclude any leaves that had suffered injury and any of unusual size. The fresh weight of each sample was recorded to

the nearest gram, and the leaves designed for culture in the light were supported in V-shaped troughs by means of thin board extensions of the trough walls of a size sufficient to accommodate nearly the entire length of the leaf. The bases of the leaves were held securely in the water in the bottom of the troughs by glass rods laid on top of them after all were in place. The troughs held 20 leaves each when the leaves were placed alternately lying on the right or left sides, and, by careful arrangement, overlapping of the leaf surfaces was reduced to a negligible minimum. Owing to the movements of the leaves as their turgidity changed during culture, the leaf surfaces were prevented from coming into close contact with each other, and ample circulation of air over the upper surfaces and most of the lower surfaces was provided.

The culture troughs were set up in a greenhouse, the glass of which had been heavily whitewashed, and a continuous record of the temperature and humidity was taken. The temperature varied from approximately 18° from midnight to sunrise, and increased to 32 or 33° at noon and during the early afternoon. The humidity was relatively constant at 90 percent except during the hottest part of the day when it temporarily dropped to 50 percent or less. Artificial light from an adequate number of 500-watt Mazda bulbs was provided between sunset and sunrise.

The distilled water in the troughs was maintained at a level which insured that the bases of all the leaves should be immersed. It was changed every two days and a careful inspection of all leaves was made for evidence of infection at the base. When necessary, a thin layer of tissue was scraped from the base.

The 60-leaf samples designed for culture in the dark were distributed in pails in such a manner that the bases were immersed in a shallow layer of water and the surfaces were as free from contact with each other as possible. Although the leaves were somewhat crowded, there was sufficient room for circulation of air. The cultures were set up in a dark room and were exposed to a dim red light, for only a few minutes daily, when inspections were made. The water was changed every two days and at the same time the bases were examined for infections and scraped, if necessary. The temperature of the dark room was constant at $25 \pm 1°$ and the humidity remained uniform at about 71 percent. Ample ventilation was provided.

At intervals of approximately 24 or 48 hours, a sample both from the light and from the corresponding dark culture series was removed for preparation for analysis. Ten leaves were selected in such a manner as to form a subsample which represented the condition of the entire set as closely as possible. The weights of the 10-leaf subsample and of the remaining 50 leaves were ascertained, and the 50-leaf sample was placed in a drying oven which provided a rapid circulation of air maintained at 85°; drying was complete within two hours. The dried leaves were then weighed ("crude dry weight"), crushed by hand and bottled, and were subsequently ground to a fine powder for analysis.

The 10-leaf subsample was cut with scissors into strips immediately after being weighed, and the tissue was immersed in ether for 30 minutes. The ether was strained off through a small piece of cotton gauze which was then folded around the leaf tissue, and the whole was enveloped in stiff canvas press cloth and pressed between steel plates in the hydraulic press. The residue was washed twice with water, being pressed thoroughly each time, and was then dried. The ether was washed twice with water,

the washings being combined with the extract from the press, and the extracts, wash fluids, and rinsings of the apparatus were combined and made to one liter. The preparation of this extract occupied approximately two hours, and samples of it were immediately analyzed for ammonia and amino nitrogen in order to provide data to serve as a control on the ammonia and amino nitrogen determinations subsequently made on the dried whole leaf tissue.

The leaves used in the glucose culture experiment were picked July 24, 1934, from plants in the same rows in the field that provided the leaves of the first experiment. The leaves taken were the fourth, fifth, and sixth, counting from the bottom of the plant, the lower leaves having been removed the previous week. Collection was complete by 9 A. M., and the cultures were set up three hours later. The culture solution in which the leaves were placed was a 0.02 M (0.36 percent) solution of glucose in distilled water. The management of the cultures and the preparation of the samples for analysis were carried out as already described.

The leaves for the ammonia culture experiment were picked July 31 from the same lot of plants, the seventh, eighth, and ninth leaves being taken. The lower leaves had been previously removed. Collection of the material was completed by 9.30 A. M. and the cultures were set up within three hours.

The culture solution employed was modified from one of Tottingham's solutions by the substitution of calcium chloride for calcium nitrate, and by doubling the concentration of ammonium sulfate. The composition was KH_2PO_4 0.143, $CaCl_2$ 0.233, $MgSO_4 .7H_2O$ 0.875, and $(NH_4)_2SO_4$ 0.555 gm. per liter. A sufficient volume was prepared for the whole experiment and was then treated with enough N potassium hydroxide solution to bring the reaction from pH 6.28 to pH 6.4. The resulting mixture was designed to have an osmotic pressure not widely different from 0.5 atmos.

The hydrogen ion activity of the culture solutions was examined from time to time. The solutions used in the cultures in the light tended to become slightly more acid; thus after 24 hours the reactions in two cases were pH 5.54 and 5.99; after 48 hours such values as 5.34, 5.64, and 5.87 were obtained. The cultures in the dark, on the other hand, changed but little in the early stages, the 24- and 48-hour values being pH 6.28 and 6.52. The last sample cultured in the dark became slightly more alkaline, pH 6.87, after being used for 48 hours, but in this case the main veins of the leaves had become infected.

No evidence of absorption of ammonia from the culture solution was obtained from analyses of the solution before and after use. The nitrogen content of the original solution was calculated to be 0.1176 gm. per liter. Determinations before and after 24 hours of use gave 0.117 and 0.118 gm. per liter. Nevertheless, as will appear, the analyses of the leaves showed that a small amount of nitrogen was absorbed.

DESIGNATION OF SAMPLES

A method of labeling the samples was adopted which served as a guide to the treatment each had received. The three sets cultured in water, glucose, or ammonia solution were designated by the key letters W, G, or N, which appeared on each label. The two conditions of culture, in light and in dark, were referred to by the letters L and D. The successive samples of leaves cultured in light were numbered from 1 to 7,

while the successive samples cultured in the dark were lettered from A to E or F. Thus sample LW1 was the first sample removed after culture in water in the light, sample DNB was the second sample removed after culture in ammonia solution in the dark.

The control samples of fresh leaf material were taken in duplicate for each experiment, and were designated FLa and b. With the addition of the key letter for the culture solution used, FLWa and FLNb are the designations of one of the fresh leaf samples used as a control for culture in water, and for a sample of fresh leaf material used as a control for the culture in the ammonia-containing salt solution. These designations apply to all of the dried leaf samples which consisted in each case of 50 leaves. The extracts prepared from the ten-leaf subsamples were designated according to the same system with the addition of the word "extract".

METHODS OF ANALYSIS

The analytical methods employed in this laboratory have been described in considerable detail in the previous bulletin (92) of this series, and in various published papers. Continuous development of the technics is, however, being made in the interests of convenience or accuracy, and slight changes are introduced from time to time. A number of difficulties were encountered in the present work, particularly in the determination of the organic solids and the ash, and the data obtained by the methods outlined in Bulletin 374 (92) are not entirely satisfactory. Study is being given to these points, however, and it is hoped that improvements can be devised.

In order to indicate as briefly as possible the methods employed, it is necessary to recall that two portions of each sample of leaves were available for analysis, the fresh leaf extract prepared from a 10-leaf subsample and the air-dry powder prepared from the remaining 50 leaves. The fresh leaf extract was analyzed for ammonia and amino nitrogen immediately after preparation and was then discarded. The dry leaf samples were analyzed later.

The determinations of water-soluble components were made on a water extract prepared from the dried leaf tissue. Careful study of the conditions under which accurate and reproducible values can be obtained has indicated that the following convenient and rapid method gives results indistinguishable from those obtained by more thorough methods of extraction.

The *dry tissue extract* is prepared by weighing 2.500 gm. of air-dry, carefully mixed, powdered leaf tissue and transferring this to a beaker in which it is mixed to a paste with a small volume of water; water to make approximately 85 ml. is then added. The beaker is heated for 10 minutes in a water bath at 80° with continuous stirring, and the contents are washed into a heavy-wall test tube graduated at 100 ml., cooled, and made to volume, and are thoroughly mixed by being shaken. The tube is then centrifuged and the clear extract is poured off through a small plug of glass wool in a dry funnel into a stoppered container in which it is preserved, if necessary, with a layer of toluene. Extracts prepared in this way have given analytical results which agreed within the limits of precision of the methods with results obtained on solutions prepared by making exhaustive extractions of the tissue.

For most purposes, an extract of 2.500 gm. of tissue made to 100 ml. volume gives a solution suitable for aliquoting for determinations of ammonia, amides and many other factors. In some cases 5 gm. of tissue are taken, in others, 1 gm.

An *alcohol extract* of the tissue is prepared for certain of the determinations, particularly the carbohydrates, by extracting 2.5 gm. or 5 gm. of dry tissue placed in a paper extraction thimble in a continuous extraction apparatus of the syphoning type in which it is extracted with 75 percent alcohol, usually overnight. The extract is then concentrated *in vacuo* to remove alcohol and is made to 100 ml. with water. The carbohydrates were determined essentially as described in Bulletin 374 (92, p. 569) save that the titration was carried out according to Schaffer and Somogyi (69).

The tissue residue from the alcohol extraction is employed for determinations of the protein. For this purpose, the dry weight is obtained in order to secure a factor for the conversion of analytical data to the basis of the dry weight of the original tissue. A weighed portion is then extracted with boiling water, is filtered on a Gooch crucible and thoroughly washed, and is transferred to a Kjeldahl flask for determination of the total nitrogen. For the determination of the amino nitrogen after acid hydrolysis, duplicate 0.250-gm. portions are extracted with hot water and are then boiled with 10 ml. of 10 N sulfuric acid under reflux for 24 hours. The hydrolysates are diluted to 50 ml., centrifuged, and the humin is washed on a Gooch crucible and nitrogen is determined in it. The two solutions are combined and aliquots are taken for the determination of amide nitrogen, the residue from the distillation being acidified with acetic acid and employed for the amino nitrogen determination.

The *organic acid fraction*, which is also employed for the determination of nitrate nitrogen, is prepared as described by Pucher, Vickery and Wakeman (62) and is employed for the determination of citric, malic, and oxalic acids and of the total organic acids (63, 60, 64).

The statement in Table 1 will make clear the analytical procedure for the individual determinations not already mentioned.

TABLE 1. ANALYTICAL METHODS

Solids	Dry tissue, 1.0 gm.	(92, p. 565)
Ash	Residue from solids	(92, p. 565)
Soluble solids	Dry tissue extract, 10 ml.	(92, p. 569)
Soluble ash	Residue from solids	(92, p. 569)
Total N	Dry tissue, 0.3 to 0.5 gm.	Kjeldahl
Water-soluble N	Dry tissue extract, 10 ml.	Kjeldahl
Alcohol-insoluble N	Residue from alcohol extract, 0.100 gm.	Semi-micro Kjeldahl
Protein N	Residue from alcohol extract after hot water extraction, 0.100 gm.	Semi-micro Kjeldahl
Nitrate N	Organic acid fraction, 10 ml.	(62)
Nicotine N	Dry tissue, 0.5 to 1.0 gm.	(2)
Ammonia N	Dry tissue, 0.5 gm.	(61)
Ammonia N	Fresh leaf extract, 5 to 25 ml.	(61)
pH 7.0 Ammonia N	Dry tissue extract, 5 ml.	(91)
Amide N	Dry tissue extract, 5 ml.	(61)
"Extra ammonia N"	Dry tissue extract, 5 ml.	Special method, p. 811
Amino N	Dry tissue extract, 10 ml.	(92, p. 567)
Amino N	**Fresh leaf extract, 5 to 25 ml.**	**(92, p 567)**
Peptide N	Dry tissue extract, 5 ml.	(92, p. 567)

ORGANIZATION OF THE DATA

In order to calculate the analytical data to a basis of grams of the constituent in 1 kilo of original fresh leaf, it is more economical of time to employ factors to convert the raw data for each sample to the desired basis. The analyses of the fresh leaf extracts, expressed in grams per liter of solution, represented 10 leaves selected from 60. These, in turn, had been subjected to culture, and their weight, as removed from the culture solution, differed, in general, from the original weight of the sample before culture, owing to change in the water content. Accordingly, the extract analysis was multiplied by a factor calculated from the weight of the leaves as removed from the culture (RL), the weight of the leaves selected for extraction (EL), and the fresh weight of the original 60 leaves before culture (CL). This factor was calculated as follows:

$$\text{Extract analysis} \times \frac{RL \times 1000}{EL \times CL} = \text{grams per kilo of fresh leaf.}$$

Similarly the analyses of the dry leaf samples were calculated by converting the crude dry weight of the individual samples into the crude dry weight per kilo of original fresh leaf as follows:

$$\text{Crude dry weight} \times \frac{RL \times 1000}{DL \times CL} = \text{crude dry weight per kilo of fresh leaf,}$$

where DL is the fresh weight of the 50 leaves that were dried. This factor was then used to convert the quantity of each analytical constituent contained in one gram of each individual sample of dried tissue into the number of grams per kilo of original fresh leaf.

The factors were calculated for each sample and were tabulated as logarithms. It was then a simple matter to express each set of analytical data on the common basis chosen by setting up a table which gave the fundamental data for the set of samples, and the analytical data, both in logarithmic form. When prepared in this way, checking by another calculator was reduced to an easy routine.

Some use was also made of two additional factors, namely, one to convert the weight of leaves removed from culture solution to grams per kilo of fresh leaf, and one to convert the number of leaves cultured (fifty) to the number of leaves in 1 kilo of original fresh leaf.

GENERAL BEHAVIOR DURING CULTURE

The general behavior of the leaves cultured in light in water and in glucose was similar and is shown in some detail in Table 2. They became very turgid and stiff, and remained apparently normal for 72 hours. Yellowing then began at the tips and margins and slowly extended downward and inward; a few leaves began to pass into the brown stage after eight days. The leaves cultured in nutrient salt solution in the light, on the other hand, did not become turgid, but wilted somewhat and showed a tendency to curl forward. Chlorophyll breakdown was, however, much delayed in this series and, even after ten days, the yellowing was not pronounced save in leaves the midribs of which had become infected.

The leaves cultured in the dark behaved quite differently from those in the light with respect to the regions in which chlorophyll breakdown first occurred. The yellow coloration began at the tips and near the main veins and extended outward from these areas. The set cultured in salt solution did not become flaccid, although the turgidity was not so great

as that of the leaves in glucose or in water. It was to be expected that bacterial infection should become serious after a few days of culture in salt solution, and this factor probably places some restriction on the value of the analytical data obtained in the later stages of such experiments. Nevertheless, in this type of experimentation we are interested chiefly in the behavior of the cell constituents themselves, and structural damage to the main channels of communication with the stem is of little significance. Accordingly the failure to maintain sterile conditions during the later periods of culture has no great importance for the conclusions we wish to draw.

TABLE 2. BEHAVIOR OF LEAVES IN CULTURE

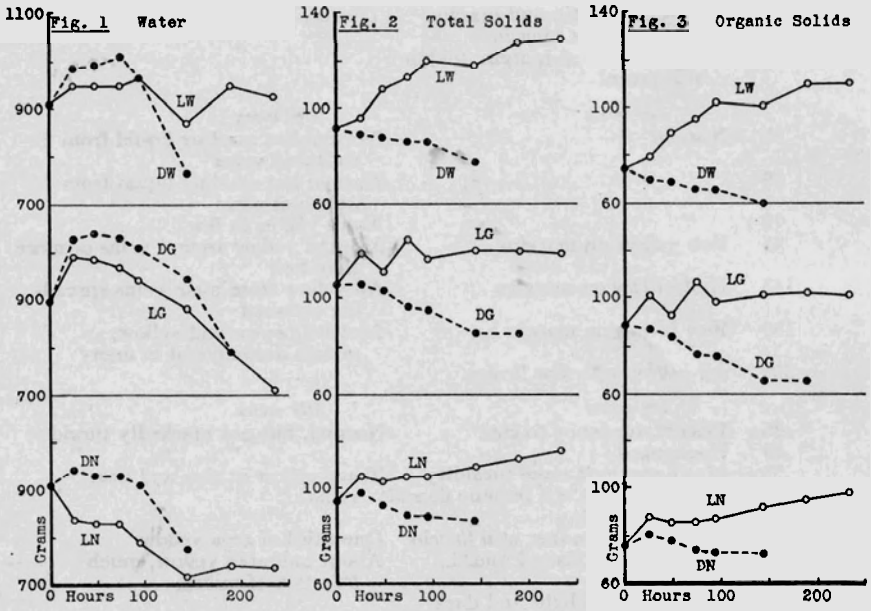
Hours	LW series	DW series
25	Normal, turgid	Normal, turgid
49	" "	" "
73	Trace yellow in few	Nearly all yellow at margin and near veins
95	" " " "	Yellowing more extensive
143	Few yellow at tip and margin	Tips brown, rest yellow
190	Many yellow at margin	
235	Mostly yellow at margin, middle still green	
	LG series	**DG series**
25	Normal	Normal but exuding liquid from ruptured veins
49		Normal but exuding liquid from ruptured veins
73	"	Trace yellow in few
95	Few yellow on margins	Marked yellow around veins of more than half
143	Half yellow on margins	All yellow from main veins spreading outward
190	Few brown on margin	Mottled brown and yellow, midrib decomposed in many
235	All yellow with few brown	
	LN series	**DN series**
25	Flaccid, tendency to curl	Normal, but not markedly turgid
49	Unchanged	" " " " "
73	Many regained some turgidity during night but became flaccid during hot day	Trace yellow at tips and near veins
95	Trace yellow in few, still flaccid	One-third of area yellow
143	Same, many infected midribs	About half area yellow, much infection of midrib
190	Many mottled light and dark green, more infection	
235	5 discarded due to infection, about one-third of the rest show definite yellowing at tip and margins, remainder still green	

The general behavior of the tissues in the three experiments was in most respects so similar that for many purposes the data may be regarded as those of a single experiment on water-cultured, excised leaves done in triplicate on leaves of slightly different ages and position on the plant. Specific effects of the glucose or of the ammonium salt are, save in a few instances, difficult to distinguish with certainty.

It is, of course, recognized that comparisons between leaves picked a week apart from different parts of the stalk of the plant are not necessarily valid. Obviously, under these conditions, no certainty can exist with respect to the evidence of differences between the behavior of the cultures on different media. On the other hand, most of the types of chemical change are common to all of the cultures, and these accordingly may be emphasized inasmuch as the experiments are mutually confirmatory.

WATER AND ORGANIC SOLIDS

A clear picture of the gross changes that occurred in the leaves during the culture period is shown by the curves of Figures 1, 2, and 3[1]. Figure 1 shows the changes in water content. The imbibition of water soon after the leaves were placed in five of the culture solutions is clearly evident, while the prompt wilting of the LN series is reflected in the loss of water from the start. As will appear in what follows, this behavior of the LN series had its effect in reducing the velocity of many of the chemical changes that occurred. Whether the wilting was a specific effect of the



salt solution, or was due to some chance combination of temperature and humidity, or to some more deep-seated physiological cause, is not known; but the behavior of the LN series, as contrasted with the other cultures in the dark, suggests that the effect is more likely to have its origin in the salt solution employed. The enormous imbibition in the DG series, which resulted in exudation of water from many of the small vessels of the leaves, is particularly well shown.

All three lots of leaves in culture in the dark passed through a period of maximal water content and then, as the breakdown of chlorophyll progressed, lost water rapidly. No particular significance can be attached

[1] Data for all figures are given in Table 20, p. 828.

to the period of survival—we have carried out water cultures of leaves that retained their turgidity for as long as 200 hours in the dark, but there probably is a correlation between the onset of extensive chlorophyll degeneration and the loss of water-holding capacity. The age or relative maturity of the leaves may be of significance with respect to the period during which they will remain in apparent good condition when subjected to culture.

In Figure 2, the increase in solids of the leaves cultured in the light contrasts sharply with the loss of solids from the leaves in the dark. The data for the water culture series give particularly smooth curves and it is of interest to inquire somewhat more closely into the nature of the substances that accumulated in these leaves during culture in the light.

The increase in organic solids (Figure 3) in the LW series amounted to 35.7 gm. or 47.4 percent of the weight of the organic solids of the fresh leaves. The corresponding figures for the LG and the LN series are 12.6 gm. (14.0 percent) and 21.6 gm. (28.3 percent). The unusually low value for the LG series is probably connected with the fact that these leaves were appreciably larger and more fully developed, and also chanced to contain a large proportion of soluble carbohydrate at the start.

Study of the analytical data to be presented below shows that definite increases in the quantities of a number of substances took place. The results for the LW culture series were:

		gm.
Fermentable sugar	12.9	16.3
Unfermentable sugar	3.4	
Starch		0.8
Organic acids (66 milliequivalents x 0.06)		4.0
Asparagine		1.3
Glutamine		1.9
		24.3

Thus it is apparently possible to account in chemical terms for approximately two-thirds of the mass of the newly-formed organic matter in the leaves. The assumptions involved in this accounting are numerous, and are by no means uniformly well established. There is little doubt with respect to the sugar and starch. These substances are universally taken to represent the products of photosynthetic action, and the contrasting losses of sugar and starch in the cultures carried out in the absence of light furnish evidence for this view. But there is no evidence whatever that the organic acids were formed by synthesis from carbon compounds newly acquired by the leaf tissue during the period of culture in the light. The evidence with respect to asparagine is distinctly in the other direction, inasmuch as from two to four times as much asparagine was ultimately synthesized in the dark as in the light. Glutamine, however, almost certainly arose from newly synthesized carbon compounds. Accordingly, of the 36 gm. of newly formed organic solids in the leaves of the LW series, 18 gm. at most are probably due to the synthesis of carbohydrates, and even on the most favorable assumptions, at least one-third of the new material is of wholly unknown nature. The inference is clear that detached leaves in water culture in light are the seat of a wide variety of highly complex

chemical reactions of which the synthesis of carbohydrates according to the classical views of photosynthesis probably form only the preliminary steps.

It is of interest to pursue this theme a little further. The LW series of leaves contained, after 235 hours of culture, 35.7 gm. of newly formed organic solids. The increase in *soluble* organic solids was 37.5 gm. and this close agreement between the increase in total solids and the change in soluble organic solids occurs also in the LG and the LN series, in which the corresponding figures are 12.6 gm. against 14.8 gm., and 21.6 gm. against 20 gm., respectively. The inference from this, that the newly formed solids are exclusively soluble organic solids, is not entirely justified however. Demonstration of this can be obtained from the data for protein nitrogen (Figure 5). The loss of protein nitrogen—that is, the total nitrogen which remains insoluble after successive extraction of the dried leaf samples with alcohol and with hot water—was, in the LW series, 0.71 gm. Hence the loss of protein (N x 6.25) is of the order of 4.4 gm., and there should be an increase of soluble solids and a loss of insoluble organic solids of this order. The data for the LW series are not as satisfactory as those of the other two light series in this respect, but all three show a loss in insoluble organic solids that is definitely smaller than the loss that must have taken place due to the digestion of protein. The figures in grams per kilo of fresh leaf are:

	Loss insoluble solids	Loss protein
LW	2.9	4.4
LG	1.3	6.6
LN	1.5	4.6

This suggests that a part of the loss due to digestion of protein must have been compensated by the synthesis of new organic compounds that are insoluble in alcohol and in hot water. The quantity is not large, but the possibility that even a small amount of organic solids insoluble in hot water is synthesized during culture in the light is important, in that it provides an example of how far the synthetic activity of leaves under these conditions may extend.

Although speculation on the nature of the newly synthesized substances other than sugar and starch is probably of little value at this time, there is one possibility that should be mentioned. The tobacco leaf contains very considerable quantities of complex carbohydrates and a part of the increase of water-soluble substances may be due to an increase in these.

The behavior of the solids of the leaves cultured in the dark was profoundly different from that of the solids in the light cultures. In all three experiments, there was a distinct loss of organic solids, although this was small in the DN series. The actual losses were 14.6 and 22.3 gm. in the DW and DG series, and these were distributed about equally between the soluble and the insoluble organic solids.

The behavior confirms our earlier experiment in water culture in the dark (93) although the magnitude of the relative loss (19 percent and 25 percent of the total organic solids in the two cases mentioned) was less than that observed in the more prolonged previous experiment. The phenomenon is, of course, to be accounted for in terms of the conversion of organic substances in the tissues into volatile decomposition products, probably mainly water and carbon dioxide, i.e. respiration; and the mag-

nitude of the loss illustrates how extensive such decomposition reactions may be. The nature of the substances decomposed will be discussed more fully below.

THE INORGANIC CONSTITUENTS

Study of the analytical data on the weights of the total inorganic constituents of the samples shows a marked variability. None of the curves which express these data is satisfactory save possibly that for the LW series, and the curves are accordingly omitted.[1] Two factors contribute to this. The analytical determination of the ash weights of plant tissue is by no means an easy matter. In spite of the greatest care in control of the temperature of the muffle furnace, duplicate determinations of an ash of approximately 15 percent frequently varied by as much as 1 percent. Further investigation of the point has shown that the exact time of heating in the muffle, and of cooling in the dessicator, as well as care in the time occupied in weighing the ashes, are all matters of importance. It happens that the determination of the ash of the tobacco plant presents peculiar problems not all of which are as yet solved.

The other factor which affects the smoothness of the curves for a series of presumably identical samples is the actual biological variation from sample to sample. Leaves collected from plants growing in a field, even when thoroughly mixed and sampled at random, cannot be expected to give results of the degree of precision that would be obtained from leaves of plants grown under controlled conditions in culture solutions. Chance variations in the composition of the soil have their effect upon individual plants, and this will be revealed in their composition.

In view of these considerations, the data for the inorganic composition of the samples cannot be clearly interpreted. It happens that the LW series data show a satisfactory degree of constancy throughout, and that, with the exception of two points, the LG series is likewise constant. The corresponding cultures in the dark are, however, not satisfactory.

The cultures in an inorganic salt solution which provided nitrogen in the form of ammonium ion showed no definite evidence of absorption of significant amounts of inorganic constituents. The LN series data, with the exception of two points, fall on a line which slopes definitely upward, but the final ash weight is less than the initial, so that the inference that absorption occurred cannot be definitely drawn. The DN culture data slope definitely downward, suggesting loss of inorganic constituents throughout—a conclusion that will require further support before it can be regarded as established.

NITROGENOUS CONSTITUENTS

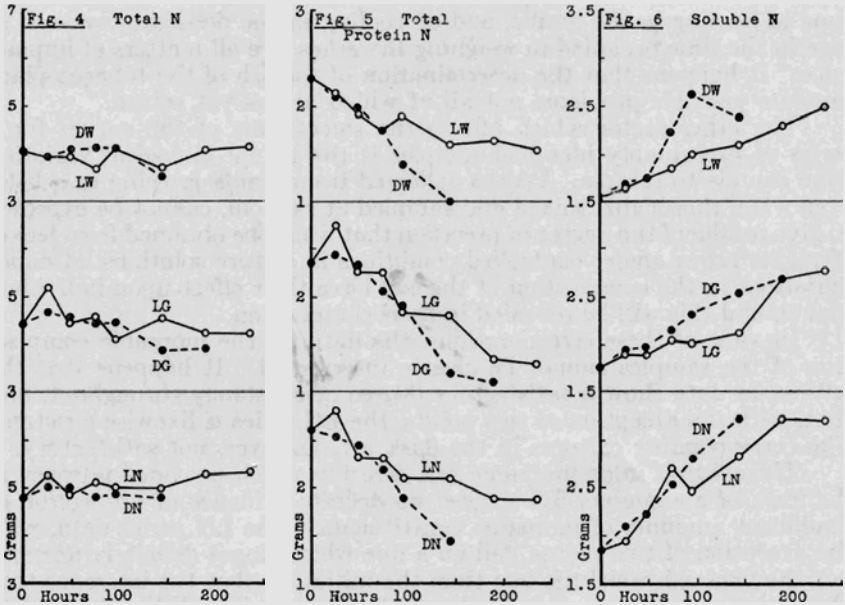
Total Nitrogen

The total nitrogen of the samples of leaves plotted in Figure 4 provides a further example of the variability in composition of presumably identical samples. When the best straight line that expresses the data for the light experiments, as calculated by the method of least squares, is plotted, that for the LW series is practically horizontal, that for the LG series slopes slightly downward to the right and that for the LN series slightly upward. In both the latter cases, however, it is clear that the departure of these

[1] For data see Table 20.

lines from the horizontal is mainly due to the presence of one or two abnormal values, the bulk of the data falling very close to a mean calculated with the omission of these values. Accordingly, it is evident that the data as a whole support the view that, within the error of the measurements, neither loss nor gain of nitrogen occurred during culture in light. A similar conclusion can be drawn from the data of the cultures in the dark.

This conclusion is in agreement with previous work (93) and, indeed, is to be anticipated from the nature of the experiments. The only opportunity for loss of nitrogen that is provided is through the attack of bacteria on the bases of the leaves with the production of soluble products that might diffuse into the culture solution. This source of error was minimized by the technic of the experiment.



On the other hand, the acquisition of nitrogen by the cultures of the N series was to be expected, but the evidence that an absorption of nitrogen occurred, in quantities that could be determined by the Kjeldahl method as here applied, is not convincing. Nevertheless, as will be shown later, small amounts of ammonia did enter the tissues but these are well within the analytical error of the present data.

Protein Nitrogen

The total nitrogen determined on the residue of dry leaf tissue, after this had been thoroughly extracted with alcohol and with hot water, may not represent exclusively protein nitrogen, but there is every reason to believe that by far the greater part of this nitrogen is so combined. Moreover the changes in this quantity, as culture proceeded, are with little doubt to be attributed chiefly to alterations in the quantity of protein. Further evidence in support of these statements is to be found in the data

for the proportions of amino and amide nitrogen that result from hydrolysis, and in the results of the effects of proteolytic enzymes, the details of which are given below.

The changes in protein nitrogen of the six lots of cultured leaves are shown in Figure 5. It is clear that digestion of the protein into soluble products soon became a significant factor, and it is noteworthy that the rate of digestion in the early stages of culture (75 to 100 hours) was closely similar in all cases regardless of culture solution or illumination of the leaves. Later, the rate of digestion in the illuminated leaves fell behind the rate in the darkened leaves so that, at the expiration of 143 hours, the average loss of protein from the three dark cultures was 1.2 gm., that from the light cultures 0.6 gm. Protein digestion is thus ultimately considerably more extensive in the dark than in the light; in fact the residual protein in the illuminated leaves after 150 hours appeared in general to resist further extensive digestion.

The fact that the leaf proteins rapidly undergo partial conversion into soluble products during the early stages of culture in the light is particularly noteworthy. Owing to the uncertainty regarding sampling errors, it is not possible to assert that the transformation was evident immediately at the beginning of the culture period, that is to say that the digestion observed is merely a continuation of a reaction which is proceeding in normal leaves still attached to the plant, but it is quite clear that the transformation was initiated before the expiration of 48 hours and continued during the greater part of the culture period.

These experiments lead to a result different from those of Zaleski (97). This investigator studied the stems of young etiolated bean plants (*Vicia faba* Windsor); equal fresh weights of his experimental material were floated on nutrient solutions that contained 5 or 10 percent of sucrose in addition to inorganic salts, and were placed in the light. In the course of four days he observed an increase in dry weight and an increase in protein nitrogen as determined by Stutzer's method, the order of magnitude of the increase being 16 percent of the original protein nitrogen. It is difficult to interpret his results, however, as similar increases were obtained whether the nutrient salt solution contained nitrogen or not, and also because no control experiments in the absence of sugar were conducted. It is impossible to tell whether the increase in dry weight resulted from photosynthetic reactions in the light of a nature analogous to the reactions we have observed in our tobacco leaves cultured in water in the light, or whether the increase is due to assimilated sugar withdrawn from the nutrient solution. Furthermore, the function of the nitrogen in the culture solution is obscure. No evidence was provided that this was utilized in the synthesis of the protein. The culture solutions he employed were probably much more concentrated than ours; the actual concentration of the nutrient salts is not stated, but in earlier work of a similar nature (96) he used 3 percent Knop's solution with 4 percent of sugar added.

Chibnall's demonstration that the protein nitrogen of bean leaves diminishes during the night (14) is more closely related to the present experiments. Using the paired leaflet method, and basing his conclusions on changes expressed as percentage of the fresh weight which a review of the literature (13) had shown to be the most reliable method of calculation, Chibnall showed that the protein nitrogen decreases during the night by a quantity that greatly exceeds the probable error of the meas-

urements. This implies a mechanism of protein digestion in the normal leaf which can be demonstrated during the period when photosynthesis is suspended. Our own experiments indicate that this digestive process operates both in light and in the dark at a rate which, in the former case, completely overbalances any formation of protein that may occur. The objection may be raised that the leaves we have studied are excised and are therefore in an entirely abnormal environment. This is true, but they are nevertheless capable of carrying on many chemical reactions which can be regarded as those of normal leaves; for a considerable period they are still actively metabolizing tissue.

The rate of disappearance of protein from the leaves cultured in the dark was approximately constant throughout the experimental period; the rate of disappearance from the leaves cultured in light, however, diminished materially after the expiration of about 100 hours, and became much slower thereafter. Several factors may enter into the explanation of this behavior, although discussion of them must be highly speculative. It may be assumed that protein synthesis continues in the leaves from the start, and that the loss of protein is a net loss owing to the preponderance of digestive over synthetic reactions. In the later stages, however, the presence of higher concentrations of the products of protein digestion may give rise to conditions under which the synthetic reactions become increasingly significant, and the rate of net loss of protein is thereby diminished. Another view is that the proteins of the tissue differ in their stability to the digestive enzymes, and that the phenomena observed in the light cultures represent the sum of two or more rates of reaction some of which run more rapidly than others. Still other suggestions may be advanced; exhaustion of the activity of the proteolytic enzymes, or destruction under the influence of light, exhaustion or destruction of possible coenzymes. Owing to the complexity of the reactions represented, no choice may be made between these or other possible explanations of the behavior observed.

The quantity of protein involved in this reaction is of importance in connection with the general problem of the alteration of the solubility of the constituents of the leaves during culture. In the LW series, 0.52 gm. of protein nitrogen had disappeared at the end of 73 hours. If the conventional factor 6.25 is applied, this represents a rate of digestion of slightly more than one gm. of protein per day. An almost exactly similar rate of digestion was evident in the DW cultures. The rates of the other four culture experiments were somewhat slower, being a little under one gm. per day. A similar rate of digestion has been previously observed in tobacco leaves subjected to water culture in the dark (93).

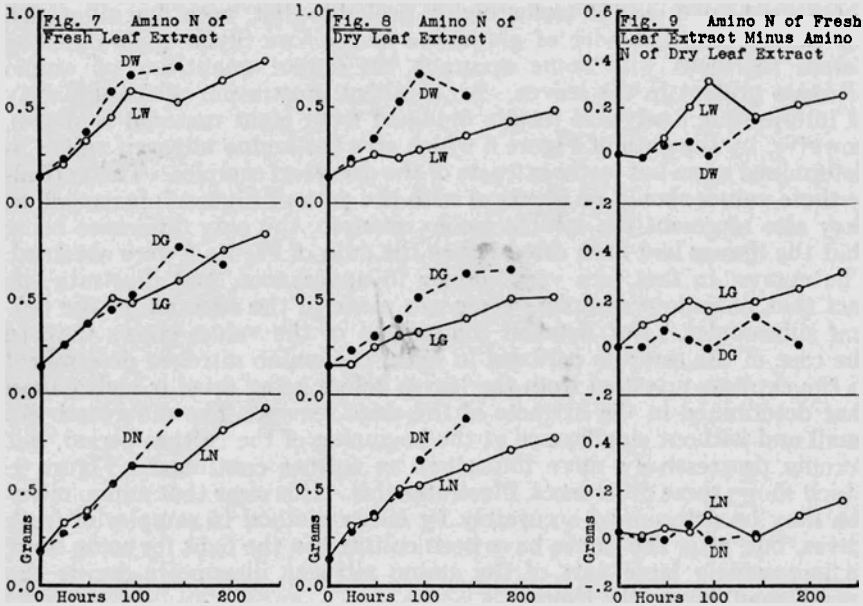
The total quantity of protein nitrogen rendered soluble over the whole culture period in each case was 0.71, 1.06, and 0.72 gm. in the three light experiments, and 1.27, 1.24, and 1.16 gm. in the three dark experiments. If mean values may be taken, these figures represent the digestion of approximately 5.2 gm. of protein in 235 hours in the light and 7.6 gm. of protein in 143 hours in the dark, or roughly, 0.5 gm. protein per day in light and 1.2 gm. per day in the dark. It is of interest next to inquire into the fate of this protein.

Soluble Nitrogen

The data for the quantities of soluble nitrogen plotted in Figure 6 show regular increases of an order of magnitude commensurate with the

losses of protein nitrogen. The three light experiments indicated an average gain of 1.1 gm. of soluble nitrogen (protein nitrogen loss 0.83), the three dark experiments a gain of 1.06 gm. (protein nitrogen loss 1.2). Accordingly, it may be inferred that much of the increased soluble nitrogen originated from the protein that underwent digestion.

What appears to be further evidence that this is the case is given by the data for increase in soluble amino nitrogen (Figure 7). The curves show the continuous production of amino nitrogen at an approximately uniform rate in all cases during the early stages of culture—an inference already drawn from the rate of loss of protein nitrogen. They also show the continuation of this high rate into the later stages of culture in the dark, but a somewhat reduced later rate in the cultures in light. It must be remembered in this connection, however, that much of the soluble amino nitrogen represents the amino nitrogen of the amides asparagine and glutamine



which were formed in increasingly important quantities as the culture period progressed. Thus the accumulation of soluble amino nitrogen is not a direct measure of the rate of production of amino acids by protein digestion. As will be shown later, a complex series of chemical reactions intervenes between the actual liberation of an amino acid from the protein and the production of what is measured as an increase in soluble amino nitrogen.

There is an interesting and suggestive constancy in the ratio between the increase in soluble amino nitrogen and the increase in total soluble nitrogen. The average value of this ratio for all six cultures is 61.8 percent, the highest and lowest values being 67.3 and 53.9 percent respectively. This ratio is of an order of magnitude that would be expected of the products of digestion of protein to amino acids, inasmuch as a part of the nitrogen so liberated might be expected to be in the form of bases or of cyclic amino acids, and the ratio would hold notwithstanding any series

of intervening reactions if the amino groups of the newly liberated amino acids only were involved in the subsequent change. The magnitude of the ratio furnishes evidence that the digestive process proceeded very nearly, if not completely, to the stage of amino acid production. The implication with respect to the proteolytic enzyme system in the tissues is clearly that enzymes not only of the tryptic type are present, but that peptidases[1] likewise share in the reactions. That such enzymes are contained in extracts from leaves has long been recognized (10), the experiments of Blood (7) with cabbage extract being particularly interesting, and in this connection the elegant demonstration of the presence of proteolytic enzymes in the rootlets of malt by Linderstrom-Lang and Holter (31) should be mentioned.

The part of the soluble nitrogen which consists of amino nitrogen can be determined in two ways. The data of Figure 7 show the quantities found in extracts prepared from a subsample of the leaves selected before these were dried, and it seems highly probable that, with due allowance for the peculiar behavior of glutamine in the Van Slyke apparatus, the values represent with some accuracy the actual quantities of amino nitrogen present in the leaves. An excellent illustration of the difficulty of interpreting analytical results obtained from plant material is shown, however, by the data of Figure 8 which give the amino nitrogen values as determined upon hot water extracts of the dried leaf samples. Theoretically these values should be identical with the data of Figure 7, inasmuch as they also represent the soluble amino nitrogen, the only difference being that the tissues had been dried before the data of Figure 8 were obtained. The curves, in fact, are very similar in appearance, and illustrate the fact that no serious sampling error was made in the selection of the ten-leaf subsamples. But detailed comparison of the values shows that, in the case of the samples cultured in light, the amino nitrogen determined in the extracts prepared from the leaves before being dried is higher than that determined in the extracts of the dried leaves. The differences are small and without significance at the beginning of the culture period, but become progressively more important as culture continued. Figure 9, which shows these differences, illustrates this. It is clear that amino nitrogen may be determined accurately by either method in samples of fresh leaves, but, after the leaves have been cultured in the light for some time, an increasingly large part of the amino nitrogen disappears during the operation of drying the leaves.

The data for the leaves cultured in the dark, plotted in Figure 9, show a quite different behavior; for the most part the differences are highly irregular and show no clear cut progression with time, and examination of the data shows only two instances in which the difference assumes really serious proportions; these are the observations at 143 hours in both the DW and DG experiments. In general, it seems probable that the irregularity of the differences in these experiments represents the order of magnitude of the errors involved in the selection of the subsamples combined with the fundamental error involved in the assumption that the original 60-leaf samples were actually originally alike in composition. The quantities of nitrogen involved are mostly very small.

[1] The first observations of the presence of proteolytic enzymes in plant tissues were made by v. Gorup-Besanez (22) in 1874, who showed that seeds of vetch, hemp, and flax, and sprouted seeds of barley, contain enzymes capable of liquefying fibrin and coagulated egg white. The early literature is fully reviewed by Butkewitsch (10) in a long paper which describes work carried out under Schulze at Zurich. Later work is reviewed by Blood (7). Butkewitsch demonstrated the formation of leucine and tyrosine during the early stages of sprouting of seeds.

The interpretation of these observations in chemical terms is impossible at present, but the more or less regularly increasing magnitude of the difference observed in the LW and LG experiments (Figure 9) suggests that we have to do with a definite reaction which takes place in leaves that have been cultured in light for some time. As a result of this reaction an appreciable part of the amino nitrogen is converted during the operation of drying the leaves either into a substance insoluble in hot water, or into a form which no longer reacts with nitrous acid. The observations clearly indicate that analytical results obtained on leaves that have been dried must be interpreted with great caution; although tissue prepared in this way has many obvious technical advantages, the possibility of the introduction of serious error in interpretation in certain instances must not be overlooked.

It may not be out of order to suggest certain chemical relationships which may help to account for the results, in spite of their purely speculative nature. It is clear, whatever it is that happens, the net result is that amino nitrogen disappears in substantial amounts during the drying of leaves that have been cultured for some time in light. Thus we may assume that the substance responsible for this change is produced directly or indirectly by photosynthetic reaction and hence is a carbohydrate or a metabolite of a carbohydrate. If small but increasing quantities of a metabolite are produced, which have the capacity to condense with amino groups, the results obtained become intelligible. Aldehydes have such properties, although the condensations of aldehydes with amino acids as usually observed are reactions that occur in alkaline solution. We do not wish to suggest that this is the actual situation in these tissues; the problem presented is, however, one of great practical significance in plant tissue analysis and warrants detailed investigation.

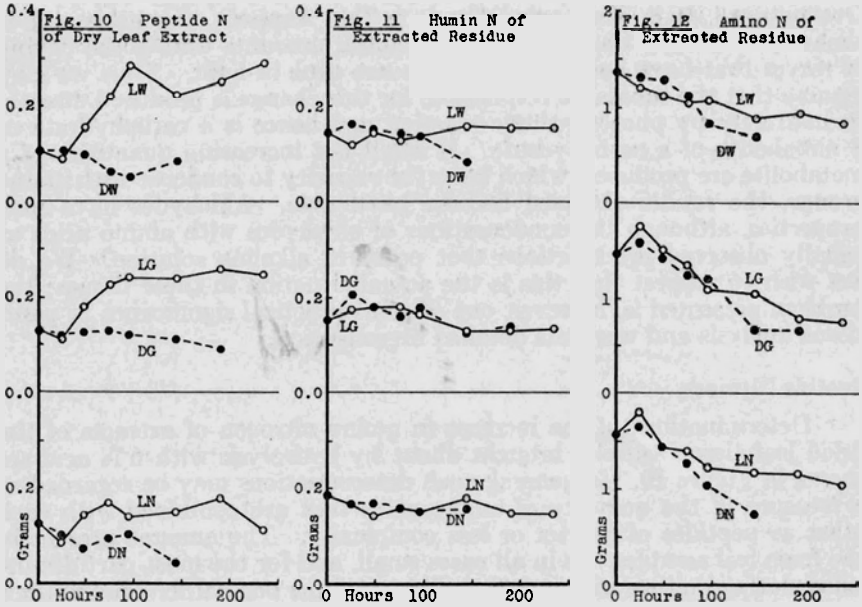
Peptide Nitrogen

Determinations of the increase in amino nitrogen of extracts of the dried leaf tissue samples brought about by hydrolysis with 6 N acid are shown in Figure 10. In general, such determinations may be regarded as a measure of the quantity of amino acids that are combined with each other as peptides of greater or less complexity. The amount present in the fresh leaf samples was in all cases small, and for the most part diminished during culture in the dark. This confirms our earlier observations on peptide nitrogen in tobacco leaves during dark culture, and illustrates the activity of the leaf enzymes. During the period studied, relatively large quantities of protein nitrogen disappeared as such, but the peptide nitrogen did not increase. The inference is obvious that the digestion of this protein was complete to the amino acid stage.

The leaves cultured in light, however, show a quite different behavior, the data suggesting that appreciable quantities of peptide nitrogen actually accumulated as culture continued. This interpretation is, however, not the only one possible and, in view of the results of the dark cultures, is by no means the most probable. It is not likely that the proteolytic enzymes were any less reactive in the leaves cultured in light than they were in the dark, and the true peptide nitrogen in these leaves was probably of the same order of magnitude throughout. There are at least two other possible sources of hydrolyzable amino nitrogen in the illuminated leaves. Glutamine, when heated in solution at pH 7, is fairly rapidly converted into

ammonia and pyrrolidone carboxylic acid, a substance in which the amino group has condensed with one of the carboxyl groups to give a ring structure. On being hydrolyzed with dilute acid, this ring opens with the production of glutamic acid, a substance that contains an amino group. Consequently any glutamine in the tissues subjected to light culture which suffered hydrolysis during the operation of drying would be represented in the extract of the dry tissue by pyrrolidone carboxylic acid, a potential source of amino nitrogen after acid hydrolysis. It would be difficult, though not impossible, to discriminate between amino nitrogen produced from this source and true peptide nitrogen.

The probable order of magnitude of this effect in the present case is, however, very small. Careful check upon the behavior of glutamine in tomato plant tissues (91) has shown that the loss of glutamine during drying under the standard conditions in our equipment is very small, and



probably does not exceed 3 or 4 percent of the amount present. The leaves subjected to culture in light in the present experiment ultimately contained in the vicinity of 0.2 gm. of glutamine amide nitrogen and, if they may be supposed to have suffered a similar loss during drying, the quantity of pyrrolidone carboxylic acid nitrogen present would be, in the most extreme case, 0.010 gm. This is very much smaller than the 0.250 gm. of apparent peptide nitrogen in these same tissues, and consequently glutamine as a probable source of any significant part of the apparent peptide nitrogen may be dismissed.

The most probable origin of this amino nitrogen produced by acid hydrolysis is to be sought in the curious behavior of the amino nitrogen of these tissues during drying, which has just been discussed. If condensation between amino acids and some metabolite of the carbohydrate occurred and the product were soluble in hot water, one would expect

to find in the extracts a form of nitrogen which gives amino nitrogen on acid hydrolysis. This is exactly what occurs. The order of magnitude of the apparent peptide nitrogen is in every case save one distinctly greater than the differences plotted in Figure 9, as it should be; actual peptide nitrogen and probably a little pyrrolidone carboxylic acid nitrogen were also probably present. If allowance is made for this, and also for the experimental errors inherent in these determinations, it is justifiable to conclude that what seems to be peptide nitrogen in the leaves cultured in the light is really the amino nitrogen that disappeared during the drying of the samples of tissue.

As will appear later, use is made of the data for amino nitrogen in the extracts from the leaves prepared after ether cytolysis in the calculation of the quantities of amino acids that are produced by the hydrolysis of the protein. The justification for using this set of data rather than the set obtained from analysis of the dry leaf extracts is provided by the uncertainty regarding the true meaning of the amino and peptide nitrogen of the dry leaf samples. Consideration of the dark culture experiments clearly indicates that the true peptide nitrogen of the tissues is probably negligibly small in amount and accordingly need not be included in the calculations.

METABOLISM OF THE LEAF PROTEIN

Much of the data discussed in the preceding sections has had to do with the asssumption that the insoluble nitrogen of dry leaf tissue consists largely of protein, a substantial part of which becomes soluble as culture proceeds, and gives rise to an increase in the water soluble amino nitrogen of the leaf tissue. The different lines of evidence mutually support each other and leave little doubt of the general nature of the processes that take place. Nevertheless it seems worth while to digress for a moment to provide direct evidence that most of the nitrogen of the alcohol-extracted residues actually does consist of protein. This evidence has been obtained in two ways.

By extraction of such residues with hot alcoholic alkali, most of the nitrogen can be brought into solution, and the extract obtained, when neutralized, deposits a flocculent precipitate that can be shown by means of various tests to consist largely of protein, although, to be sure, in an altered and partially decomposed form. Furthermore, treatment of the alcohol-extracted residues with proteolytic enzymes causes a large part of the nitrogen to pass into solution, a change in solubility that can hardly be explained on other grounds than the assumption that the dissolved nitrogen was originally present as protein.

To illustrate the nature of the observations, the data in Tables 3 and 4 are given. A sample of alcohol-extracted fresh leaf tissue was thoroughly extracted several times, first with hot water and then with hot, 60 percent alcohol which contained 0.2 percent of sodium hydroxide. The alkaline extracts were then treated with dilute acetic acid until a maximal precipitate was produced which was centrifuged off, washed with dilute alcohol, and dehydrated with absolute alcohol. The preparation gave the usual protein color reactions. The residual tissue, the filtrate from the neutralization precipitate, and the precipitate were then all examined for amino and amide nitrogen before and after complete hydrolysis with hydrochloric acid.

Table 3 shows that the order of magnitude of the proportion of nitrogen soluble in hot water, after the dried tissue had been thoroughly extracted with alcohol, is small, and the last line in Table 4 shows that much of this nitrogen is in a form that is converted into ammonia by hydrolysis but that the increase in amino nitrogen is relatively small. This fraction, therefore, is definitely not protein. The neutralization precipitate contained only about half of the remaining nitrogen of the tissue, inasmuch as the treatment with hot alkali brought about considerable hydrolysis. The proportion of amino nitrogen in it was low, but was increased to a rather high level by acid hydrolysis. The evidence for the presence of amide groups is also very clear, and there is no question that

TABLE 3. NITROGEN IN ALCOHOL EXTRACTED TOBACCO LEAF RESIDUES
(Figures are percent of total nitrogen)

Experiment	1	2	3
Water-soluble fraction	6.3	6.2	7.0
Neutralization precipitate	47.7		47.7
Filtrate from precipitate		38.7	37.9
Tissue residue		2.7	1.8

TABLE 4. BEHAVIOR OF THE FRACTIONS ON ACID HYDROLYSIS
(Figures are percent of the nitrogen of each fraction)

	Before hydrolysis		After hydrolysis	
	Ammonia N	Amino N	Ammonia N	Amino N
Neutralization precipitate		1.5	5.7	67.0
Filtrate from precipitate	2.7	4.2	11.1	57.7
Tissue residue			6.3	53.5
Water-soluble fraction	0.28	1.52	2.6	2.3

this material is essentially protein in nature. Three preparations contained 14.8, 14.1, and 15.4 percent of nitrogen on the dry, ash-free basis figures that are low when compared to the nitrogen content of seed proteins, but of the same order of magnitude as those of ordinary crude preparations obtained from leaves. The humin nitrogen after acid hydrolysis was 2.7 and 1.8 percent in two of these preparations, suggesting the presence of a carbohydrate impurity.

The filtrate from the neutralization precipitate contained substances of a higher amino nitrogen ratio than the precipitate itself, as might be expected, but not so high as to indicate a very extensive hydrolysis of the tissue protein during the process of alkali extraction. After hydrolysis, the amino nitrogen reached a proportion to be expected of material of protein origin, and the amide value suggests that the dicarboxylic amino acids are to a certain extent concentrated in this more easily hydrolyzed part of the protein.

The tissue residue contained nitrogen that very evidently represents protein which was not brought into solution by the hot alkali. After hydrolysis, the level of amide nitrogen was close to that of the neutralization precipitate and the amino nitrogen was somewhat low.

The qualitative evidence that most of the nitrogen of the alcohol-extracted leaf tissue is essentially protein is therefore perfectly clear. No other type of nitrogenous material could be expected to produce amino and amide nitrogen after acid hydrolysis in just the proportions found. Additional evidence on this point was obtained by treating samples of the alcohol-extracted residue with pepsin or with trypsin preparations under the proper conditions, and allowing them to digest at 38° for various periods. The samples were then centrifuged and the nitrogen in the insoluble residues was determined. Controls in the absence of the enzyme were carried out simultaneously and the data obtained are shown in Table 5.

For these experiments, 0.250 gm. of tissue were suspended in centrifuge tubes in 15 ml. of 0.4 percent sodium bicarbonate, and 5 ml. of 0.5 percent trypsin solution were added. The tubes were incubated at 38° and, at daily intervals, were centrifuged, and the residues were washed twice with 20 ml. of hot water. New enzyme and bicarbonate were added to all save two tubes, the contents of which were transferred to a Kjeldahl flask and the nitrogen was determined. The pepsin experiments were carried out in a similar manner save that 15 ml. of 0.055 N hydrochloric acid were substituted for the bicarbonate. The controls contained water in place of the enzyme solutions.

The data show that the protein is rendered soluble under these conditions quite rapidly and very extensively. The controls showed that from only 10 to 14 percent of the nitrogen was rendered soluble in the absence of the enzyme. Accordingly, it seems established that at least 87 percent of the nitrogen of these residues consists of protein, and it is probable that the correct value is even higher.

To return to the consideration of the leaf culture experiment: It is next of interest to inquire whether the proteins which remained undigested during the culture period also undergo a series of chemical changes that can be detected by the methods available for the study of proteins mixed with the other insoluble constituents of leaf cells.

TABLE 5. EFFECT OF PROTEOLYTIC ENZYMES ON ALCOHOL-EXTRACTED LEAF TISSUE
(Figures are percent of the total nitrogen rendered water-soluble.)

Hours	Trypsin	Control	Hours	Pepsin	Control
24	55.6	5.	21	47.1	7.
47	77.4	5.5	45	72.0	6
170	87.0	11.2	116	83.0	13.
			207	86.0	

The validity of the conclusions we shall draw from our data rests upon the admissibility of the technical process of hydrolysis of a protein with strong acids when the protein is mixed with other substances of non-protein nature. When even the purest obtainable preparation of a protein is boiled for 24 hours with 20 percent hydrochloric acid, the resulting solution almost invariably contains a certain amount of a black, insoluble material, and also a highly tinctorial black or brown material in solution. The former can be removed by dilution and filtration, much or all of the latter by treatment of the solution with decolorizing carbon in liberal amounts. Both types of product are referred to as humin, and little distinction is usually made between them. Both are nitrogenous, and the

total quantity of nitrogen in this form of combination is usually of the order of 1 percent of the protein nitrogen. Humin is regarded as a product of decomposition of one or more of the amino acids under the conditions of hydrolysis, and the amino acid thought to be chiefly involved is tryptophane.

The quantity of humin obtained, as measured by the determination of the humin nitrogen, is materially influenced by the presence of carbohydrates in the protein preparation hydrolyzed. In recent years, it has become clear that most proteins contain a moderate proportion of substances of carbohydrate nature as an integral part of the molecule (81,82). One or two amino sugars and a number of true sugars have been isolated from presumably homogeneous protein sources, and it has also been observed that the proportion of humin nitrogen that can be derived from purified protein preparations can be increased several fold by subjecting them to hydrolysis in the presence of carbohydrates or aldehydes (21). In these circumstances, the humin nitrogen may readily be increased to 5 percent or more of the total nitrogen, and it is clear that the increased humin nitrogen must be derived from amino acids which otherwise would have survived the hydrolysis unchanged.

Some data which illustrate the kind of behavior encountered when proteins are hydrolyzed in the presence of carbohydrates are shown in Table 6. The experiments were conducted by treating 0.1 gm. of a very pure specimen of hempseed edestin with 10 ml. of 8 N sulfuric acid for 24 hours in a boiling water bath. Various carbohydrates or derivatives of carbohydrates were added in the amounts indicated. After hydrolysis, the humin was centrifuged off, the nitrogen in it was determined, and samples of the hydrolysates were freed from ammonia and analyzed for amino nitrogen.

TABLE 6. AMINO AND HUMIN NITROGEN OF EDESTIN AFTER COMPLETE HYDROLYSIS IN THE PRESENCE OF CARBOHYDRATES AND OF FURFURAL

Carbohydrate gm.			Amino N %	Humin N %
	None		58.7±1.	trace
0.116	furfural		53.0	9.7
0.058	"		56.8	5.7
0.100	glucose		58.5	1.8
0.200	"		58.7	2.5
0.100	levulose		59.3	3.1
0.100	starch		59.3	2.5
0.200	cellulose		58.8	trace
0.200	lemon pectin		57.3	4.9
0.200	" "	**+0.200 glucose**	**57.1**	**6.6**
0.200	" "	+0.200 cellulose	58.4	5.7

Of the substances studied, furfural and lemon pectin alone brought about any significant decrease in the amino nitrogen ratio and these substances also promoted the maximum humin formation. The sugars had little or no measurable effect on the amino nitrogen ratio although they did give rise to the formation of an appreciable quantity of humin nitrogen. It would seem that pentose sugars are more to be feared for their effect upon the amino nitrogen ratio than hexose sugars or their polymers. The absence of any marked effect from cellulose is particularly striking.

The leaf tissues we have studied undoubtedly contain appreciable quantities of substances that may be expected to give rise to furfural on being heated with strong acid. Accordingly, there is little doubt that the amino nitrogen values we have obtained are somewhat low, and the humin nitrogen values are, of course, considerably magnified. The operation of protein hydrolysis in the presence of such substances is obviously one to be avoided if possible.

Granting all this, however, it is clear that the process of humin formation can involve only a limited proportion of the total products of hydrolysis of the protein, and it would seem perfectly proper to inquire into the nature of the substances that survive the hydrolytic reactions. Furthermore, in an investigation of serial samples of tissues in which the differences between one member of the series and another are of importance rather than the absolute amounts in each, there is considerable probability that each member of the series will be affected in a similar manner, and the magnitude of the difference between any two members will have significance.

It is with these considerations in mind that the residues of dried leaf tissues, after thorough extraction with alcohol and with boiling water, have been studied with respect to their behavior on complete acid hydrolysis.

The relative proportions of protein and non-protein organic solids in these residues at the start of the culture experiment can be roughly estimated from the quantity of insoluble organic solids and from the protein nitrogen (Figure 5). The average quantity of insoluble organic solids of the fresh leaf samples was 48 gm. (see data table 20), the average protein (N $\times$ 6.25) was 15 gm. Accordingly, the extracted residues from the fresh leaf samples consisted of protein mixed with rather more than twice its weight of non-protein organic solids (average 33 gm.) much of which must have been of essentially carbohydrate nature (cellulose, hemicellulose, etc.). After the completion of the culture period, the relative proportion of protein was diminished inasmuch as the non-protein solids were not materially changed.[1]

Although the conditions under which these samples of leaf protein were hydrolyzed were thoroughly disadvantageous from the standpoint of strict protein chemistry, the data (Figure 11) indicate a fair degree of constancy in the amount of humin produced, regardless of whether the leaves had been cultured in light or in the dark. This is of interest when it is remembered that the total quantity of protein decreased materially. As a result, the relative proportion ot humin nitrogen, calculated as a percentage of the protein nitrogen, increased. In all cases save the LN series, the humin nitrogen increased from an average of 6.6 percent of the total nitrogen to approximately 10 percent. There was no detectable increase of humin nitrogen in the LN series.

A clear explanation of this result is impossible in view of our ignorance of the chemistry of the reactions involved. It may be mentioned, however, that a series of parallel determinations in which the alcohol-extracted residues were hydrolyzed without being previously extracted with hot water showed distinctly higher absolute amounts of humin in each case

[1] If the protein nitrogen is multiplied by the conventional factor, 6.25, an approximate estimate of the quantity of protein in the extracted residues is obtained. When this quantity is subtracted from the insoluble organic solids as determined in these residues a remarkably constant figure is obtained. The average value for the fresh leaf samples was 33.0 gm.; the average of each set of samples varied from 30 to 37 gm., and the grand average of all determinations was 34.4 gm. Accordingly it may be concluded that the culture conditions brought about very little change in the quantity of insoluble organic substances with the exception of the protein.

and also higher proportions of humin nitrogen. Hot water extraction of the tissues, therefore, resulted in the removal of alcohol-insoluble substances (possibly pectins) that contributed materially to the formation of humin during the acid hydrolysis. The amount of humin is, therefore, dependent upon the nature of the non-protein substances present, and changes that took place during culture in the proportion or amount of the precursors of the humin probably reflect changes in the nature and proportion of the non-protein substances rather than of the protein.

Evidence has been presented in connection with the increase in soluble amino nitrogen that the proteins of the leaf undergo a fairly rapid digestion during the period of culture either in light or in the dark. The gradual conversion of insoluble protein nitrogen into soluble nitrogen much of which was present as amino nitrogen should be accompanied by a diminution in the amino nitrogen which can be liberated by hydrolysis of the insoluble protein-containing residues. That this is the case is clear from the data in Figure 12 in which the amino nitrogen of these residues after hydrolysis with acid has been plotted. The curves closely resemble those of Figure 5 which show the total protein nitrogen, and, like these, indicate the digestion of protein at a similar rate in all six cultures during the early stages of the experiment. They also suggest the diminished rate of digestion of the three light cultures during the later stages in contrast to the undiminished rate in the cultures in the dark.

The ammonia produced during the hydrolysis of proteins is for the most part derived from amide groups which form a part of the structure of the molecule. The quantities of ammonia obtained from the hydrolysis of the extracted tissue residues are shown in Figure 13, and further illustrate the similarity of the digestive process in the early stages of culture in all the series; the lagging behind of the rate of hydrolysis of the protein in the light cultures in the later stages is also apparent.

The ratio of the amide to the total nitrogen plotted in Figure 15 is remarkably constant in all save the DW series, and shows that the overall composition of the residual protein is similar to the part of the protein which underwent digestion. There is little if any suggestion that the part of the protein of the leaves which was digested differed in amide ratio from that which remained, and, in this respect, the data differ from those obtained by extracting the residual leaf protein with alkali. It is also of interest to note that the order of magnitude of the amide nitrogen ratio is similar to that observed by Miller and Chibnall (36), in a series of leaf proteins from various species, i.e., in the vicinity of 6 percent.

There is a marked difference between the results obtained by hydrolyzing the alcohol and hot water-extracted leaf residues and by hydrolyzing the residues that have been extracted with alcohol but not with hot water. Data plotted in Figure 14, when compared with Figure 13, show that the water extraction removed a considerable amount of material from the leaves cultured in the dark that yielded ammonia on hydrolysis with strong acid. The findings are in agreement with the view that appreciable quantities of this ammonia-yielding material are formed during the later stages of culture in the absence of light. The curves of Figure 16, in which the ratio of apparent amide nitrogen to total nitrogen is plotted, show this even more clearly when they are compared with those of Figure 15. Although the actual magnitude involved is small—of the order of 0.1 gm.—it is evident that we have here the evidence for a definite synthetic

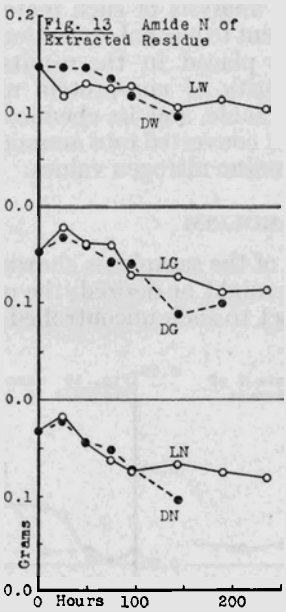


Fig. 13 Amide N of Extracted Residue

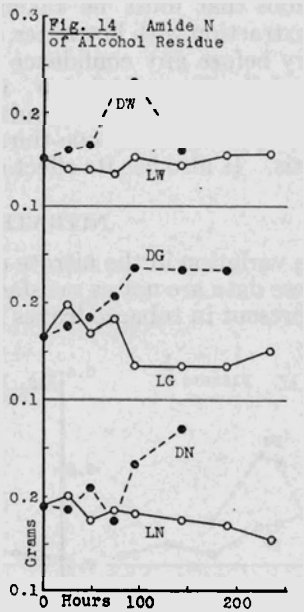


Fig. 14 Amide N of Alcohol Residue

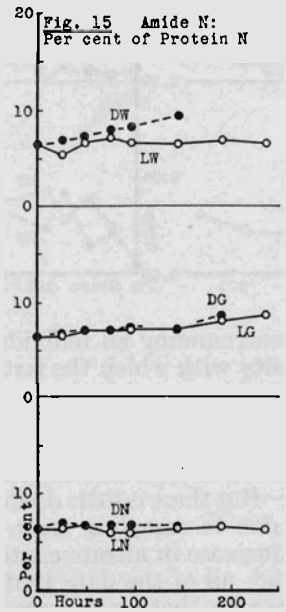


Fig. 15 Amide N: Per cent of Protein N

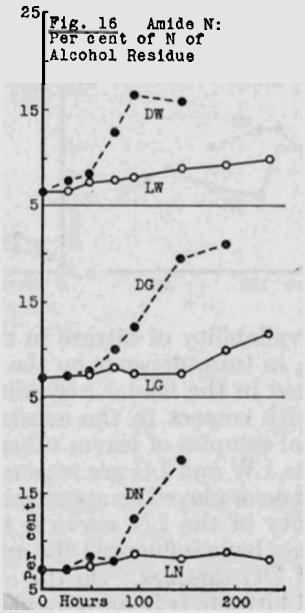
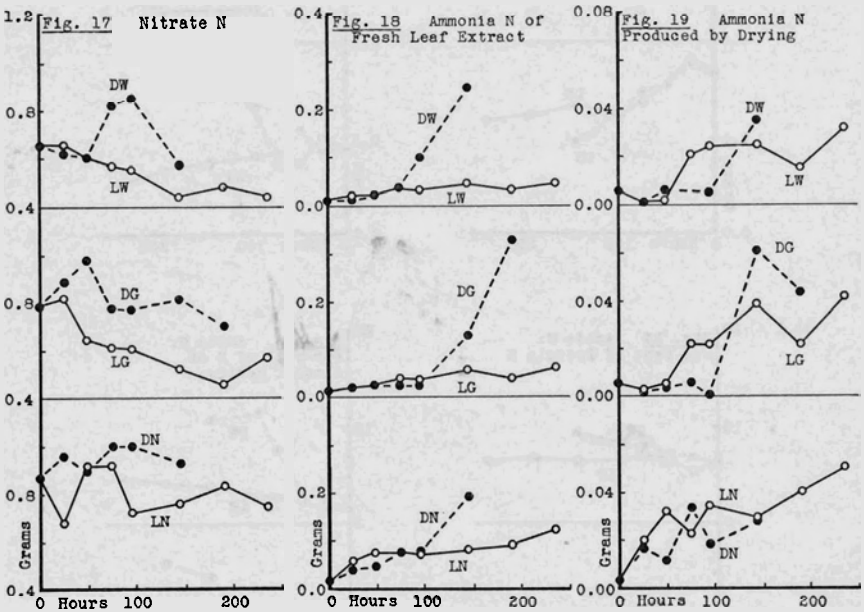


Fig. 16 Amide N: Per cent of N of Alcohol Residue

reaction which is promoted in the dark cultures. The data illustrate the precautions that must be taken in the analysis of such material. The step of extraction with hot water, subsequent to alcohol extraction, is clearly necessary before any confidence may be placed in the results. As will be more fully discussed below, the quantity of non-protein nitrogen removed by this treatment is quite appreciable, and its chemical nature is such that approximately one-third of it is converted into ammonia by acid hydrolysis. It also has its effect on the amino nitrogen values.

NITRATE METABOLISM

The variation in the nitrate content of the samples is shown in Figure 17. These data are not as satisfactory as might be desired; the quantity of nitrate present in tobacco leaves is subject to such uncontrolled influences



as the availability of nitrate in the soil surrounding an individual plant, and this, in turn, depends on the uniformity with which the fertilizer was distributed in the field. Accordingly, there is a certain degree of uncertainty with respect to the constancy of the actual initial nitrate in the individual samples of leaves taken from a field crop. The curves for experiments LW and LG are reasonably smooth and give no suggestion that sampling error played an appreciable part. But there is little doubt that the irregularity of the LN curve is in part due to sampling error, and this factor may have influenced the apparent increase in nitrate content of the DW and DG samples. On the other hand, all of the data that we have collected hitherto is in agreement with the view that an increase in nitrate actually occurs in tobacco leaves during culture in the dark. The matter is to be investigated more thoroughly later; for the present the data will be accepted at face value, and it may then be assumed that a decrease

from the maximum, which occurred in all three dark culture experiments, took place owing to the reduction of a part of the nitrate to ammonia. As will be shown later, there is reason to suppose that this conversion actually occurred, and the subsequent fate of the ammonia so produced can be made fairly clear.

The data for the LW and LG experiments are quite consistent and suggest that reduction of nitrate began promptly and proceeded at a roughly steady rate throughout the period of study. The irregularity of the data for the LN experiment is too great to permit a clear inference but it also suggests a certain amount of reduction. The quantities of ammonia produced from this source may be roughly estimated from the difference between the initial and final values in each case and are shown in Table 7.

The apparent increase in nitrate content that occurred in each of the three dark experiments raises questions not only of the origin of this nitrate but also of its subsequent fate. For the present, we do not feel justified in making any statement with regard to the origin. We have previously pointed out that an increase in nitrate implies an equilibrium between nitrate and its immediate metabolic products which is temporarily reversed under the conditions that obtain in darkened leaves. The disappearance of nitrate toward the end of the experiment may, however, as in the case of the illuminated leaves, also be attributed to reduction to ammonia, and, on this assumption, the estimates given in Table 7 show the quantities of nitrogen which may be involved.

As will be made clear below, the chief value of these figures is to show a possible origin of between 0.2 and 0.3 gm. of ammonia nitrogen in connection with the discussion of the amide metabolism.

TABLE 7. DECREASES IN NITRATE NITROGEN DURING CULTURE
(Figures are grams per kilo of fresh leaf)

LW	0.198
DW	0.28
LG	0.214
DG	0.28
LN	0.122
DN	0.06

AMMONIA METABOLISM

The amide metabolism of leaf tissue is intimately involved with the presence of ammonia in the cell sap. Early work in this field, reviewed in the introduction, provided evidence of a close association between the synthesis of the amide asparagine and the advent of ammonia, and has given rise in recent years to the view (56, 57) that the function of the amides in plants is that of detoxication of ammonia. It is obvious that the dehydration of the ammonium salt of a carboxylic acid to the amide removes ammonium ion from the system with the production of a neutral compound, and the economy of this method, from the standpoint of the plant, is evident, as the amides so formed may in turn serve as sources of nitrogen for subsequent synthetic reactions. Ammonia, therefore, stimulates a reaction in leaf tissue that in turn results in a diminution of the

quantity of ammonia present. It would not be expected to accumulate until the conditions became such that the postulated reaction could no longer continue.

In Figure 18 are plotted the data for the quantities of ammonia found in the cold water extracts of the fresh leaf subsamples. The LW and LG series show a slow, although very small, increase in the quantity present starting from the low level of approximately 12 mg. (0.3 percent of the total nitrogen) and increasing about fourfold during the entire culture period. The LN series, however, shows an immediate increase within 24 hours from 15 mg. to 60 mg. and subsequently an increase to 75 mg. at which point the ammonia remained constant until nearly the end of the experiment. This increase is very probably due to the acquisition of ammonia from the nutrient solution and is a clearly defined effect of culture in this medium. The entire group of data on the light cultures indicates, however, that, if substantial quantities of ammonia do appear in the tissues, they are promptly disposed of; the reactions that lead to amide synthesis are highly efficient, and adequate amounts of the necessary precursors were present.

The three cultures in the dark present a marked contrast in the later phases of the culture period. During the first 73 hours, there is little, if any, evidence of accumulation of ammonia in quantities greater than those in the corresponding light cultures. Later, however, and the time corresponds with the beginning of the destruction of chlorophyll and loss of water-retaining capacity in these cultures, ammonia began to accumulate at a rapid rate so that, for example, the final sample of the DW series contained 250 mg. of ammonia nitrogen or 6.3 percent of its total nitrogen in this form. The inference may be drawn that the mechanism which tends to remove ammonia from the system ultimately broke down in the leaves cultured in the dark, and permitted an accumulation to occur. The most probable explanation is that the precursor necessary for asparagine synthesis was exhausted.

The actual amounts of ammonia in these tissues are small, and their accurate determination presents considerable difficulty. The determinations upon the cold water extracts of the 10-leaf subsamples (Figure 18) give a series of values which, when plotted, fall upon relatively smooth curves and suggest that no serious sampling error occurred. Ammonia determinations were likewise made upon the main samples of dried leaf tissue. The data in all cases fall slightly below the values from the fresh leaf extracts. Like these, they can be represented by relatively smooth curves which conform closely to the curves of the ammonia values in the fresh leaf extracts.

The problem arises as to which of these sets of data more closely represents the true ammonia content of the cultured leaves. As will appear later, the use of either set in the calculation of the amide values is permissible inasmuch as the differences are small and no essential change is involved with respect to the conclusions that can be drawn from the amide data. But the existence of this invariably higher value for the ammonia in a cold water extract of the leaves than in similar leaves that have been dried suggests that there is an indication here of a chemical reaction that may have significance in the interpretation of such experiments.

The lower value in the dried leaves may represent actual volatilization of a portion of the ammonia during the operation of drying. Such be-

havior has been reported by Prianischnikow (57) in the case of certain lupine seedlings. Against this view is the fact that the hydrogen ion activity of the leaves we have studied is such that no appreciable part of the ammonia is present uncombined with acid. Another and more likely possibility is that a reaction took place between a part of the ammonia and some other constituent of the tissue during the operation of drying, with the result that the quantity of ammonia present was reduced. Some color is lent to this explanation by the observation that the dried leaf samples can be shown to contain a substance that yields ammonia under conditions of hydrolysis quite different from those employed for the decomposition of amides. This will be discussed below as "extra ammonia nitrogen".

A plot of the differences between the two sets of ammonia values is shown in Figure 19 and reveals several well-defined regularities in the phenomenon. The actual quantities in fresh leaf samples and in those cultured in water or in glucose solution, either in light or dark, are negligibly small during the first 50 hours. Subsequently there is a striking increase which is shown most clearly by the fairly consistent values for the light cultures, but which seems to be even greater in the dark cultures. On the other hand, the leaves cultured in the salt solution that contained ammonia give evidence of a marked increase in this factor from the start, and no distinction can be drawn between the light and the dark cultures. In all cases the quantity present in the later samples is significant, being frequently of nearly the same order of magnitude as the free ammonia determined in the dry leaf tissue.

No clear interpretation of this behavior of the ammonia can at present be made. If decomposition of glutamine occurred during drying, one would expect higher ammonia values in the dried specimens. The presence of the phenomenon of apparent disappearance of a part of the ammonia on drying, even when this is carried out under conditions that have been demonstrated to be harmless to the unstable amide group of glutamine (91), suggests that caution must be exercised in the examination of tissues that have been dried. It is obviously desirable, if not necessary, to control the results of such examination by analyses of suitably prepared samples of fresh tissues.

AMIDE METABOLISM

The quantity of amide nitrogen in a plant tissue is obtained by subtracting the ammonia nitrogen already present from that present after mild acid hydrolysis. A division of this amide nitrogen into two parts, one of which may be attributed to glutamine, the other to asparagine, can be made in view of the fact that glutamine is readily and completely hydrolyzed on being heated for a few hours to 100° at a reaction close to pH 7, whereas asparagine is not hydrolyzed appreciably unless a considerble excess of acid is added. Accordingly, the quantities of ammonia at the start, after hydrolysis at pH 7, and after hydrolysis in 1 N sulfuric acid, are determined; the difference between the first and last figure is taken as the total amide nitrogen, and the difference between the first and second figure as the glutamine amide nitrogen. Asparagine is calculated by difference. The determinations of glutamine, then, depend upon a relatively specific property of the glutamine molecule and are probably trustworthy. Asparagine, being calculated by difference, necessarily includes

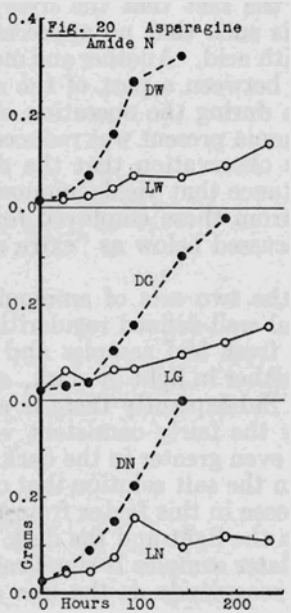


Fig. 20 Asparagine
Amide N
DW
LW
DG
LG
DN
LN
Grams
0.4
0.2
0.0
0 Hours 100 200

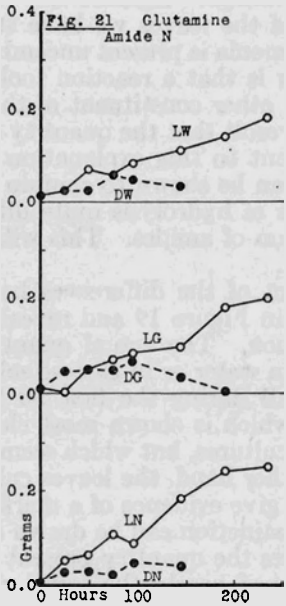


Fig. 21 Glutamine
Amide N
LW
DW
LG
DG
LN
DN
Grams
0.4
0.2
0.0
0 Hours 100 200

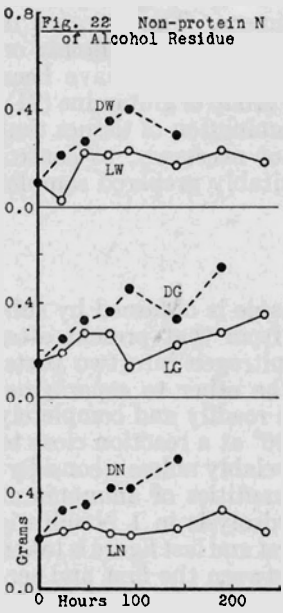


Fig. 22 Non-protein N
of Alcohol Residue
DW
LW
DG
LG
DN
LN
Grams
0.8
0.4
0.0
0 Hours 100 200

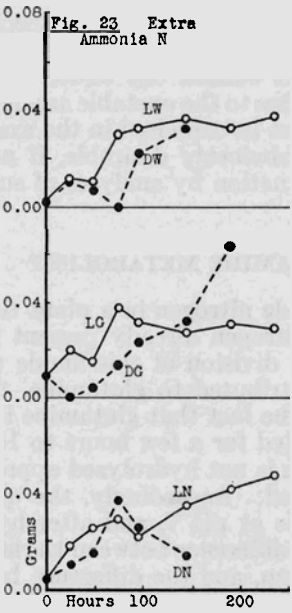


Fig. 23 Extra
Ammonia N
LW
DW
LG
DG
LN
DN
Grams
0.08
0.04
0.00
0 Hours 100 200

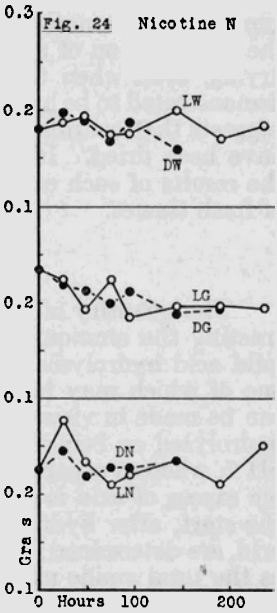


Fig. 24 Nicotine N
LW
DW
LG
DG
DN
LN
Grams
0.3
0.2
0.1
0 Hours 100 200

any amide other than glutamine that may be present and, as has been pointed out elsewhere (88), our qualitative knowledge of the tobacco plant is not yet sufficiently extensive to permit assurance that no amide other than these occurs in this plant.

A further restriction on the accuracy of the data is due in the present case to the uncertainty as to which set of free ammonia values should be employed in calculating the amide values. We have chosen to use the determinations made on the cold water extracts of the fresh tissue, not only because these were made on material that had been subjected to less drastic treatment, but also because the actual values are higher and therefore tend to reduce the magnitude of the changes that occurred in the amides. These changes were so profound, however, that the minor differences in the ammonia values had no significant effect upon them.

The asparagine and glutamine amide nitrogen values are plotted in Figures 20 and 21. The effect of light upon the formation of these two substances is striking. Asparagine is promptly and rapidly formed in the cultures in the dark, but its formation is retarded and is much less extensive in the light. On the other hand, glutamine is synthesized in the light at a rate somewhat greater than the asparagine, but is scarcely formed at all in the dark, Light, therefore, promotes the formation of glutamine in the tobacco leaf, but in the absence of light, asparagine is almost exclusively formed.

These conclusions are of such significance in connection with the general problem of amide metabolism that the data will be examined in considerable detail.

The formation of asparagine in the dark cultures was initiated very early in the experimental period; within 48 hours the quantities present had more than doubled, and at the expiration of 143 hours, 0.302, 0.285, and 0.383 gm. of asparagine amide nitrogen had appeared respectively in the water, glucose, and salt cultures. The initial value was in all cases close to 0.02 gm. so that the enrichment in asparagine in the three experiments was 15-, 14-, and 19-fold. The greater enrichment in the third experiment is very probably an evidence of the assimilation of a little ammonia from the culture solution. In the other two experiments, where no outside source of ammonia was available, it is clear that a quantity of some 0.3 gm. of nitrogen must have been rendered available for the synthesis of the amide group. Accordingly, in order to account for the formation of the asparagine in the dark cultures, approximately 0.6 gm. of nitrogen which disappeared from some other form of combination must be sought.

The transformation of protein nitrogen into asparagine, when leaves are cultured in water, was discovered by Borodin (8), was early studied by Schulze and Bosshard (77) and by Butkewitsch (11), and in more recent years by Chibnall (15) and by Mothes (37). In our previous work (93) we have also observed this relationship and, because of the similarity of the quantities of nitrogen involved, were able to point to the probability that it is the amino nitrogen of that part of the protein that undergoes digestion during the culture period which ultimately is converted into amide nitrogen. Calculations from the present data clearly indicate that there is insufficient amino nitrogen rendered available to account for the whole of the asparagine nitrogen, but there is sufficient to account for half of it, that is, for the amide group alone, a confirmation of the earlier data.

The calculation involves a comparison of the soluble amino nitrogen, exclusive of that of asparagine and glutamine themselves, with the amino nitrogen derived from the digestion of the protein, the latter quantity being estimated from the hydrolyzable amino nitrogen of the protein in the extracted leaf residue. Obviously, if no other reaction intervenes, the hydrolyzable amino nitrogen which disappeared from the protein, as culture proceeded, should be represented by an increase in soluble amino nitrogen inasmuch as the amino acids that result from protein digestion are soluble substances.

The correction of the soluble amino nitrogen of the tissues for that due to the two amides themselves involves a consideration of the behavior of asparagine and glutamine towards nitrous acid in the Van Slyke apparatus. Asparagine yields half its nitrogen under the usual conditions of the determination, glutamine yields 90 percent of its nitrogen. Asparagine therefore reacts only with its α-amino group; but glutamine reacts not only with its α-amino group but its amide group also shares in the reaction, 80 percent of this being converted to nitrogen gas by the reagents. Accordingly, in order to correct the soluble amino nitrogen for the nitrogen derived from these two substances, it is necessary to subtract a quantity equal to the sum of the amide nitrogen of asparagine and 180 percent of the amide nitrogen of glutamine. The difference, then, is equal to the amino nitrogen of amino acids other than these two amides, and the observed increase in the quantity so calculated is obviously due to the digestion of protein with the liberation of soluble amino acids.

The data in Table 8 show the calculations for the DW and DG experiments. The second and sixth columns contain the soluble amino nitrogen values after correction for the amino nitrogen derived from the asparagine and glutamine. The third and seventh columns are obtained from the data plotted in Figure 12 and represent the amino nitrogen produced by the digestion of the protein which should have appeared in soluble form, the figures being secured by subtracting each individual value from the value at the start.

TABLE 8. RELATIONSHIP BETWEEN AMINO NITROGEN THAT DISAPPEARED AND THE GAIN IN AMIDE NITROGEN IN DARK CULTURE EXPERIMENTS

(Figures are grams per kilo of fresh leaf)

Hours	DW				DG			
	Soluble amino N corrected	Amino N from protein	Δ 3–2	Asparagine amide	Soluble amino N corrected	Amino N from protein	Δ 7–6	Asparagine amide
1	2	3	4	5	6	7	8	9
25	0.066	0.050	−0.016	0.006	0.023	−0.120	−0.143	0.009
49	0.163	0.080	−0.080	0.050	0.128	0.030	−0.098	0.021
73	0.227	0.240	0.013	0.139	0.127	0.210	0.083	0.088
95	0.209	0.534	0.325	0.250	0.131	0.240	0.109	0.137
143	0.221	0.670	0.449	0.302	0.286	0.786	0.500	0.285
190					0.188	0.791	0.603	0.357

If our assumptions are sound, the differences shown in columns 4 and 8 must, therefore, represent amino nitrogen that had been rendered soluble by digestion of protein and which had been in turn converted into some other form. At the 143 hour point, 0.302 and 0.285 gm. of aspara-

gine amide nitrogen had been formed in the two experiments—quantities obviously smaller than the amounts of amino nitrogen of protein origin which had disappeared from the system. It is obvious that there is sufficient nitrogen derived from this source to account for the synthesis of the amide groups of the asparagine, but that there is not sufficient to account for the whole of the nitrogen of the asparagine formed; some other form of nitrogen must therefore have played a part in the synthesis. This is not a matter for surprise; the intermediate compound is undoubtedly ammonia, and this may arise in ways other than by the deamination of amino acids.

That an excess of ammonia was actually present towards the end of the experiment is clear from the data for free ammonia in Figure 18. It is probable that the limiting factor in the synthesis of asparagine was the inadequacy of the supply of the non-nitrogenous precursor of this substance; and the problem, in the case of the leaves cultured in the dark, is not so much a matter of accounting for the asparagine formed as it is to account for the total ammonia metabolism. Not only must the origin of the asparagine amide and amino groups be provided for, but also the free ammonia that accumulated in the tissues as the leaves became yellow.

The origin of a part of this ammonia has already been suggested, namely the deamination of amino acids; another possible source is the reduction of nitrate. It has already been shown that a diminution in nitrate occurred in the later phases of both the DW and DG experiments and, if it is assumed that reduction of nitrate to ammonia occurred, a fairly satisfactory account can be given of the total ammonia metabolism in these two culture experiments. In the DW experiment after 143 hours, the increase in asparagine nitrogen was 0.604 gm. (twice the asparagine amide nitrogen). In addition, there had been an increase of 0.233 gm. of free ammonia; accordingly 0.837 gm. of nitrogen had presumably passed through the stage of ammonia. The data in Table 8 show that 0.449 gm. may have been derived from deamination reactions, and the data in Figure 17 show that, in the interval between 95 and 143 hours, 0.277 gm. of nitrate had disappeared. The sum of these is 0.726 gm. This is not quite enough to account for the total ammonia metabolism, but it is close to the order of magnitude.

In the DG experiment the increase in asparagine nitrogen at 143 hours was 0.570 gm., at 190 hours, 0.714. The respective ammonia values were 0.112 and 0.314, so that the total ammonia metabolism at these points was 0.682 and 1.03 gm. From deaminization (Table 8) the values were respectively 0.500 and 0.603 gm., from nitrate reduction 0.165 and 0.284 gm; accordingly, an ammonia metabolism of 0.665 and 0.887 gm. can be accounted for, figures which are again within the same order of magnitude.

It is possible also that a little of the asparagine formed in the dark cultures arose directly from hydrolysis of the protein. This point is discussed more fully below where it is shown that the whole of the glutamine formed during the dark cultures may have had this origin. For this reason, the small amount of glutamine in these samples has been ignored in the present discussion. If asparagine did originate in this way, the total amount must have been very small—perhaps of the order of 0.02 to 0.04 gm. —and, if this is the case, the total amide metabolism is that much less and is in even closer agreement with the quantities of ammonia presumably derived from deaminization of amino acids and reduction of nitrate. Furthermore, other sources of ammonia are also possible. There is a con-

siderable proportion of the total nitrogen of the tissue combined in forms of which we know nothing. The calculated values for this so-called "rest nitrogen" contain the errors of all the analytical determinations which contribute to them, and so do not have any high degree of accuracy, but we have previously shown (93) that the qualitative composition of this fraction of the nitrogen undergoes very material change during the culture period. That some of this nitrogen may have contributed to the ammonia metabolism is far from improbable.

The above discussion, then, reduces to the statement that the greater part, at least of the nitrogen in the leaves subjected to culture in the dark which was converted either into asparagine or remained as free ammonia, probably originated from the deamination of amino acids and the reduction of nitrate. These assumptions are, at any rate, within the bounds set by the data. Proof that such reactions occurred cannot be had until a technic is devised whereby the interconversions of ammonia that contains deuterium instead of hydrogen, or of carbon compounds containing isotopic carbon, can be followed. This type of experimentation has already yielded valuable results in animal physiology (71) and there is no obvious reason why it should not be equally successful in studies of plants.

The glutamine content of the fresh leaf samples was in all cases extremely low, accounting for less than 0.5 percent of the total nitrogen. Culture of the leaves in the dark gave rise to only a very small increase in glutamine amide nitrogen, and in no case did the proportion rise to more than the equivalent of 1.5 percent of the total nitrogen. Because of the low initial level of glutamine amide nitrogen, the relative increase was striking, amounting to a four- to six-fold increase in the DW and DG experiments; nevertheless the synthesis of this substance can hardly be regarded as a quantitatively important reaction of tobacco leaves during culture in the dark. The total amount formed was of the order of one-tenth that of the asparagine amide nitrogen synthesized under the same conditions towards the end of the culture period.

The situation in the leaves cultured in light was, however, quite different. Both glutamine and asparagine were rapidly synthesized, the quantity of glutamine in general exceeding the quantity of asparagine (Figures 20 and 21) by 50 to 100 percent of the latter. The rates of synthesis of the two amides were quite similar, the impression given by the data being that asparagine synthesis lagged a little behind glutamine synthesis.

As is shown in Table 9, the total quantity of amide nitrogen formed in the early stages of the light experiments was almost precisely the same in the first 49 hours of the light as in the dark experiments and differed

TABLE 9. SUM OF ASPARAGINE AND GLUTAMINE AMIDE NITROGEN
(Figures are grams per kilo of fresh leaf)

	Fresh leaf	25 hrs.	49 hrs.	73 hrs.	95 hrs.	143 hrs.	190 hrs.	230 hrs.
LW	0.0317	0.0431	0.0933	0.0989	0.148	0.174	0.229	0.318
DW	0.0317	0.0456	0.0927	0.214	0.320	0.357		
LG	0.0300	0.0651	0.0906	0.129	0.149	0.189	0.303	0.358
DG	0.0300	0.0734	0.0944	0.158	0.223	0.341	0.383	
LN	0.0308	0.098	0.112	0.184	0.244	0.283	0.359	0.357
DN	0.0308	0.083	0.123	0.177	0.274	0.445		

to only a minor extent in 73 hours. Subsequently, however, the total amide synthesis in the illuminated leaves dropped rapidly behind that in the darkened leaves.

Any attempt to account for these phenomena is, of course, highly speculative in the present state of our knowledge. It nevertheless seems worth while to point out a few fairly obvious relationships and to suggest a possible, although only partial, explanation. It will be recalled that the rate of protein digestion during the first 73 hours was not appreciably different in the illuminated leaves from the rate in the darkened leaves (Figure 5). This implies that amino acids were liberated at essentially the same rate under both conditions. If it be assumed that decomposition of these amino acids with the production of ammonia kept pace with their liberation, it is possible to account for the production of amides, either in light or dark, likewise at equal rates during the early phase of the experiments. The reactions of protein digestion and of deamination are enzymatic in nature, and there is no *a priori* reason to assume that they would be affected by light under the conditions of these experiments; and if ammonia actually is produced in this way, the formation of equal total quantities of amides either in light or in dark could be predicted.

The questions of which amide is formed, and of the relative proportions of the two amides synthesized in illuminated leaves, obviously have to do with the availability of the necessary precursors of the asparagine and glutamine. This is a matter which is profoundly affected by light energy, and our observations strongly suggest that the two precursors are non-nitrogenous, and that one of them is newly synthesized in the leaves of the light experiments.

That the precursor of the glutamine synthesized in the root of the beet when the plant is treated with large quantities of ammonium sulfate is non-nitrogenous follows from the work of Vickery, Pucher and Clark (90). It was shown that the increase in soluble nitrogen of the root was quantitatively accounted for by the increase in total glutamine nitrogen. The extraneous nitrogen must therefore have been assimilated in two ways, one of which resulted in the formation of an α-amino group, the other in an amide group, and the compound must have arisen *de novo* from ammonia on the one hand and the non-nitrogenous precursor which contained the proper carbon chain on the other.

The present experiments demonstrate that very little of the precursor suitable for the synthesis of glutamine was present in the tobacco leaves at the start of the culture experiments, inasmuch as very little glutamine was formed after these leaves were deprived of light. Adequate amounts were present in the leaves exposed to light, and the inference is clear that the hypothetical substance must have been formed by photosynthetic action during culture in the light. At exactly what stage in the elaboration of this substance nitrogen entered into combination is not clear, but the beet experiments suggest that the actual precursor must be derived from a fairly highly elaborated and stable material, inasmuch as a bountiful store of it is present in the beet root. The evidence points to a substance of carbohydrate nature.

The relationship that exists between the precursor of glutamine and the precursor of asparagine is by no means clear. On the simplest assumption, the precursor of asparagine would contain a chain of four carbon atoms and the precursor of glutamine would contain five; the relative

proportions of the amides synthesized would depend on the relative concentrations of the two precursors present, since there was no excess quantity of ammonia present at the end of the experiment. It seems evident that both amides share in the ammonia detoxifying action in the illuminated leaves.

Although the total amide synthesis was the same during the first 73 hours, whether the leaves were in light or in the dark, the total synthesis in the light subsequently dropped behind that in the dark. This correlates with the rate of digestion of protein which was also delayed in the later phases of the light experiments as compared with the dark experiments. Calculations of the amino nitrogen which disappeared as such during the later phases of the culture period are shown in Table 10 in columns 4 and 8, and the increase in amide nitrogen due to the synthesis of glutamine and asparagine is shown in columns 5 and 9. The order of magnitude of the quantity of amino nitrogen that disappeared is appreciably greater towards the end of the culture period, than the order of magnitude of the amide nitrogen synthesized. Accordingly, it may be concluded that sufficient amino nitrogen was metabolized to account for all the amide nitrogen that appeared. There was not sufficient, however, to account for the total nitrogen of the asparagine and glutamine actually formed.

TABLE 10. RELATIONSHIP BETWEEN AMINO NITROGEN THAT DISAPPEARED AND THE GAIN IN AMIDE NITROGEN IN LIGHT CULTURE EXPERIMENTS
(Figures are grams per kilo of fresh leaf)

Hours	LW				LG			
	Soluble amino N corrected	Amino N from protein	Δ	Amide N	Soluble amino N corrected	Amino N from protein	Δ	Amide N
1	2	3	4	5	6	7	8	9
25	0.039	0.160	0.121	0.012	0.077	−0.300	−0.377	0.035
49	0.082	0.230	0.148	0.062	0.121	−0.04	−0.160	0.060
73	0.199	0.300	0.101	0.067	0.208	0.10	−0.11	0.099
95	0.269	0.270	0.001	0.117	0.147	0.36	0.213	0.119
143	0.141	0.470	0.229	0.143	0.231	0.40	0.169	0.159
190	0.199	0.410	0.211	0.198	0.195	0.65	0.455	0.270
235	0.179	0.520	0.341	0.287	0.207	0.64	0.483	0.328

As before, it is possible to render a more exact account for this nitrogen by assuming that reduction of nitrate to ammonia occurred. It has been shown that approximately 0.2 gm. of nitrate nitrogen disappeared in both the LW and LG experiments. In the LW experiment, 0.34 gm. of amino nitrogen disappeared along with 0.20 gm. of nitrate, a total of 0.54 gm.; during the same time 0.287 gm. of amide nitrogen made its appearance. Twice this is 0.57 gm. and the agreement is very close. Similarly in the LG experiment, 0.48 gm. of amino and 0.21 gm. of nitrate disappeared, a total of 0.69 gm.; twice the amide nitrogen found is 0.656 gm.—again a close agreement. The evidence is therefore in favor of the view that both amino nitrogen and nitrate nitrogen were ultimately converted into the nitrogen of the amides glutamine and asparagine, the intermediate being in each case ammonia. Too much emphasis must not be placed upon these figures, however. The assumption is implied that nitrogen from no source

other than amino acids and nitrate was converted into ammonia, and on this point, of course, we have no information. That substances other than amides which yield ammonia on acid hydrolysis exist in the leaves is certain. For example, the alcohol-extracted residues of the dried leaf tissues, on severe acid hydrolysis, yielded considerably more ammonia than did these same residues after they had been extracted with hot water. The hot water evidently removed substances which, on severe acid hydrolysis, yield ammonia. The nature of these substances is entirely unknown, save that they were not protein and were insoluble in hot, 75 percent alcohol. The total quantity of nitrogen other than protein nitrogen in these residues is shown in Figure 22. The general behavior of the curves suggests that there was little change in the amount present during culture in light, but that a definite increase occurred in this type of nitrogenous substance during culture in the dark. It may be inferred that this nitrogen represents an accumulation of alcohol-insoluble products of various reactions that occurred chiefly in the absence of light. The total quantity of nitrogen involved is not great, being of the order of 0.3 gm. in the dark experiments over the entire period of culture, but it forms an increasingly significant proportion of the total alcohol-insoluble nitrogen. Towards the end of the culture period it exceeds 20 percent of this; it forms approximately 10 percent of the alcohol-insoluble nitrogen in the illuminated leaves, save in one experiment in which this value was considerably exceeded.

THE AMMONIA CULTURE EXPERIMENT

Discussion of the amide metabolism in the two experiments in which the leaves were cultured in a nutrient solution that contained ammonia has been reserved in order to emphasize the difference in the effects observed. Table 11 shows the details of the calculation of the amino nitrogen which disappeared together with the increase in amide nitrogen and the data for the comparison of the increase of nitrogen combined as amides with the ammonia produced by metabolic processes are collected in Table 12.

TABLE 11. RELATIONSHIP BETWEEN AMINO NITROGEN THAT DISAPPEARED AND THE GAIN IN AMIDE NITROGEN IN EXPERIMENTS ON CULTURE SOLUTIONS THAT PROVIDE A SOURCE OF NITROGEN

(Figures are grams per kilo of fresh leaf)

Hours	LN				DN			
	Soluble amino N corrected	Amino N from protein	Δ	Amide N	Soluble amino N corrected	Amino N from protein	Δ	Amide N
1	2	3	4	5	6	7	8	9
25	0.032	−0.024	−0.056	0.067	0.023	−0.011	−0.034	0.052
49	0.091	0.120	0.029	0.081	0.076	0.100	0.024	0.092
73	0.127	0.160	0.033	0.153	0.203	0.290	0.087	0.146
95	0.170	0.330	0.161	0.213	0.197	0.520	0.323	0.243
143	0.049	0.390	0.341	0.252	0.279	0.780	0.501	0.414
190	0.121	0.390	0.269	0.328				
235	0.235	0.510	0.275	0.326				

TABLE 12. MAXIMUM CHANGES IN THE AMMONIA CULTURE EXPERIMENTS
(Figures are grams per kilo of fresh leaf)

	LN	DN
Amino N loss	0.275	0.501
Nitrate N loss	0.122	0.070
Sum (metabolic ammonia)	0.397	0.571
Amide N gain x 2	0.652	0.828
Free ammonia N gain	0.111	0.181
Sum	0.763	1.009
Difference of sums	0.366	0.438

It is obvious that the ammonia produced from amino acids and nitrate is in both cases inadequate to account for the amide synthesis and, when the free ammonia values are also considered, the influence of the ammonia derived from the culture solution is very apparent. Whereas the discrepancy between the sum of the glutamine, asparagine, and ammonia nitrogen on the one hand, and the metabolic ammonia on the other hand, is of the order of magnitude of 0.1 gm. in each of the other four experiments, the discrepancy in these two is of the order of 0.4 gm. and clearly points to the advent of ammonia from the culture solution. It is now obvious that the quantity of ammonia which reached the tissues from this source is of the order of 0.3 to 0.4 gm., and the failure of this nitrogen of extraneous origin to show up on the curves of total nitrogen of the tissues (Figure 4) is comprehensible. This quantity is of the same order of magnitude as the irregularities in total nitrogen due to sampling errors.

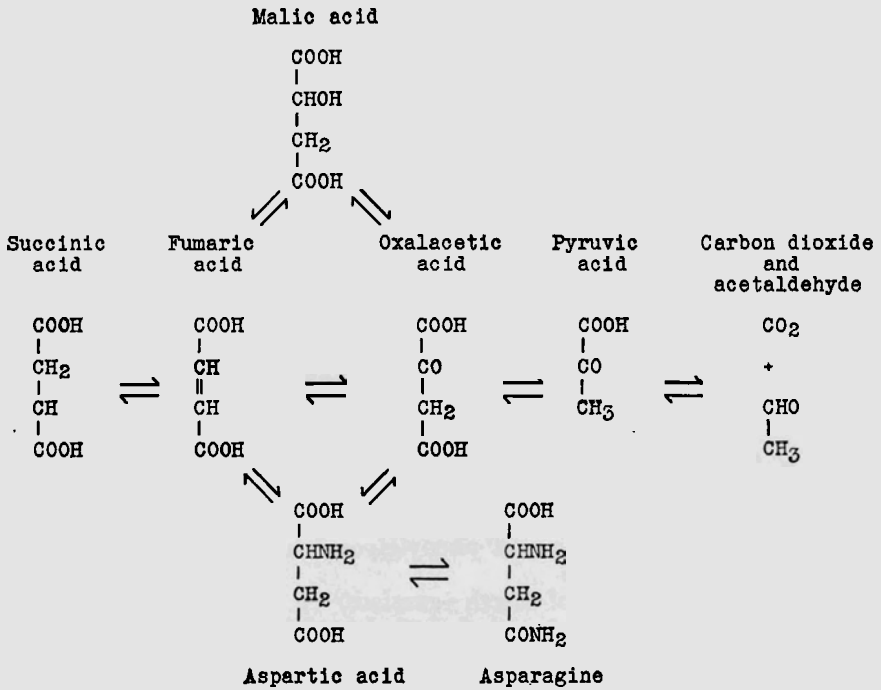
SPECULATIONS REGARDING THE PRECURSORS OF THE AMIDES

Before turning to the consideration of other portions of the data, it may be helpful to discuss various chemical hypotheses of the nature of the substances which, in the presence of ammonia and under the influence of enzymes, may give rise to the synthesis of the amides. It must be emphasized that these hypotheses are entirely speculative; the reasoning involved is based upon what may be imagined to happen if certain chemical substances are present. In the present case there is no evidence that certain of the key substances involved are present at all, nor is there evidence that the enzyme systems which may be invoked to bring about the changes occur in this particular tissue. Nevertheless this type of thinking has been of the most signal service in elucidating the chemical reactions of yeast fermentation, or the activities of muscle tissue, and the field of amide metabolism appears to be one in which it may also render assistance.

The fundamental facts upon which hypotheses may be erected are as follows. Amides are formed when ammonia appears in the tissues in excess of a certain very low concentration, and synthesis continues until either the ammonia is exhausted or until some other substance likewise necessary is no longer available. The other substance, or amide precursor, is non-nitrogenous, and in the case of glutamine, is produced in tobacco leaves by photosynthesis, or results from subsequent conversions of some product of photosynthesis. A precursor suitable for the synthesis of asparagine is present normally in tobacco leaves in appreciable amounts, and is used up during culture in the dark. Whether more of it is supplied during culture in the light does not appear.

General information on the subject of amide metabolism indicates that the precursors both of glutamine and asparagine are derived from carbohydrates, and that oxidation processes are involved; it may accordingly be assumed that aldehydes, hydroxy acids, and particularly ketonic acids will figure in the system. The literature contains many suggestions that certain of the common organic acids of leaf tissue, especially malic acid and fuamric acid, are involved in the synthesis of asparagine (56). There are certain attractive features to this assumption because of the close relationship in chemical structure, and Mothes (40) has observed that the infiltration of the ammonium salts of malic or succinic acid into living leaf tissue results in the synthesis of asparagine. Schwab (79) has questioned the inference from this experiment that the malate or succinate ion contributed to the synthesis of the amide. His own observations indicated that, provided the solution employed were dilute enough, amide synthesis occurred equally well whether the acid supplied were malic or sulfuric, and consequently the conclusion that there is a genetic connection between the carbon chains of the malic acid and of the asparagine is not justified.

Recent experimental work designed to reveal such a relationship has thus indicated that none exists. Nevertheless, it may be of interest to point out certain possibilities inherent in the structural relationships of some of these substances. Robinson (68) has suggested a scheme of reactions of which the following is a slight modification.



Many of these reactions are known to occur in animal tissues or in bacteria under certain circumstances. The enzyme fumarase (95) provides for the conversion of fumaric to malic acid, fumaric dehydrogenase (1) can

oxidize fumaric acid to oxalacetic acid, and is held by Green (23) to be identical with the enzyme which converts malic acid to oxalacetic acid, the so-called fumaric dehydrogenase reaction being merely the combined action of malic dehydrogenase and fumarase. Furthermore fumaric acid can dismute anaerobically to form succinic and oxalacetic acids in the presence of a hydrogen acceptor, the enzymes involved being succinic and malic dehydrogenase.

The equalibria between α-keto acids and amino acids involve the presence of ammonia as well as a catalyst. Knoop and Oesterling (26) have shown that many α-keto acids, when reduced with hydrogen and palladium, or platinum catalysts in the cold and in the presence of ammonia, are converted into α-amino acids, and they regard this transformation as the closest approach yet made to a simulation of the conditions under which amino acids are produced in nature. The reaction is regarded as a reduction of an imino acid produced by condensation of the keto acid with ammonia.

$$\text{R-CO-COOH} \longrightarrow \text{R-C(=NH)-COOH} \longrightarrow \text{R-CH(NH}_2\text{)-COOH}$$

The transformation of α-hydroxy acids into amino acids in the liver has been known since the work of Embden and Schmitz (20), and the actual intermediate in this case is thought to be the α-keto acid. Finally, the dehydration of an ammonium salt of an amino acid to the amide has been shown to occur in kidney, brain cortex, and retina tissue by Krebs (28) who has demonstrated that these tissues contain an enzyme which converts ammonium glutamate into glutamine, and also one that hydrolyzes the amide group of glutamine. Amidases which specifically hydrolyze the amide group of asparagine and glutamine are also known.

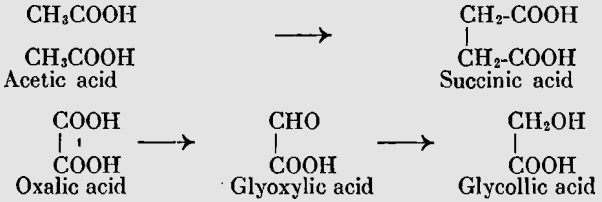
The reactions suggested by the scheme are thus all well-recognized possibilities in various animal tissues. Unfortunately the investigation of analogous reactions in plant tissues has been much less extensive, and in only a few cases have the essential enzymes been detected. Various dehydrases are known in yeast and bacteria, however, as well as in such tissues as sprouted seeds, and the enzyme systems present in the tobacco leaf have been specifically studied by Neuberg and Kobel (42). They have obtained evidence of systems which can bring about the following reactions:

a. The production of acetaldehyde
b. The decomposition of sugars and their compounds (hexose-diphosphatase, saccharase)
c. The transformation of phenylglyoxal to *l*-mandelic acid (keto-aldehyde mutase)
d. The hydrolysis of starch (amylase)
e. The production of methyl alcohol (esterase)

Doubtless many other systems are also present.

The present discussion can be reduced to the statement that enzyme systems are known which provide for a direct relationship between malic acid and asparagine. The most important intermediate in these reactions is oxalacetic acid, the α-keto acid corresponding to aspartic acid, and this

in turn is related to acetic acid, oxalic acid, and pyruvic acid. Acetic acid in turn can be converted by the well known Thunberg reaction (reduction) to succinic acid, and oxalic acid can be converted to glyoxylic and glycollic acids by reduction.



Hence all of these compounds are more or less directly related to each other, and several of the individual substances are in turn known to be related to the sugars, for example pyruvic and glyoxylic acids, the reactions in each case being relatively simple ones.

Although the steps from malic acid to asparagine appear simple and direct, this does not necessarily mean that it is the mechanism actually employed in nature. Until actual evidence has been obtained of the conversion of malic acid to asparagine, the only conclusion that can at present be drawn is that the precursor of this substance in leaf tissue is still unknown, but that it is in all probability a metabolite of carbohydrate. The structural relation to malic acid merely means that this substance is also related to carbohydrate metabolism, and that some of the intermediate steps in its formation are analogous to those by which asparagine is formed.

The synthesis of glutamine in leaf tissues provides a considerably more difficult problem than the synthesis of asparagine, inasmuch as the hypothetical intermediate substances with five carbon atoms, analogous to oxalacetic acid, fumaric acid, and malic acid, are scarcely, if at all, known in nature. A few hypotheses have been suggested, however, and brief consideration of two of these may be of assistance. By analogy with the asparagine scheme, one would expect to find that the key intermediate substance would be α-keto glutaric acid. Mayer (34) has shown that sprouted peas contain a keto-aldehyde mutase that converts methyl-glyoxalyl acetic acid into d,α-hydroxyglutaric acid in almost quantitative yield, and in a condition of high optical purity. There are two features of this reaction that warrant especial comment. In the first place an optically inactive substance is converted into an optically active substance in high yield, that is, an asymmetric synthesis has been effected. Secondly, the active compound produced belongs to the *d* series, that is, the optical isomer of the substance produced by the action of nitrous acid on *l*(+)glutamic acid, and is accordingly the unnatural isomer. The significance of this is not apparent, but Weil-Malherbe (94) has recently shown that the succinic acid dehydrogenase of brain tissue will oxidize *d*(−)glutamic acid but fails to oxidize the natural isomer, *l*(+)glutamic acid. Consequently in this group of related compounds, there is some as yet obscure biochemical relationship to the substances of the extremely uncommon *d* series.

The structural relationships of these substances to carbohydrates on the one hand, and to the amino acids on the other, can best be appreciated from the formulae.

$CH_2OH\text{-}CHOH\text{-}CHOH\text{-}CHOH\text{-}CHOH\text{-}CHO$	glucose
$\downarrow$	
$CH_3\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}COOH$	levulinic acid
$\downarrow$	
$CH_2Br\text{-}CO\text{-}CHBr\text{-}CH_2\text{-}COOH$	dibromlevulinic acid
$\downarrow$	
$CHO\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}COOH$	methyl-glyoxalyl acetic acid
$\downarrow$	
$COOH\text{-}CHOH\text{-}CH_2\text{-}CH_2\text{-}COOH$	α-hydroxy-glutaric acid

The conversion of glucose to levulinic acid is carried out experimentally by heating the sugar with dilute hydrochloric acid. Bromination of levulinic acid and subsequent hydrolysis yields methyl-glyoxalyl acetic acid, and the last step is a dismutation effected by the enzyme reaction just mentioned. It is highly improbable that methyl-glyoxalyl acetic is produced in plants by any such sequence of reactions. Sugar decompositions in nature are usually characterized by the production of two substances, each with three carbon atoms, or of two substances, one with two and the other with four carbon atoms. The formation of a five-carbon atom chain is far more likely to be brought about by the condensation of a three-carbon atom substance with a two-carbon atom substance. Thus one might suppose that acetic and pyruvic acid can combine by a reaction analogous to the Thunberg reaction to form α-keto-glutaric acid.

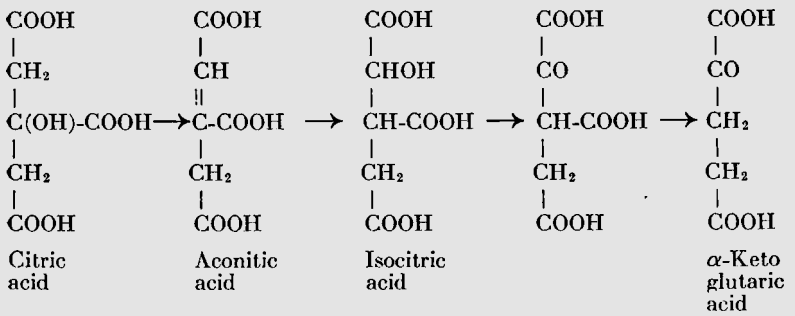
$$\begin{array}{l} COOH \\ | \\ CH_3 \\ \\ CH_3 \\ | \\ CO \\ | \\ COOH \end{array} \quad \xrightarrow{-2H} \quad \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array}$$

The important point in connection with the present discussion is that there is an enzyme mechanism in plants that with the proper precursor can react to give a compound allied to glutamic acid. The fact that this enzyme produces the unnatural isomer in a given case may be considered in the light of the observation of Binder-Kotrba (5) that a keto-aldehyde mutase present in peas converts phenyl-glyoxal into the natural *l*-mandelic acid, and that Neuberg and Kobel have demonstrated that a similar enzyme is present in tobacco leaves.

$C_6H_5\text{-}CO\text{-}CHO$	$\longrightarrow$	$C_6H_5\text{-}CHOH\text{-}COOH$
Phenylglyoxal		Mandelic acid

In any case, the key substance in the formation of the amino acid is the α-ketonic acid, and this is readily produced *in vitro* by oxidation of the keto-aldehyde with bromine and can doubtless also be produced in tissues. The amination of the keto acid is probably brought about by reduction in the presence of ammonia. Knoop and Oesterlin have in fact prepared glutamic acid from α-keto glutaric acid in this way.

A totally different hypothesis of the origin of glutamine in plant tissues is implied in some recent work of Martius and Knoop (33). These investigators have observed that citric acid can be converted in part into α-keto glutaric acid by means of liver dehydrase. The series of reactions they suggest to account for this is of especial interest inasmuch as the intermediate substances, the formulae of which are shown, are well known as plant constituents.



The loss of the carboxyl group in the β-position to a keto group in the last step is a reaction of normal type and occurs *in vitro* with great ease. The implication of a relationship of glutamine metabolism to citric acid metabolism is of great importance. There is no biological evidence of such a relation and, in fact, the present study of glutamine formation in tobacco leaves is directly opposed to such a view. As we have shown, glutamine is only produced in light in the tobacco plant and is therefore derived from a product of photosynthesis. The tissues in which this reaction occurs already contain considerable citric acid and there is little if any change in the citric acid content during the synthesis of the glutamine (see below). The relationship of glutamine metabolism to citric acid metabolism is, however, from the structure chemistry point of view, analogous to the relationship of asparagine to malic acid metabolism, and although neither of these has been shown to be valid in the actual case, the analogies suggest that all of these substances are related biologically to each other, the common denominator probably being a hexose sugar.

AMIDES DERIVED FROM PROTEIN BY ENZYMATIC HYDROLYSIS

If digestion of the leaf protein occurs by the breaking of peptide bonds without decomposing the amide groups, both asparagine and glutamine may arise in the tissues without the intervention of a synthetic process. That such a possibility can actually be realized *in vitro* has been demonstrated by Damodaran (16) and by Damodaran, Jaaback, and Chibnall (17). If it be assumed, therefore, that the leaf protein which underwent hydrolysis suffered no decomposition of the amide groupings, the total quantities of glutamine and asparagine which might thus appear in soluble form can be deduced from the data plotted in Figure 13, which give the amide nitrogen of the residual protein of the leaf cells. A summary of the pertinent data is given in Table 13 in which column 2 shows the total amide nitrogen present in the tissues at the end of the culture period, and column 3, the amide nitrogen which may have been derived from the digested protein. The difference between these values in column 4 is the amide nitrogen that must have been synthesized *de novo* or have been present initially; if the fresh leaf values are deducted (column 5), the total quantities of amide nitrogen synthesized are obtained.

The amide nitrogen which may have been derived from the digestion of the protein forms a remarkably constant proportion of the total amide present, being approximately 14 percent. There is no significant difference between the light and the dark experiments. The obvious conclusion to be

drawn from the figures is that amide nitrogen of direct protein origin can account for only a relatively small part of the total amide nitrogen present.

TABLE 13. AMIDE NITROGEN CORRECTED FOR AMIDES DERIVED FROM PROTEIN DIGESTION
(Figures not otherwise designated are grams per kilo of fresh leaf)

	Total amide	Protein amide	Δ	Amide synthesized	Protein amide in % of total amide
1	2	3	4	5	6
LW	0.318	0.045	0.273	0.241	14.2
DW	0.358	0.052	0.306	0.274	14.5
LG	0.358	0.039	0.319	0.289	10.9
DG	0.383	0.054	0.329	0.299	14.1
LN	0.357	0.049	0.308	0.277	13.7
DN	0.445	0.070	0.375	0.244	15.7

A consideration of the probable chemical nature of the amide nitrogen contributed by the protein suggests that the greater part of it consists of glutamine. Although no data on the composition of isolated tobacco leaf proteins have been recorded, Miller's analysis (35) of cocksfoot protein indicates that this substance yields 13.1 percent of glutamic and 5.3 percent of aspartic acid. Proteins in general yield more glutamic acid than aspartic acid, and this may safely be assumed to be the case for the tobacco leaf protein. An interesting inference from this assumption is that the whole of the newly formed glutamine observed in the case of the leaves cultured in the dark may well have arisen from the digested protein. The data in Table 14 illustrate this; column 1 shows the total amount of amide nitrogen which may have been derived from digestion of the protein at the end of 143 hours, and column 2 shows the increase in glutamine amide nitrogen that occurred during the same period. The relative order of magnitude is approximately what would be expected if the whole of this glutamine had its origin in the protein, and it is also clear that the amount of asparagine that could have arisen in the dark cultures in this way is of the order of magnitude of 0.02 to 0.04 gm.—a negligible quantity in comparison to the total asparagine synthesis.

TABLE 14. RELATION BETWEEN AMIDE NITROGEN OF PROTEIN AND INCREASE IN GLUTAMINE AMIDE NITROGEN
(Figures not otherwise designated are grams per kilo of fresh leaf)

	Amide N from protein	Glutamine amide N	Ratio percent
DW	0.052	0.024	46
DG	0.065	0.026	40
DN	0.070	0.036	51

"EXTRA AMMONIA NITROGEN"

In addition to amide and ammonia nitrogen, extracts of *dried* tobacco leaves contain a small amount of a substance which yields ammonia on mild *alkaline* hydrolysis. The substance responsible for this is prob-

ably an artifact inasmuch as it has not been detected in fresh leaves. We have no information regarding its chemical nature, and the method of determination adopted is highly arbitrary—further investigation is needed to define the best conditions. For the present purpose, this factor was determined by subjecting a sample of dry leaf extract to hydrolysis with N acid to decompose the amides completely. The sample was then made alkaline by the addition of 3 ml. of 5 N sodium hydroxide to a total volume of 6 ml. of N acid, and the resultant alkalinity therefore represented approximately 1 N sodium hydroxide. The mixture was refluxed gently for five minutes in the distillation apparatus, the delivery tube being immersed in the absorbing acid; and the rate of heating was then increased and the ammonia was distilled for six minutes. The difference between the quantity of ammonia found under these conditions and that found if the acid hydrolyzed sample were merely neutralized with sodium hydroxide and distilled in the presence of excess alkaline borate buffer (pH 10-11) represents what has been designated "extra ammonia nitrogen". The quantities found are shown in Figure 23. It is the ammonia liberated by the short treatment at (100°) with N sodium hydroxide. Studies conducted for the purpose have shown that the quantity found is not significantly increased by a several-fold increase in the alkalinity of the solution nor by a somewhat longer period of heating.

This ammonia is not derived from amide nitrogen, inasmuch as it is not liberated by mild acid hydrolysis. It can hardly represent arginine or a guanidino or urea grouping, inasmuch as the treatment with alkali was not severe enough to decompose such compounds appreciably. Moreover, if this were the case, an increase in the apparent amount would be found if the alkalinity, or the time of heating, were increased as there is undoubtedly considerable arginine present in these extracts.

It will be recalled that determinations of the ammonium nitrogen in cold water extracts of plasmolyzed fresh leaf tissue invariably gave slightly higher results than those made upon the same tissues after being dried. The orders of magnitude of the difference between the two ammonium nitrogen values (Figure 19) and of the so-called "extra ammonia nitrogen" (Figure 23) are approximately the same—the average values for the entire series of experiments being 22 and 20 mg. respectively. It is not impossible that the ammonia which disappears during the drying of the tissue is the same as the ammonia that is liberated by the mild alkaline hydrolysis, although the agreement in amount in the present experiments may be entirely fortuitous.

NICOTINE NITROGEN

The metabolism of nicotine in the tobacco plant presents a problem that has not yet received a definite answer. Mothes (38) regards nicotine as a possible product of protein decomposition, but a chemical relation between nicotine metabolism and that of protein is by no means clear. According to this investigator, synthesis of the alkaloid occurs almost equally well in leaves grown in the dark as in normal leaves, and the proportion of nicotine in the leaves appears to be a function of age, being higher in the older leaves.

The data on the nicotine nitrogen content of the leaves subjected to culture, shown in Figure 24, do not admit of any simple generalization. When analyzed by the method of least squares, the LW, LN, and DN data

yield straight lines scarcely, if at all, inclined from the horizontal, and accordingly the individual sets of data indicate that no change in the nicotine content of these samples occurred during the culture period. The data of the LG, the DG, and the DW samples, however, give straight lines which incline appreciably in such a manner as to suggest that the nicotine content diminished slightly during the culture period, the loss being of the order of 10 to 15 percent of the amount originally present during 200 hours in each case. It seems probable that sampling errors may account for an apparent loss of this order of magnitude in view of the relatively high accuracy with which nicotine may be estimated. In any case, however, it is clear that decomposition of nicotine during culture of tobacco leaves, if it occurs at all, is an entirely minor matter, and nicotine cannot be regarded as an active metabolite.

ORGANIC ACID METABOLISM

Owing to the lack of suitable analytical methods, the changes that occur in the organic acid composition of leaves during culture have only recently been open to detailed investigation. The difficulties that beset many of the methods that have been proposed for the determination of individual acids have been discussed in previous publications from this laboratory (86, 87, 59) and there seemed little prospect of progress in this field until new and accurate methods to determine these substances had been developed. Much time has therefore been devoted to the problem, and we are now in a position to obtain accurate information regarding the total quantity of organic acids present and also with respect to the individual substances, oxalic acid, malic acid, and citric acid.

It has been shown in connection with our previous work that these three acids make up a large part of the total organic acidity of tobacco leaves. For example, in leaves of plants from 35 days to 110 days old, the three acids usually make up from 80 to 85 percent of the total acidity (92). These figures refer, however, to the entire mass of leaves growing on one plant, and include not only the mature basal leaves but also the relatively much younger top leaves. As yet we have no data on the acid distribution in the leaves of different age on a single plant. There is little doubt, however, that variations occur. Leaves of seedlings may contain as much as 35 percent of their acidity as acids other than the three mentioned (92), and wide variations in the relative proportion of unknown to known acids are readily produced by varying the conditions under which the plants have been grown (unpublished observation).

It is to be noted that the three sets of leaves employed for culture in water, in dilute glucose, and in dilute inorganic salt solutions, both in light and in dark, differed in position on the plant and in age. Accordingly, the absolute quantities of the various constituents present differed from set to set. We have been interested chiefly in the behavior of these constituents during the culture periods and, in general, have observed very little fundamental difference in this behavior in the separate sets of leaves regardless of the composition of the culture solution or of the age and origin of the leaves. Whether such differences as occur are to be attributed to the nature of the culture solution or to more fundamental differences in the tissues chosen for study remains to be determined. In the interpretations that are given below, however, these possibilities have been held in mind.

The total organic acidity of the three sets of leaves during culture is shown in Figure 25. The initial acidity of the W series was 311 milliequivalents per kilo, that of the G series was 326 and it will be recalled that these were somewhat larger leaves from a higher position on the plant. The acidity of the N series was, however, lower, being only 248 in spite of the fact that these still higher leaves were of about the same size as those of the G series.

The three cultures in light behaved somewhat differently from each other. The LW series showed a distinct increase in total acidity, amounting to more than 15 percent in 150 hours, and maintained the higher level of acidity to the end. The change was probably greater than the experimental error, and suggests that acidic substances were synthesized in appreciable amounts. The LG series, however, showed only a minor increase at 150 hours, a little over 5 percent, and the final acidity dropped to approximately the initial level. The magnitude of the change was no greater than the experimental error and the conclusion is warranted that no appreciable synthesis of organic acids occurred in spite of the fact that large quantities of carbohydrates were synthesized during this period. The same conclusion can be drawn from the LN series. Thus two of the three experiments showed no change; the third showed only a minor increase in organic acidity.

The three experiments in the dark showed a different behavior. Both the DW and DG series showed a loss of about 12 percent of the initial acidity during the entire period of culture, but the DN series showed no change. The soluble carbohydrates of the leaves diminished to a very low level during this period but it is evident that the end products were not organic acids. The experiments in general, then, indicate that any change that occurs in the total organic acidity during culture either in light or in dark is of a minor nature. Synthesis of small relative amounts appears to be possible in the light, and loss of small amounts may occur in the dark.

The data for oxalic acid in Figure 26 show a considerable degree of constancy throughout the culture period whether in light or in dark. Two samples in the DW series indicate a temporary increase of about 15 percent of oxalic acid as does one sample in the DN series. The rest of the data can be satisfactorily expressed by straight lines that depart very little from the horizontal. Oxalic acid, therefore, is apparently not involved to any appreciable extent in the metabolism of tobacco leaves during culture. This conclusion may be correlated with our previous observations (87) that upwards of 80 percent of the oxalic acid of tobacco leaves is present in a form insoluble in hot water, probably as calcium oxalate. A general conclusion that oxalic acid is wholly inactive in the leaf metabolism cannot be maintained, however, inasmuch as we have shown that appreciable quantities of this substance are withdrawn from the leaf during the maturation of the seeds (92).

The observations on malic acid, plotted in Figure 27, show a profound change in the organic acid composition of tobacco leaves when these are cultured in the dark, although little or no significant change occurred in the light. Within a period of 143 hours, 70, 64, and 64 percent of the malic acid originally present had disappeared from the tissues in the three sets of samples. This enormous change in the malic acid content implies a rapid conversion of malic acid into some other substance. The maintenance

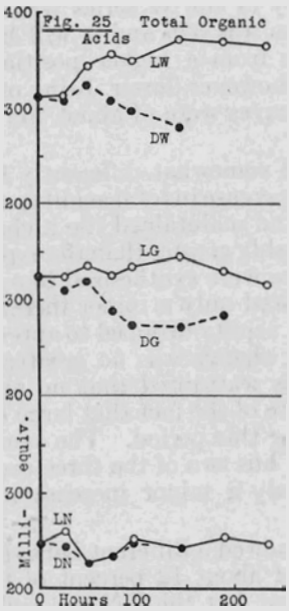


Fig. 25 Total Organic Acids
LW
DW
LG
DG
LN
DN
Milli- equiv.
0 Hours 100 200

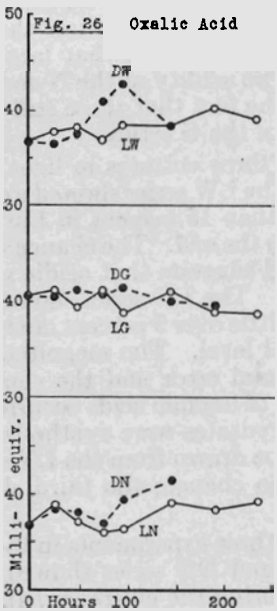


Fig. 26 Oxalic Acid
DW
LW
DG
LG
DN
LN
Milli- equiv.
0 Hours 100 200

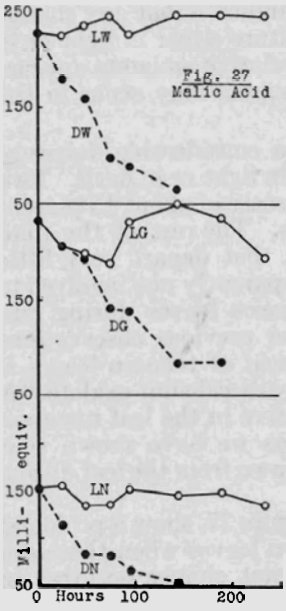


Fig. 27 Malic Acid
LW
DW
LG
DG
LN
DN
Milli- equiv.
0 Hours 100 200

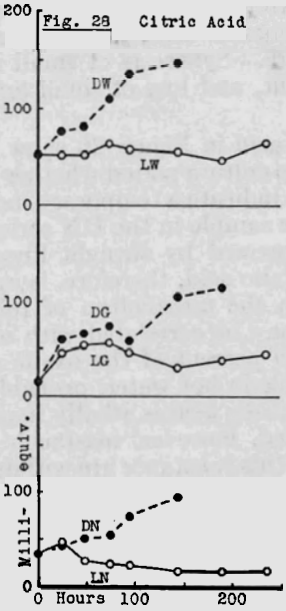


Fig. 28 Citric Acid
DW
LW
DG
LG
DN
LN
Milli- equiv.
0 Hours 100 200

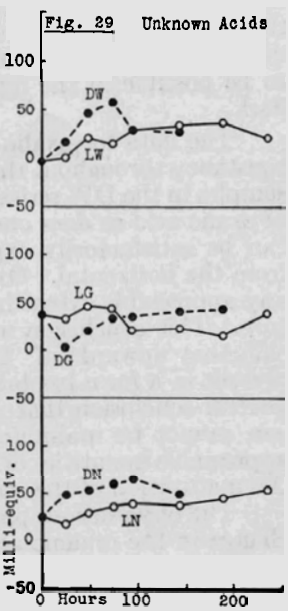


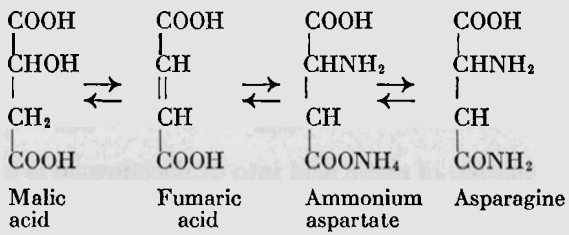
Fig. 29 Unknown Acids
DW
LW
LG
DG
DN
LN
Milli-equiv.
0 Hours 100 200

of the total organic acidity at a level that diminishes only slightly indicates that the direct or indirect product of the transformation must likewise be acid and, as will shortly appear, evidence for a contemporaneous synthesis of citric acid has been obtained.

A clearer idea of the order of magnitude of this conversion can perhaps be obtained from a consideration of the quantity of acid involved in it expressed in grams and the relationship of this to the total organic solids of the leaves.

The data of Figure 27, which are expressed in milliequivalents, can be converted to grams by multiplication by the factor 0.067. The fresh leaves of the W series contained 15.14 gm. of malic acid per kilo. Reference to Figure 3 shows that they contained 74.6 gm. of organic solids, hence approximately 20 percent of the organic solids consisted of malic acid. After being cultured in the dark for 143 hours, the leaves contained only 4.48 gm. of malic acid, and 60.0 gm. of organic solids. Thus the loss of 10.7 gm. of malic acid involved a reduction in the proportion present from 20 percent to 7.4 percent of the organic solids. The conversion of malic acid to other products therefore affected a very considerable part of the total solids of the leaves and may well be ranked as one of the most important reactions that occurred.

Before discussing the evidence regarding the fate of the malic acid, further attention should be directed to the hypothesis mentioned in a preceding section which is occasionally suggested to account for the synthesis of asparagine in leaves, namely that asparagine arises through the interaction of malic acid or some transformation product of malic acid and ammonia. This is a change which involves loss of water and addition of ammonia, the last step being the dehydration of the ammonium salt to the amide.

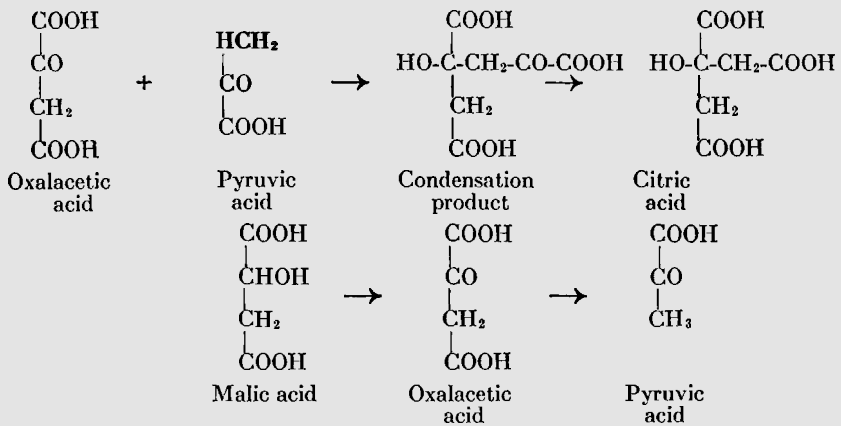


The data in Table 15 give the magnitudes of the quantities of asparagine formed during the entire period of culture in the dark. In column 4 are given the equivalent quantities of malic acid on the assumption that this was the origin of the asparagine, and column 5 gives the total quantity of malic acid that disappeared. The last column indicates that considerably more malic acid disappeared than can be accounted for by the asparagine that was formed. Thus the conversion of malic acid to asparagine, while possible on the basis of the quantities involved, accounts for only a small part of the change in the malic acid that took place. In any case it is necessary to find other reactions to account for the disappearance of most of the malic acid, and it is also necessary to account for the fact that the total acidity did not change significantly.

TABLE 15. RELATIONSHIP BETWEEN ASPARAGINE AMIDE NITROGEN AND MALIC ACID
(Figures are grams per kilo of fresh leaf)

	Asparagine amide N	Asparagine increase	Malic acid equivalent	Actual malic acid loss	Ratio 5:4
1	2	3	4	5	6
DW	0.302	2.85	2.89	9.65	3.34
DG	0.285	2.69	2.73	10.6	3.67
DN	0.383	3.61	3.67	6.5	1.77

The most probable fate of the malic acid, according to the data we have obtained, is a conversion into citric acid. The possibility that such a reaction may occur has recently been pointed out by Knoop and Martius (25), who have shown that condensation occurs when pyruvic acid is allowed to react in alkaline solution at room temperature with oxalacetic acid for some time. If the mixture is then oxidized with hydrogen peroxide under proper conditions, a yield of 35 percent of citric acid may be obtained. The reaction may be formulated as follows, the oxidation step being designed to decarboxylate the ketonic acid condensation product.



The transformation of malic acid into oxalacetic acid is a well known reaction brought about by malic dehydrogenase, an enzyme widely distributed in animal organs and also found in plant seeds (23, 84). Pyruvic acid may arise from oxalacetic acid by decarboxylation, and it is also a well known product of carbohydrate metabolism. Neuberg and Kobel (43) have demonstrated the presence of an enzyme in pea sprouts which transforms the phosphoglyceric acid derived from the fermentation of glucose into pyruvic acid. Accordingly the biochemical reactions necessary for the formation of citric acid from malic acid are well established possibilities.

The data which lend a certain degree of probability to this hypothesis of the fate of the malic acid in cultured tobacco leaves are shown in Figure 28. It is clear that little or no change took place in the citric acid content of the leaves cultured in light. The LG series, the initial value of which was unusually low, show an indication of a small synthesis at the start of the experiment, but this may be only an expression of a sampling error

in the fresh leaf samples; no significant change occurred after the first day. The LN series suggest a slight loss early in the experiment but constancy thereafter. The three sets of leaves cultured in the dark, however, give evidence of a prompt synthesis of citric acid which continued throughout the experimental period. The quantities involved, expressed in grams, are shown in Table 16.

If it be assumed that the citric acid is formed from malic acid according to the transformations implied in the equations of Knoop and Martius, it is clear that 2 mols of malic acid yield 1 mol of citric acid, a theoretical yield of 71.6 percent. The citric acid gain has been calculated on this assumption (column 5), and the actual citric acid gain is compared in the last column with the calculated. The agreement of the three experiments is surprisingly good and the yield is suggestively close to the theoretical requirement. This must not be understood as evidence in favor of the hypothesis, however. In making the calculation, it is assumed that both oxalacetic acid and pyruvic acid arose from transformations of the malic acid. This is not at all necessary inasmuch as other possible sources of pyruvic acid are well recognized.

TABLE 16. RELATION BETWEEN MALIC AND CITRIC ACIDS
(Figures not otherwise designated are grams per kilo of fresh leaf)

	Citric acid gain	Malic acid loss	Ratio citric : malic acids	Citric acid gain calculated	Yield in percent
DW	6.20	10.66	0.582	7.63	81.3
DG	5.82	9.92	0.586	7.10	81.9
DN	3.91	6.54	0.598	4.68	83.4

That the citric acid formed can, in fact, exceed the quantity calculated from the malic acid lost is evident from data of an earlier experiment on water culture of tobacco leaves in the dark. This particular experiment was carried out under essentially the same conditions and likewise showed evidence of transformation of malic to citric acid. The data necessary for comparison are shown in Table 17, the last two columns showing the changes that occurred expressed in grams during the periods designated.

The theoretical yield of citric acid, calculated on the above assumptions from the decrease in malic acid at 159 hours, is 5.11 x 0.716 = 3.66 gm. which exactly agrees with the actual yield of citric acid. The calculated yield at 279 hours is 6.31 gm. which is materially less than the actual yield of 7.58 gm. and implies that some source of pyruvic acid other than malic acid must have been drawn upon, if the general outline of the series of reactions given above is to serve as an explanation of the experimental facts.

TABLE 17. WATER CULTURE OF TOBACCO LEAVES IN THE DARK
(Figures are milliequivalents or grams per kilo of original fresh weight)

	Fresh leaf m.e.	159 hrs. m.e.	279 hrs. m.e.	Δ 159 hrs. gm.	Δ 279 hrs. gm.
Citric acid	23.2	80	141.6	3.63	7.58
Malic acid	177.2	100.9	45.6	5.11	8.82

Accordingly, although our results are strongly in favor of a transformation of malic into citric acid during culture of tobacco leaves in the dark, and a mechanism to account logically for this transformation has been suggested, the matter must still be regarded as a speculation which, however, conforms fairly closely with the experimental data.

Certain clear inferences may be drawn, however. It is obvious that malic acid loss and citric acid gain are connected in some way, direct or indirect. There is no significant loss of malic acid in the light, and correspondingly no gain of citric. Where there is a loss of malic, as in the dark experiments, the ratio of citric acid gain is 0.582, 0.586, and 0.598 in the three experiments at 143 hours. This constancy in the relationship between the quantities of the two acids is an argument against the view that malic acid is associated with amide synthesis. It will be recalled that different proportions of the malic acid would be required in each case to account for the asparagine formed, the third experiment in particular being quite different from the other two. There could hardly be a constant relationship in all three experiments between malic and citric acids if a part of the malic acid had been utilized for asparagine synthesis, this part being different in each case.

It is of some interest to indicate the relationship between the gains of citric acid in the dark cultures and the organic solids of the tissues. The data in Table 18 show that citric acid occurs in moderate proportions in fresh tobacco leaf tissue making up from 1 to over 4 percent of the organic solids. After culture in the dark, however, the concentration of citric acid in terms of organic solids increases very materially and this substance assumes major importance as a constituent. The increase of 6 gm. of citric acid in the DW experiment, associated with the loss of 15 gm. of organic solids, gives rise to a condition in which citric acid alone ultimately accounts for one-eighth of the organic solids. It will be recalled that the malic acid, expressed in these same terms, diminished from 20 to 7 percent of the organic solids, and it is clear that the conversions that the two acids undergo are of great quantitative importance. The profound changes that occur when the leaves are cultured in the dark suggest that these acids are among the most reactive metabolites of the tissues under such conditions.

TABLE 18. RELATION BETWEEN CITRIC ACID AND ORGANIC SOLIDS
(Figures not otherwise designated are grams per kilo of fresh leaf)

	Citric acid in fresh leaf	Citric acid at 143 hrs.	Increase	Organic solids in fresh leaf	Organic solids at 143 hrs.	Percentage citric acid in fresh leaf	Percentage citric acid at 143 hrs.
DW	3.25	9.45	6.20	74.6	60.0	4.37	15.7
DG	1.02	6.84	5.82	88.6	66.2	1.15	10.3
DN	2.23	6.14	3.91	76.2	72.5	2.92	8.5

The changes in the unknown acids plotted in Figure 29 indicate that, in two of the three experiments, an increase took place both in light and in dark, the increase in the dark being somewhat greater than that in the light. The glucose culture experiment did not show evidence of any consistent change. These data include the experimental errors of all the individual determinations of the acids in addition to the sampling error and, in

any case, the changes are small relative to the total organic acids, being of the order of 5 to 10 percent of this, save in the DN experiment. In this, the unknown acids increased from a level of 10 to 26 percent of the total acidity and it is possible that this has significance.

In general, one might expect the curves for unknown organic acids to indicate an increase. If the amino acids that result from the digestion of the protein undergo deamination, as seems to be the case from the evidence on amide synthesis, the residual carbon compounds should be essentially acidic in nature and a portion of them at least should become evident on the curve for unknown organic acids. The order of magnitude of this effect can be deduced from the data for protein nitrogen. The effects on the curves for unknown organic acids in the DW and DN cultures are mainly within the 95-hour period. From Figure 5 it can be seen that 0.87 and 0.73 gm. of protein nitrogen were digested in this period in these two experiments. The quantities of protein represented are therefore of the order of 5.4 and 4.5 gm. Deducting the nitrogen leaves roughly 4.5 and 3.8 gm. of organic solids to be accounted for in terms of unknown organic acids. There is no way to calculate the actual quantity of substance represented by an increase in 30 milliequivalents of unknown organic acids, which is the approximate value of the change that occurred in the unknown acidity in the DW and DN experiments during 95 hours; but if it be assumed that the acidic substances have a molecular weight of the order of that of malic acid (134) and are monobasic, the factor is 0.13, whereas if they have half this molecular weight, it is 0.06. Thus the quantity of acidic substances represented by 30 milliequivalents may be estimated to be within the limits 3.9 gm. and half this amount, and it may be concluded that the increase in unknown organic acids is of the same order of magnitude as the quantity of acidic organic residues which might be expected to be derived from protein decomposition. Such inferences are as yet, however, highly speculative and only merit attention inasmuch as they represent a possibility in certain cases.

Calculations of the distribution of the total acidity among the various organic acids serve to emphasize the relative constancy of the malic, citric, and oxalic acids during culture in the light, and the magnitude of the apparent interconversion of malic to citric acid during culture in the dark. Table 19 gives the average and the minimal and maximal values. The average values for malic and citric acid in the three dark experiments have no significance on account of the magnitude of the changes that occurred, but it is clear that the oxalic acid was substantially constant in all experiments, and that the malic and citric acids were substantially constant in the three light experiments.

TABLE 19. ORGANIC ACIDS AS PERCENT OF TOTAL ORGANIC ACIDITY

	Oxalic acid			Malic acid			Citric acid		
	Av.	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.
LW	11.0	12.1	10.3	67.9	72.8	64.4	15.7	17.5	12.7
DW	12.7	13.6	11.5	(44.2)	72.8	23.8	(33.4)	52.6	16.3
LG	12.1	12.6	11.5	65.6	72.7	57.7	12.4	16.7	4.9
DG	13.7	15.1	12.4	(51.3)	71.1	29.8	(24.5)	40.8	4.9
LN	15.4	16.3	14.5	59.1	60.8	55.1	10.8	18.2	7.2
DN	16.1	17.2	14.9	(38.1)	60.8	22.2	(24.5)	39.8	14.1

CARBOHYDRATE METABOLISM

The total soluble reducing carbohydrate calculated as glucose is shown in Figure 30. The curves indicate a rapid synthesis of soluble carbohydrate in light at a constant rate in each case. The leaves in the LW experiment synthesized approximately 16 gm. in 190 hours. The LG leaves, which started at a slightly higher initial value, synthesized 9.4 gm. in the same period. Although the leaves in the LN series were somewhat flaccid throughout the period of study, they synthesized almost the same amount, viz. 8.3 gm.

It is an unfortunate chance that the leaves employed for culture on glucose solution should have contained a high proportion of reducing carbohydrate at the start. Owing to this, it is not possible to demonstrate an absorption of glucose from the culture solution, and, if any absorption did take place, it is not evident from a comparison of the slopes of the LW and the LG curves. Nor is there any apparent lag in the rate of disappearance of the carbohydrate in the dark experiments. The picture presented is clearly one of photosynthesis in light, and decomposition of carbohydrate in the dark, both reactions proceeding at relatively smooth rates.

The curves for fermentable sugar, in Figure 31, call for little additional comment. The greater part of the reducing carbohydrate is fermentable, and the increase of sugar in light and decrease in the dark follow smooth curves. There is no indication of an increased sugar metabolism on the glucose medium—in fact the quantities involved in the changes that ocurred are distinctly less than those in the leaves in water culture.

The curves for unfermentable carbohydrate in Figure 32 present a phenomenon of considerable interest. The magnitude measured is the total reducing carbohydrate less the fermentable sugar. There is an appreciable amount of this type of material in normal tobacco leaves, and the recent observations of Schlenker (70) indicate it is also present in other species. It is obvious that a prompt synthesis began in the leaves cultured in light and continued for about 100 hours in both the LW and LG series; subsequently very little more of this component accumulated. In the LN series, possibly owing to the wilting of the leaves with consequent closure of the stomata, the rate of synthesis was slower and the maximal amount corresponding to that observed in the other two experiments was not reached.

The three dark experiments show a slight diminution of the unfermentable carbohydrate in the DW experiment, practically no change in the DN experiment, but a slow and steady loss in the DG experiment. This was the only specimen of leaves that contained more than traces of this component in the fresh condition and its behavior is precisely similar to that noted in our earlier work (93).

Attention has already been directed in the section on organic solids to the fact that the synthesis of total solids far exceeded the synthesis of soluble carbohydrates. In view of the quantities of substance involved, this probably means that carbohydrates entered into other reactions subsequent to their formation. Thus, for example, in the LW series approximately 13 gm. of fermentable sugar and 3.5 gm. of unfermentable carbohydrate were formed in 190 hours, but the total organic solids increased about 36 gm., suggesting that the total synthesis of sugar must have been about twice that indicated by the determination of total reducing power. The nature of the products of these later conversions is problematical.

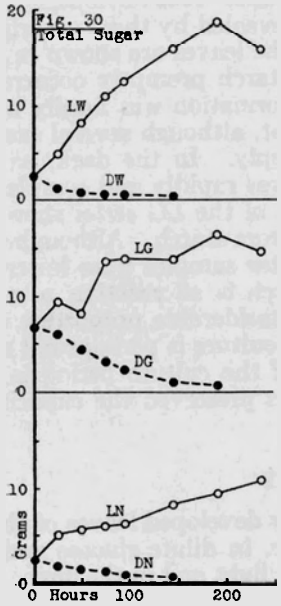


Fig. 30
Total Sugar
LW
DW
LG
DG
LN
DN
Grams
0 Hours 100 200

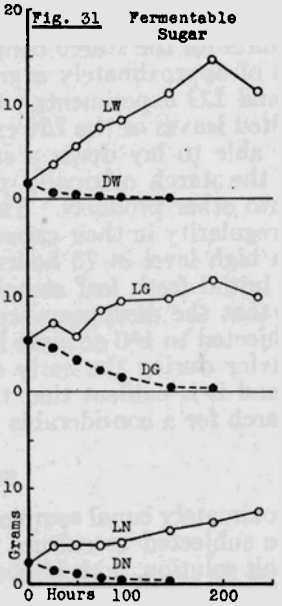


Fig. 31 Fermentable Sugar
LW
DW
LG
DG
LN
DN
Grams
0 Hours 100 200

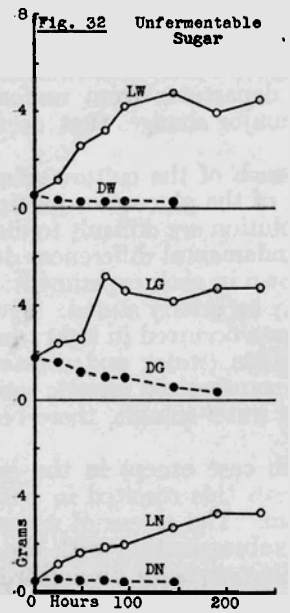


Fig. 32 Unfermentable Sugar
LW
DW
LG
DG
LN
DN
Grams
0 Hours 100 200

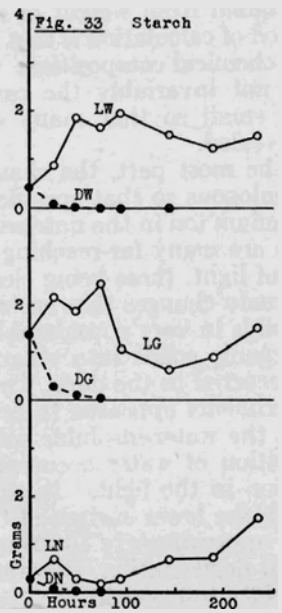


Fig. 33 Starch
LW
DW
LG
DG
LN
DN
Grams
0 Hours 100 200

Determinations of hemi-celluloses, pectins, uronic acids, and other substances of this general nature were not made. It is obvious, however, that a wide field for further investigation is revealed by these experiments.

The data for the starch content of the leaves are shown in Figure 33. Synthesis of approximately a gram of starch promptly occurred in both the LW and LG experiments. Starch formation was hardly appreciable in the wilted leaves of the LN experiment, although several samples were evidently able to lay down a small supply. In the dark, as would be expected, the starch originally present was rapidly and completely metabolized into other products. The leaves of the LG series showed a good deal of irregularity in their capacity to store starch. Although the starch reached a high level in 73 hours, the later samples were lower in starch than the initial fresh leaf sample. Starch is so reactive a metabolite, however, that the disappearance of a considerable proportion of it from leaves subjected to 100 or more hours of culture is perhaps not surprising. The behavior during the early stages of the culture period is more significant, and it is evident that the leaves preserved the capacity to synthesize starch for a considerable period.

SUMMARY

Approximately equal samples of fully developed leaves of the tobacco plant were subjected to culture in water, in dilute glucose and in dilute nutrient salt solution, both in continuous light and in the dark. Analyses of samples removed from time to time show the progress of many of the chemical changes that take place under these conditions. The analytical data are expressed on a common basis of grams of each constituent per kilo of original fresh weight of leaf tissue. The assumption involved in this method of calculation is that each sample of leaves originally possessed the same chemical composition. Although the results show that this was probably not invariably the case, the departures from uniformity are relatively small so that many of the major changes that occurred are clearly revealed.

For the most part, the changes in each of the culture solutions are closely analogous so that specific effects of the glucose or particularly of the ammonium ion in the nutrient salt solution are difficult to distinguish. But there are many far-reaching and fundamental differences due to the influence of light, these being clearly shown in each experiment.

The main changes that occurred may be briefly stated. Synthesis of organic solids in very considerable amounts occurred in light; decomposition of organic solids into volatile products (water and carbon dioxide chiefly) occurred in the dark. The newly synthesized organic solids in the light experiments appeared to be mainly water-soluble, there being little change in the water-insoluble solids.

Inbibition of water occurred in each case except in the culture on salt solution in the light. In several cases this resulted in guttation of water from the lower surface of the leaves. The excess of water was retained for approximately 100 hours, but subsequently, with the onset of chlorophyll degeneration, the water holding capacity diminished and the leaves rapidly lost weight.

Digestion of protein with the production of amino acids proceeded rapidly and at approximately the same rate for 72 hours in all experiments. Later, the rate of digestion diminished in the leaves cultured in light, but

was maintained in the leaves cultured in the dark. Corresponding with the digestion of the protein, there was an increase in the quantity of water-soluble nitrogen, and the composition of the water-soluble fraction underwent material change. These changes have chiefly to do with the amide metabolism of the leaves. Ammonia accumulated in notable amounts towards the end of the experiment in the leaves cultured in the dark, but there was only a moderate increase in free ammonia in the leaves in the light. The synthesis of the two amides, asparagine and glutamine, appears to be closely associated with this behavior. Very considerable amounts of asparagine accumulated during dark culture, but the amount of glutamine formed was small. On the other hand, both amides were rapidly synthesized in the light, the quantity of glutamine appreciably exceeding that of asparagine. The total amide synthesis at any point in light was materially less than the total amide synthesis in the dark.

The observations can be accounted for according to the detoxication hypothesis of Prianischnikow by the assumption that amide synthesis is a result of the liberation of ammonia in the tissues. The precursor necessary for the formation of asparagine was already present in the tissues at the beginning of the experiment and was drawn upon for the formation of asparagine during culture in the dark. Nevertheless there was an inadequate supply of this hypothetical substance present to care for all of the ammonia produced, and consequently, after 100 hours, the excess of ammonia accumulated. There was little, if any, of the precursor necessary for the synthesis of glutamine present and little of this amide was formed. In the light, however, photosynthetic reactions provided a bountiful supply of the precursor of glutamine with the result that this amide was synthesized in rather greater amount than asparagine, and the total amide metabolism was competent to care for all of the ammonia produced in the tissues so that little accumulated.

With respect to the source of the ammonia, it is shown that the total nitrogen of the two amides may have arisen from ammonia produced in two ways. If it is assumed that oxidative deamination of amino acids occurred, and that reduction of nitrate to ammonia likewise took place, the whole of the nitrogen of the amides, together with the excess of free ammonia, can be accounted for from the disappearance of amino nitrogen of amino acids derived originally from the protein by enzymatic digestion, and from the disappearance of nitrate nitrogen. The sums of the two respective quantities agree within the experimental error. The data, therefore, support the ammonia detoxication view of the origin of amides and account with some accuracy for the actual quantities of amides produced.

No information with respect to the chemical nature of the non-nitrogenous precursors of the amides has been obtained with the exception of the demonstration that the precursor of glutamine is synthesized during culture in the light, and is, therefore, probably a carbohydrate, or a metabolite of a carbohydrate.

The total organic acidity of the tissues changed but little during culture, there being evidence for a slight increase in light and a slight decrease in the dark. The oxalic acid content also remained essentially constant throughout. Although both malic and citric acids remained constant during culture in light, the quantity of malic acid diminished very rapidly in the dark, the change being one of the most extensive in terms of actual

quantity of substance involved that has been noted. Contemporaneously with the rapid decrease of malic acid, there was an increase in citric acid of equivalent significance. The evidence points to a direct or indirect conversion of malic to citric acid under these conditions, and certain suggestions as to possible chemical mechanisms to account for the interconversion have been advanced.

Both fermentable sugar and unfermentable carbohydrate were synthesized throughout the period of culture in the light, although both disappeared fairly completely from the tissues during culture in the dark. The total quantity of carbohydrate synthesized accounts, however, for considerably less than the total increase in organic solids, and it is inferred that a substantial part of the newly synthesized products of photosynthesis were converted into other and non-reducing metabolites. It is reasonable to assume that the total increase in organic substance is a measure of the total photosynthesis, consequently the difference between this increase and the measured increase in the reducing carbohydrate represents the extent of metabolism of the carbohydrate subsequent to its synthesis.

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TABLE 20. COMPOSITION OF TOBACCO LEAVES DURING CULTURE
(Figures not otherwise designated are grams per kilo of fresh leaf)

Hours	0	25	49	73	95	143	190	235
Water (Figure 1)								
LW	908	947	944	947	963	871	947	925
DW	908	986	994	1011	966	764		
LG	894	989	984	968	940	881	789	712
DG	894	1027	1037	1030	1008	942	789	
LN	905	838	830	831	787	717	740	735
DN	905	940	925	925	904	775		
Total solids (Figure 2)								
LW	92.0	96.0	107.7	112.4	119.6	117.9	127.1	128.4
DW	92.0	89.8	87.8	86.4	86.3	76.9		
LG	106.2	119.2	111.5	125.0	116.5	120.0	120.0	118.9
DG	106.2	106.2	103.1	96.9	95.4	85.8	85.5	
LN	94.8	105.0	102.8	104.9	104.7	109.3	113.1	116.0
DN	94.8	97.5	93.5	89.3	88.9	87.4		
Organic solids (Figure 3)								
LW	74.6	78.8	89.7	95.1	102.4	100.8	109.6	110.3
DW	74.6	70.2	68.9	66.1	65.7	60.0		
LG	88.6	100.3	93.2	107.3	98.0	100.5	102.1	101.2
DG	88.6	86.9	83.8	77.0	75.9	66.2	66.3	
LN	76.2	88.1	85.6	86.2	87.3	91.9	95.2	97.8
DN	76.2	81.2	77.9	73.9	73.1	72.5		
Inorganic solids (ash) (see p. 777)								
LW	17.5	17.3	18.0	17.4	17.2	17.1	17.5	18.1
DW	17.5	19.6	18.9	20.3	20.6	16.9		
LG	17.6	18.9	18.3	17.7	18.5	19.5	17.9	17.7
DG	17.6	19.7	19.3	19.8	19.5	19.7	19.1	
LN	18.6	16.9	17.2	18.7	17.4	17.4	17.9	18.2
DN	18.6	16.3	15.7	15.4	15.8	14.8		
Total nitrogen (Figure 4)								
LW	4.03	3.97	3.95	3.66	4.07	3.72	4.11	4.16
DW	4.03	3.94	4.11	4.15	4.16	3.53		
LG	4.43	5.19	4.45	4.56	4.11	4.56	4.23	4.23
DG	4.43	4.67	4.57	4.42	4.46	3.86	3.92	
LN	4.77	5.24	4.84	5.12	5.04	4.99	5.30	5.34
DN	4.77	4.98	4.99	4.81	4.86	4.80		
Total protein nitrogen (Figure 5)								
LW	2.27	2.13	1.92	1.75	1.89	1.59	1.65	1.56
DW	2.27	2.09	1.98	1.71	1.40	1.00		
LG	2.35	2.75	2.27	2.27	1.83	1.79	1.35	1.29
DG	2.35	2.45	2.34	2.07	1.92	1.20	1.11	
LN	2.61	2.81	2.32	2.31	2.09	2.08	1.88	1.89
DN	2.61	2.54	2.44	2.17	1.88	1.45		
Soluble nitrogen (Figure 6)								
LW	1.55	1.64	1.75	1.86	2.01	2.09	2.31	2.49
DW	1.55	1.69	1.75	2.24	2.62	2.38		
LG	1.75	1.90	1.89	2.04	2.02	2.14	2.70	2.77
DG	1.75	1.95	1.99	2.15	2.34	2.55	2.73	
LN	1.85	1.95	2.23	2.69	2.47	2.83	3.23	3.21
DN	1.85	2.10	2.22	2.54	2.82	3.23		

Hours	0	25	49	73	95	143	190	235
Amino nitrogen of fresh leaf extract (Figure 7)								
LW	0.137	0.197	0.326	0.441	0.580	0.518	0.636	0.737
DW	0.137	0.224	0.370	0.582	0.666	0.699		
LG	0.147	0.253	0.363	0.500	0.472	0.589	0.751	0.834
DG	0.147	0.240	0.373	0.434	0.514	0.764	0.673	
LN	0.172	0.323	0.393	0.533	0.623	0.618	0.809	0.928
DN	0.172	0.267	0.360	0.536	0.647	0.899		
Amino nitrogen of dry leaf extract (Figure 8)								
LW	0.135	0.207	0.251	0.237	0.271	0.358	0.426	0.484
DW	0.135	0.235	0.333	0.530	0.671	0.557		
LG	0.149	0.159	0.247	0.305	0.323	0.401	0.505	0.520
DG	0.149	0.237	0.303	0.398	0.520	0.641	0.658	
LN	0.145	0.308	0.354	0.503	0.525	0.616	0.714	0.777
DN	0.145	0.273	0.375	0.476	0.658	0.882		
Amino nitrogen of fresh leaf extract minus amino nitrogen of dry leaf extract (Figure 9)								
LW	0.002	−0.010	0.075	0.204	0.309	0.160	0.210	0.253
DW	0.002	−0.011	0.037	0.052	−0.005	0.142		
LG	−0.002	0.094	0.116	0.195	0.149	0.188	0.246	0.314
DG	−0.002	0.003	0.070	0.036	−0.006	0.123	0.015	
LN	0.027	0.015	0.039	0.030	0.098	0.002	0.095	0.151
DN	0.027	−0.006	−0.015	0.060	−0.011	0.017		
Peptide nitrogen of dry leaf extract (Figure 10)								
LW	0.106	0.091	0.156	0.220	0.285	0.223	0.250	0.292
DW	0.106	0.106	0.098	0.059	0.049	0.083		
LG	0.129	0.110	0.179	0.226	0.242	0.240	0.260	0.248
DG	0.129	0.118	0.125	0.129	0.119	0.113	0.093	
LN	0.126	0.086	0.144	0.168	0.138	0.148	0.179	0.114
DN	0.126	0.101	0.074	0.095	0.101	0.045		
Insoluble organic solids (see p. 789)								
LW	46.6	43.8	46.7	47.6	51.6	43.7	48.2	45.1
DW	46.6	42.9	41.0	40.2	38.6	36.9		
LG	48.7	59.1	51.9	55.5	46.3	47.4	47.0	46.5
DG	48.7	50.4	49.4	46.9	45.4	39.1	39.1	
LN	49.0	52.5	49.2	48.5	43.8	50.4	47.9	48.6
DN	49.0	54.2	51.6	47.6	45.8	44.3		
Humin nitrogen of extracted residue (Figure 11)								
LW	0.146	0.120	0.146	0.131		0.157	0.156	0.156
DW	0.146	0.162	0.152	0.144	0.136	0.0832		
LG	0.148	0.175	0.182	0.182	0.166	0.132	0.134	0.137
DG	0.148	0.206	0.180	0.159	0.180	0.124	0.141	
LN	0.184	0.167	0.143	0.158	0.152	0.178	0.147	0.148
DN	0.184	0.175	0.169	0.162	0.154	0.157		
Amino nitrogen of extracted residue (Figure 12)								
LW	1.36	1.20	1.13	1.06	1.09	0.889	0.953	0.840
DW	1.36	1.31	1.28	1.12	0.826	0.690		
LG	1.46	1.76	1.50	1.36	1.10	1.06	0.810	0.767
DG	1.46	1.58	1.43	1.25	1.21	0.674	0.669	
LN	1.56	1.80	1.44	1.40	1.23	1.17	1.17	1.05
DN	1.56	1.67	1.46	1.27	1.04	0.778		

Hours	0	25	49	73	95	143	190	235
Amide nitrogen of extracted residue (Figure 13)								
LW	0.148	0.117	0.130	0.124	0.127	0.105	0.113	0.103
DW	0.148	0.146	0.145	0.136	0.116	0.0960		
LG	0.152	0.177	0.160	0.158	0.128	0.127	0.111	0.113
DG	0.152	0.167	0.158	0.142	0.140	0.0872	0.0980	
LN	0.167	0.183	0.156	0.138	0.125	0.134	0.126	0.118
DN	0.167	0.177	0.158	0.148	0.127	0.0968		
Amide nitrogen of alcohol residue (Figure 14)								
LW	0.150	0.140	0.141	0.136	0.152	0.144	0.156	0.156
DW	0.150	0.160	0.166	0.220	0.233	0.159		
LG	0.165	0.197	0.167	0.182	0.136	0.135	0.137	0.152
DG	0.165	0.174	0.184	0.207	0.237	0.234	0.235	
LN	0.189	0.201	0.176	0.186	0.183	0.174	0.169	0.154
DN	0.189	0.186	0.209	0.175	0.233	0.269		
Amide nitrogen in percent of protein nitrogen (Figure 15)								
LW	6.51	5.51	6.76	7.09	6.73	6.59	6.85	6.56
DW	6.51	6.98	7.34	7.93	8.25	9.60		
LG	6.47	6.45	7.03	6.97	7.00	7.12	8.20	8.69
DG	6.47	6.79	6.74	6.87	7.32	7.28	8.82	
LN	6.40	6.51	6.71	5.97	5.95	6.44	6.69	6.23
DN	6.40	6.97	6.48	6.84	6.78	6.67		
Amide nitrogen in percent of nitrogen of alcohol residue (Figure 16)								
LW	6.60	6.59	7.34	7.77	8.04	9.01	9.45	10.0
DW	6.60	7.64	8.37	12.87	16.63	15.92		
LG	7.05	7.16	7.33	8.03	7.47	7.53	10.09	11.78
DG	7.05	7.10	7.84	10.00	12.37	19.56	21.1	
LN	7.22	7.16	7.58	8.05	8.72	8.35	8.96	8.18
DN	7.22	7.32	8.54	8.08	12.39	18.52		
Nitrate nitrogen (Figure 17)								
LW	0.652	0.660	0.604	0.574	0.556	0.446	0.493	0.454
DW	0.652	0.618	0.611	0.827	0.857	0.580		
LG	0.789	0.826	0.646	0.612	0.609	0.532	0.457	0.575
DG	0.789	0.888	0.984	0.781	0.771	0.819	0.700	
LN	0.870	0.678	0.917	0.919	0.725	0.765	0.837	0.748
DN	0.870	0.959	0.899	1.000	0.998	0.932		
Ammonia nitrogen of fresh leaf extract (Figure 18)								
LW	0.0126	0.0239	0.0253	0.0378	0.0352	0.0478	0.0367	0.0501
DW	0.0126	0.0151	0.0239	0.0419	0.101	0.246		
LG	0.0162	0.0221	0.0249	0.0390	0.0375	0.0592	0.0418	0.0629
DG	0.0162	0.0195	0.0202	0.0228	0.0239	0.128	0.330	
LN	0.0147	0.0601	0.0746	0.0745	0.0721	0.0813	0.0931	0.126
DN	0.0147	0.0434	0.0473	0.0776	0.0811	0.195		
Ammonia nitrogen produced by drying (Figure 19)								
LW	0.00618	0.00218	0.00245	0.0205	0.0241	0.0247	0.0156	0.0318
DW	0.00618	0.00146	0.00734		0.0056	0.0352		
LG	0.00617	0.00299	0.00566	0.0220	0.0223	0.0388	0.0224	0.0423
DG	0.00617	0.00221	0.00387	0.00610	0.00082	0.0615	0.0444	
LN	0.00438	0.0203	0.0334	0.0228	0.0352	0.0303	0.0405	0.0507
DN	0.00438	0.0174	0.0116	0.0338	0.0185	0.0280		

Hours	0	25	49	83	95	143	190	235
Asparagine amide nitrogen (Figure 20)								
LW	0.0193	0.0197	0.0247	0.0399	0.0649	0.0621	0.0882	0.137
DW	0.0193	0.0253	0.0693	0.158	0.269	0.321		
LG	0.0200	0.0622	0.0373	0.0613	0.0661	0.0938	0.120	0.156
DG	0.0200	0.0294	0.0413	0.108	0.157	0.305	0.377	
LN	0.0254	0.0408	0.0461	0.0761	0.155	0.0968	0.119	0.108
DN	0.0254	0.0523	0.0916	0.152	0.224	0.408		
Glutamine amide nitrogen (Figure 21)								
LW	0.0124	0.0234	0.0686	0.0590	0.0834	0.112	0.141	0.181
DW	0.0124	0.0203	0.0234	0.0563	0.0508	0.0368		
LG	0.0100	0.00288	0.0533	0.0677	0.0832	0.0950	0.183	0.202
DG	0.0100	0.0440	0.0531	0.0496	0.0656	0.0363		
LN	0.0054	0.0572	0.0660	0.107	0.0889	0.186	0.240	0.249
DN	0.0054	0.0305	0.0309	0.0247	0.0496	0.0418		
Non-protein nitrogen of alcohol residue (Figure 22)								
LW	0.106	0.030	0.222	0.212	0.234	0.169	0.233	0.185
DW	0.106	0.214	0.259	0.355	0.401	0.295		
LG	0.152	0.184	0.268	0.267	0.133	0.219	0.269	0.350
DG	0.152	0.242	0.323	0.359	0.455	0.325	0.543	
LN	0.210	0.238	0.265	0.235	0.226	0.254	0.335	0.237
DN	0.210	0.330	0.349	0.417	0.412	0.546		
Extra ammonia nitrogen (Figure 23)								
LW	0.00266	0.0199	0.0111	0.0296	0.0321	0.0362	0.0324	0.0368
DW	0.00266	0.0112	0.00741	0.000	0.0219	0.0320		
LG	0.00867	0.0196	0.0151	0.0382	0.0316	0.0269	0.0305	0.0290
DG	0.00867	0.000	0.00430	0.0139	0.0227	0.0317	0.0630	
LN	0.00412	0.0183	0.0254	0.0289	0.0215	0.0355	0.0416	0.0475
DN	0.00412	0.0110	0.0155	0.0370	0.0257	0.0143		
Nicotine nitrogen (Figure 24)								
LW	0.180	0.187	0.194	0.175	0.176	0.198	0.171	0.184
DW	0.180	0.197	0.189	0.168	0.188	0.158		
LG	0.235	0.225	0.193	0.226	0.185	0.197	0.198	0.195
DG	0.235	0.219	0.214	0.201	0.212	0.187	0.194	
LN	0.225	0.277	0.233	0.211	0.221	0.237	0.212	0.252
DN	0.225	0.246	0.218	0.228	0.227	0.235		
Total organic acids in milliequivalents (Figure 25)								
LW	310.8	312.1	344.8	357.2	349.3	372.5	370.1	366.7
DW	310.8	307.7	325.1	308.4	298.9	280.9		
LG	326.6	325.4	337.1	326.3	335.9	346.4	330.1	316.2
DG	326.6	308.0	319.4	288.7	273.7	271.6	285.2	
LN	248.4	259.0	227.8	233.6	250.5	241.3	252.2	247.5
DN	248.4	243.5	225.4	233.8	246.6	241.1		
Oxalic acid in milliequivalents (Figure 26)								
LW	36.8	37.7	38.2	36.8	38.4	38.3	40.3	39.1
DW	36.8	36.4	37.5	41.0	42.7	38.2		
LG	40.6	41.1	39.3	41.1	38.7	41.0	38.8	38.7
DG	40.6	40.4	41.1	40.7	41.3	40.1	39.7	
LN	36.9	39.0	37.2	36.1	36.3	39.2	38.5	39.3
DN	36.9	38.4	38.0	37.0	39.5	41.4		

Hours	0	25	49	73	95	143	190	235
Malic acid in milliequivalents (Figure 27)								
LW	226.2	223.9	235.0	241.8	225.0	244.7	245.7	243.3
DW	226.2	178.9	159.0	97.7	89.7	66.8		
LG	232.1	206.3	198.1	188.2	230.7	251.9	236.7	193.2
DG	232.1	207.7	192.6	141.1	138.7	84.1	84.9	
LN	151.0	155.7	134.5	135.5	152.2	145.5	149.8	136.4
DN	151.0	113.3	83.6	81.5	67.0	53.5		
Citric acid in milliequivalents (Figure 28)								
LW	50.8	50.7	50.6	62.7	57.5	54.7	47.0	63.1
DW	50.8	76.3	81.1	111.3	136.6	147.7		
LG	15.9	45.5	51.5	54.6	45.3	30.6	39.5	45.0
DG	15.9	58.6	65.5	73.4	58.7	106.6	116.2	
LN	34.9	47.1	26.7	25.2	23.0	18.5	18.2	18.7
DN	34.9	42.9	51.0	54.7	73.4	96.0		
Unknown acids in milliequivalents (Figure 29)								
LW	−3.0	−0.2	21.0	15.9	28.4	34.8	37.1	21.2
DW	−3.0	16.1	47.5	58.4	29.9	28.2		
LG	38.0	32.5	48.2	42.4	21.2	22.9	15.1	39.3
DG	38.0	1.3	20.2	33.5	35.0	40.8	44.4	
LN	25.6	17.2	29.4	36.8	39.0	38.1	45.7	53.1
DN	25.6	48.9	52.8	60.6	66.7	50.2		
Total sugar (Figure 30)								
LW	2.51	4.89	8.34	10.9	12.5	15.9	18.8	15.8
DW	2.51	1.13	0.914	0.669	0.591	0.490		
LG	6.86	9.37	8.29	13.7	14.0	13.9	16.3	14.7
DG	6.86	6.05	4.39	3.13	2.37	0.977	0.672	
LN	2.63	5.11	5.74	6.02	6.24	8.33	9.63	10.9
DN	2.63	1.94	1.63	1.26	0.962	0.803		
Fermentable sugar (Figure 31)								
LW	1.95	3.74	5.75	7.69	8.30	11.13	14.81	11.31
DW	1.95	0.770	0.576	0.295	0.193	0.135		
LG	5.10	7.02	5.76	8.50	9.37	9.75	11.58	9.96
DG	5.10	4.46	3.21	2.15	1.40	0.453	0.284	
LN	2.15	3.95	4.10	4.09	4.23	5.66	6.35	7.68
DN	2.15	1.37	1.10	0.718	0.504	0.455		
Unfermentable sugar (Figure 32)								
LW	0.559	1.15	2.58	3.22	4.20	4.74	3.98	4.44
DW	0.559	0.364	0.337	0.375	0.397	0.355		
LG	1.76	2.35	2.53	5.17	4.60	4.16	4.74	4.73
DG	1.76	1.59	1.18	0.975	0.977	0.525	0.389	
LN	0.479	1.16	1.63	1.92	2.01	2.67	3.28	3.26
DN	0.479	0.572	0.537	0.541	0.458	0.448		
Starch (Figure 33)								
LW	0.432	0.869	1.85	1.64	1.95	1.51	1.21	1.47
DW	0.432	0.0976	0.0306	0.00	0.00	0.00		
LG	1.37	2.34	1.84	2.46	1.03	0.624	0.917	1.51
DG	1.37	0.271	0.113	0.0418				
LN	0.252	0.675	0.284	0.191	0.278	0.663	0.699	1.57
DN	0.252	0.0810	0.0454	0.00				