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THE METABOLISM OF AMIDES AND AMINO ACIDS IN ETIOLATED SEEDLINGS OF LUPINUS LUTEUS L.

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CONTENTS

CHAPTER I

THE PROBLEM OF THE FORMATION OF ASPARAGINE

1. OLDER OPINIONS

Although the asparagine has been known by the work of Vauquelin and Robiquet since 1806, the physiological processes, which lead to the formation of this amide, are known however in a general way for plant tissues. The study of these processes has occupied the attention of many workers, as different plants can accumulate large amounts of asparagine.

This is especially the case with etiolated seedlings of *Lupinus*. Pfeffer (1872) had already observed that sometimes so much asparagine is present in plants that it crystallizes in extracts after dilution with a small amount of alcohol.

Schulze (1898) found that the amount of asparagine, accumulated in the etiolated seedlings exceeded the amount already present in the reserve protein of the seed and he could demonstrate that the synthesis of asparagine must take place at the expense of other amino acids. For that reason he considered this amide partly as a primary and partly as a secondary reaction product.

Prianischnikow (1904) considered the asparagine almost entirely as a secondary product, derived from the protein. For this he cited some facts. During the germination more asparagine occurred than aspartic acid could be formed by the hydrolysis of the protein from the seed. Moreover seedlings of *Lupinus* of one week old contained much asparagine and a small amount of leucine, tyrosine and hexonbases. When the seedlings were three weeks old, the amount of asparagine had increased considerably, whereas the other mentioned substances had disappeared almost entirely. The formation of the asparagine proceeded surely at the expense of the other amino acids, because the amount of this compound, formed, exceeded the amount of aspartic acid, derived from the protein. Further the concentration of asparagine in seedlings was much higher than in the cotyledons, whereas this was just the reverse for the primary products of the

hydrolysis. In spite of that Prianischnikow indicated that some more substances must be involved in the synthesis.

BUTKEWITCH (1909) inhibited the development by exposing the seedlings to toluene vapour. He found less asparagine, but more ammonia and supposed that the synthesis of the amide is dependent on the amount of ammonia present.

Our knowledge of the amide metabolism has been reviewed by a number of investigators (ARCHIBALD, 1945; CHIBNALL, 1939; STEWARD, 1947; STEWARD and STREET, 1946; STREET, 1949; WAELSCH, 1952); the present discussion however has been defined to a short survey.

2. GROWTH AND INFILTRATION EXPERIMENTS

Generally it was assumed that asparagine is synthetized from ammonia and a nitrogen-free compound, which may be derived from the products of the glycolysis or the citric acid cycle. Many investigators have analyzed this in detail by growing the seedlings on nutrient solutions, to which these substances had been added, as well as by infiltrating these compounds into the plants.

SMIRNOW (1923) fed his plants with ammonia and glucose and found a considerable increase of the amount of asparagine. An objection against this investigation however is that the experiment took too long; moreover this investigator did not take into account the possibility of a metabolism of glutamine. The important participation of this substance in the formation of asparagine however was known later.

VICKERY and collaborators (1936) fed beet plants with ammonium sulfate. After some months the increase of the amount of glutamine-N corresponded to the uptake of ammonia-N. This means that not only the amide-, but also the amino-N had been built up from the ammonia.

VICKERY *et. al.* (1937) demonstrated once more that the ammonia was the external source of nitrogen for the formation of the amides, by spraying different plants, e.g. *Lupinus*, with a solution of ammonium chloride. When the plant was grown in the light, a synthesis of asparagine occurred which corresponded to the increase of the total amount of nitrogen; when *Lupinus* plants, which were in the dark, were sprayed with a solution of ammonium sulfate, the amount of asparagine decreased, whereas the ammonia concentration in the plants increased. Vickery interpreted this by assuming that the reserve of the carbohydrates was insufficient to supply the carbon-chain of this amide.

It had been demonstrated by these experiments and many others that ammonia stimulates the formation of the amides considerably; now we have to deal with the problem which substance supplies the carbon chain.

Both CHIBNALL (1939) and VICKERY *et al.* (1937) have discussed this problem in detail. It has been suggested that the amide synthesis however might occur in the presence of a specific nitrogen-free precursor; the experiments of Prianischnikow had already shown that a non-specific precursor, such as glucose, can stimulate the amide formation.

RAUTANEN (see MEISS 1952) grew seedlings of peas after removal of the cotyledons on a solution which contained ammonia, strongly diluted. During this the amount of both amides increased considerably whereas the amount of malic acid decreased. A synthesis of malic acid occurred after feeding with glutamic acid and aspartic acid. This was considered as an evidence that malic acid is an intermediate for the formation of asparagine.

MOTHES (1933, 1939/1940) applied an infiltration method, which reduced the time of the experiment. Moreover the pH remained constant during these experiments by dissolving the different stimulating substances in a phosphate buffer. He found that seedlings form glutamine and asparagine as long as they dispose of a reserve of carbohydrates, they form ammonia and urea after addition of aspartic acid or glutamic acid.

Other investigators however indicated that the nature of the nitrogen-free precursor should not be essential for the formation of the amides. SCHWAB (1936) infiltrated leaves of *Fittonia Verschaffeltii* with ammonium salts of oxalic acid, succinic acid, malic acid, aspartic acid, glutamic acid and acetic acid. All these substances caused large increases of amides, particularly ammonium acetate. There is no evidence however that the carbon compounds, supplied, are actually used in the amide synthesis, as ammonium sulfate or bicarbonate are as effective as the ammonium salts of the organic acids. MEVIUS (1928) had already arrived at this conclusion earlier.

3. THE FORMATION OF ASPARAGINE AND THE SUBSTRATE OF THE RESPIRATION

The ammonia, which is necessary for the synthesis of asparagine, may be derived from the nutrient solution, such as in the experiments mentioned above, but also from an oxidative deamination of amino acids, formed by hydrolysis of proteins.

VICKERY and collaborators (1937) determined the amounts of organic acids, carbohydrates, amides and proteins in detached tobacco leaves, placed on water for varying times. They compared leaves, placed in the light, with those, placed in the dark. In the exposed leaves asparagine and glutamine were demonstrated; the content of malic and citric acid remained constant. In the leaves, which were in the dark, glutamine did not occur practically; here a vigourous formation of citric acid and asparagine was found whereas the amount of malic acid decreased. The loss of this substance was sufficient to account for the asparagine formed.

It appears from these data that the photosynthesis is important for the formation of glutamine. Further the investigators supposed that the citric acid should arise from malic acid via the cycle of Krebs-Johnson, because the proportion of the increase of citric acid and the decrease of malic acid equals 0.5. They were not quite certain that the asparagine was formed from malic acid too.

According to CHIBNALL (1939) [1]), discussing the experiments of Vickery, we can distinguish two phases in the metabolic processes of the leaves, which are in the dark. In the second period, when the leaves had been in darkness from 73 to 143 hours or more the loss of malic acid equals the accumulation of citric acid and the ratio, just aforesaid, was about unity; during the earlier period from 0 to 73 hours however the loss of malic acid surpassed the sum of the accumulated citric acid and asparagine.

Chibnall points out the fact that in these experiments the proteins are the most prominent substrate for the respiration; the content of carbohydrates of these leaves was too small. This result is affirmed by the experiments of YEMM (1935) with leaves of barley, kept in the dark.

The proteins play an important part in the synthesis of asparagine. This followed from a further investigation of VICKERY and PUCHER (1943). Asparagine was rapidly accumulated when most protein was respired. MEISS (1952) found a considerable loss of proteins, peptides, lipids and carbohydrates and an increase of the amounts of asparagine and free amino acids during the development of etiolated seedlings of *Lupinus luteus*. From the difference of the content of carbon of both groups of substances Meiss calculated how much had been respired. During the first 6 days the loss of carbon, caused by the respiration, could be compensated by the decrease of the lipids and the carbohydrates. As the decrease of these substances after this period remained very slow in comparison to the amount, necessary for the respiration, it must be assumed that the proteins were used as a substrate of the respiration. The loss of the protein-C however was greater than the amount of CO_2-C formed, so that it was very probable that the asparagine-C has been derived from the proteins.

4. THE AMINO ACID COMPOSITION OF THE PROTEIN AND THE FORMATION OF ASPARAGINE

The investigations of SCHULZE (1898) had already shown that the amount of asparagine, accumulated in etiolated lupin seedlings, exceeded the amount in the seed. Similar results were obtained by other workers. SCHWAB (1936) found that 1 % of the total nitrogen of the seedlings was present as glutamine-N and 20 % as asparagine-N.

The amino acid composition of the protein of the seed as well as the composition of the proteins, legumine and conglutine, isolated from the seed, have been investigated by KOSTYTSCHEW (1926), HEINRICH (1941) and HANSON and KOCH (1953). Their results, of which a sample has been given in Table 1, show that the reserve proteins have been characterized by a high content of arginine and glutamic acid and by a rather high content of leucine.

It appears that during the germination the amino acids of the proteins must be interconverted to a considerable extent. According to these analyses the amino-N of the asparagine, accumulated, was

[1]) page 228–243.

TABLE 1

The amino acid composition of some proteins from seeds
Amino acid-N in % of the total-N

Amino acids	Legumine from seeds of Leguminoses according to KOSTYTSCHEW (1926)	Conglutine			Protein from seeds of *Lupinus albus* according to HANSON and KOCH (1953)
		from seeds of sweet *Lupinus luteus* according to HEINRICH (1941)	from seeds of bitter *Lupinus luteus* according to SCHWIETZER (see Heinrich 1941)	from seeds of sweet *Lupinus luteus* according to SCHWIETZER (see Heinrich 1941)	
Glycine	0				3
Alanine	1	2	1	1	
Valine	1	1	1	1	3
Leucine	6	6	6	6	8
Proline	3	3	3	3	1–2
Phenylalanine	2	3	2	2	3
Aspartic acid	2	4	3	3	4
Glutamic acid	11	16	16	17	9
Serine	0				3
Tyrosine	1	3	1	1	5
Arginine	22	26	22	24	17
Histidine	4	4	3	3	4
Lysine	4	3	3	3	5
Ammonia	11	13			
Tryptophane	present				1
Threonine					6
Methionine					1
Cystine					1

TABLE 2

The amino acid composition of seeds or seedlings of Lupinus angustifolius according to DUNN *et al.*

The result is indicated in mg amino acid per seed or seedling

Amino acids	seed	seedling, age in days						
		3	5	7	9	11	13	15
Arginine	4.7	4.5	3.2	2.8	2.6	2.2	2.1	1.9
Aspartic acid	5.2	8.4	14.7	18.0	22.0	24.0	24.8	25.5
Glutamic acid	11.8	10.1	6.4	4.3	3.4	2.4	1.5	1.5
Glycine	2.0	2.1	1.6	1.6	1.5	1.3	1.1	1.0
Histidine	1.4	1.8	1.4	1.4	1.5	1.4	1.5	1.4
Isoleucine	2.8	2.4	2.1	1.8	1.7	1.3	1.1	0.89
Leucine	3.4	3.7	3.0	2.4	2.4	1.8	1.7	1.3
Lysine	2.5	2.5	2.1	2.0	2.0	1.9	1.7	1.6
Methionine	0.20	0.24	0.22	0.20	0.21	0.18	0.16	0.15
Phenylalanine	2.2	1.9	1.8	1.7	1.8	1.6	1.5	1.4
Threonine	1.9	1.3	1.3	1.3	1.3	1.2	1.1	1.0
Valine	2.3	2.2	2.0	2.0	2.1	1.7	1.7	1.4

equivalent to the sum of the aspartic acid and glutamic acid present in the seed.

DUNN and collaborators (1948) investigated this problem more in detail and determined the amino acid composition of seeds and seedlings of *Lupinus angustifolius* microbiologically. The proteins were hydrolyzed by boiling the crude material with acid. DUNN (1947) supposed that by this no destruction of the 12 substances, investigated, had occurred.

They established that all the amino acids, except histidine, decreased after the germination (Table 2). Asparagine and aspartic acid were not determined separately; the sum of both increased especially at the expense of glutamic acid, arginine, leucine and isoleucine.

The results of the investigations of Dunn and coworkers are not as complete as may be desired; the main problems of the direct and primary precursors of the amides, accumulated, and of the physiological processes, involved in their synthesis, remained unsolved, as they had been unable to establish the reactions of these processes; the metabolic changes of twelve amino acids however have been determined. The content of asparagine, glutamine and ammonia remained unknown; both amides were converted in ammonia and the corresponding amino acids. That is why they had to restrict their conclusion to the general statement that all the amino acids, investigated, participate in the synthesis of asparagine, histidine excepted.

5. THE PROBLEM

The problem of the conversion of the amino acids in the amides during the germination of *Lupinus luteus* has been investigated further, following principally the procedure of Dunn. We were able to determine the changes of the amounts of 23 amino acids. The fractionation, developed by Stein and Moore and used in the experiment described here, permitted a determination of the amino acid content in detail.

In order to simplify the experimental conditions as much as possible, the seedlings were grown in the dark and on water; no exogenous source of nitrogen was available. The amounts of the free and combined amino acids in the seeds and in young etiolated seedlings were determined.

CHAPTER II

THE EXTRACTION OF THE NITROGENOUS COMPOUNDS

6. INTRODUCTION

For our problem it was necessary to isolate and to determine the amino acids, which occur in a free and in protein combined condition. These comprise about 72 % of the total-N.

During the isolation of the free amino acids we must prevent that the proteins go into solution. 70 % alcohol, in which the proteins of the lupin do not dissolve, is usually used for this purpose.

Now the remaining protein must be dissolved. Different methods of extraction were tested to determine their usefulness.

Generally the proteins are isolated by solutions of various salts or urea. This technique could not be used with these extracts, as we were not sure that the proteins dissolved completely. Finally these difficulties were overcome. The proteins could be dissolved by partial hydrolysis by a percolation with 20 % hydrochloric acid, partly at room temperature, partly at 100 °C. This crude extract was hydrolyzed completely after purification.

The separate amino acids, present in the different extracts, were determined, qualitatively and quantitatively after a chromatographical separation, which is described further in Ch. IV and V. During the followed process of work all the amino acids are not isolated unchanged. So the amides, which occur in a combined condition in the protein, will be split in the corresponding amino acids and ammonia during the hydrolysis. The ammonia, possibly found, in the protein hydrolysate may be derived also from other amino acids to a small degree. The investigation remains limited to the formation of the free asparagine during the germination.

The amounts of amino acids and amides were determined in seeds and etiolated seedlings of *Lupinus luteus* of respectively 7, 14 and 21 days old.

7. The extraction of the free amino acids, the amides and the combined amino acids from seeds or seedlings of *Lupinus luteus*

7.1 *The Plant-Cultures*

The seeds, used for the investigation, were selected for size and colour in order to have available an experimental material as homogenous as possible.

The seedlings were grown on sterile distilled water in the dark at a temperature of 20–22 °C.

7.2 *Pretreatment*

100 Seeds, after removal of the seedcoat, were suspended in ether and ground completely in a colloid-mill. During this procedure the fatty substances were separated from the meal. After filtration the meal was removed from the filter, dried at room temperature and then suspended in 300 ml 70 % alcohol. During the process of defatting of the seeds no loss of nitrogen occurred.

The extraction of the fatty substances can be omitted when seedlings are analysed; the plants were ground completely in the mill after addition of 300 ml 96 % alcohol.

7.3 *The Extraction of the Free Amino Acids*

After pouring the suspension into a glass tube of 50 × 3 cm, which had been closed by a plug of glass wool at the lower end, a separatory

funnel, filled with 70 % alcohol, was placed on the tube and the percolation (with a speed of 500 ml per day) continued until four drops of the effluent no longer gave a positive reaction with the ninhydrin reagent according to Stein and Moore (19.5 and 19.6); no more than 0.3 μg amino acid-N was found in this 0.2 ml.

For the complete extraction of the seeds 500 ml alcohol were necessary and for the seedlings of 7, 14 and 21 days old respectively 1300 ml, 2500 ml and 2800 ml alcohol (Extract I).

The extract of the seeds contained 5 % of the total-N.

Many investigators extract the free amino acids from the ground plant tissue with 70 % alcohol. This is preferable to an extraction with water, because in this last case a small amount of the protein dissolves. This has to be removed by an ultrafiltration through a collodion membrane. This method of separation however has the disadvantage that it takes so long that a rather strong proteolysis may occur, and that the protein precipitate can only be removed from the filter with difficulty, by which losses occur.

It has been examined, how a quantitative extraction of the free amino acids could be obtained. For this 100 powdered seeds were extracted 10 times over with 100 ml 70 % alcohol and the amount of the amino acids in each extract was determined in 0.2 ml with the reagent according to Stein and Moore (19.5 and 19.6). The tenth extract gave still a positive reaction with ninhydrin, but contained however only 3 % of the amount, present in the first extract (Fig. 1). Also the extracts

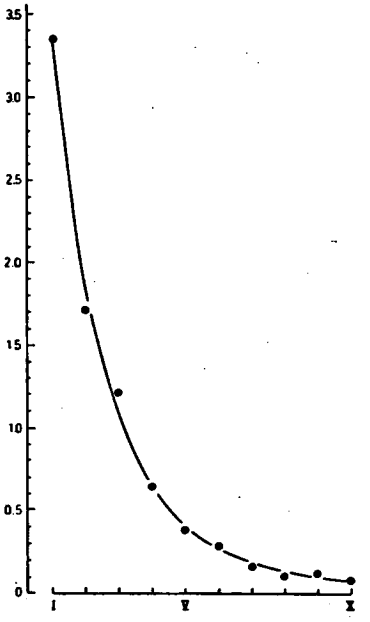


Fig. 1. The amount of free amino acids in successive extracts. Ordinate: extinction of 0.2 ml extract with 1 ml ninhydrin reagent of Stein and Moore, diluted with 5 ml of a mixture of 1 vol. n-propanol and 1 vol. water. [$E_{1\,cm}$ at 570 mμ]. Abscissa: I–X: the meal of 100 seeds was extracted 10 times with 100 ml 70 % alcohol.

6–10 contained 12 % of the amino acids in the extracts 1–5, so that the extraction might be considered practically complete.

This procedure takes up much time and may be replaced by the continuous extraction method, mentioned above, with the same result. By this a complete extraction could be obtained however with 500 ml 70 % alcohol during one day.

7.4 *Hydrolysis of the Proteins*

After extracting the free amino acids completely, we passed on to a hydrolysis of the proteins, remaining in the column. When the alcohol was percolated as far as just under the surface of the column, the separatory funnel was removed and a few ml 20 % HCl was poured on the column. Now we placed the separatory funnel filled with 20 % HCl on the tube and percolated for 3 days till 4 drops of the effluent did not show a purple colour with the ninhydrin reagent after neutralization with 6 n NaOH to phenolphtaleine. For the extraction of the seeds 2000 ml were necessary, for the seedlings of 7, 14 and 21 days old, respectively 1500, 1000 and 500 ml.

After this the hydrolysis was continued at 100 °C. A jacket of water had been fitted round the column and was heated to 100 °C; the further extraction was continued by a 20 % HCl-solution till the reaction on the effluent with ninhydrin was negative again; this procedure took about one hour. For the seeds 500 ml was necessary and for the seedlings of 7, 14 and 21 days old respectively 400, 250 and 125 ml.

Both HCl effluents were added together (Extract II).

In samples of the extracts I and II the total-N was determined twice.

It could be remarked that a more simple method would have been to hydrolyze the ground plant tissues and to fractionate the amino acids afterwards.

However it is generally known that various amino acids undergo changes by the presence of carbohydrates during the hydrolysis of the proteins. By the quantitative determinations of the amino acids the recovery was insufficient. Only a few papers, dealing with this problem, are mentioned. LUGG (1933) observed a loss of cystine during hydrolysis of a protein in the presence of sugars and cellulose; substances, such as humines, are formed. According to BAILEY (1937) this occurs especially under the influence of arabinose. ROXAS (1916) investigated the reactions of tyrosine and tryptophane with small amounts of carbohydrates in an acid solution during heating. These amino acids were broken down; the formation of humines was stimulated especially by furfural, originating from the sugars.

It appeared indeed that no reliable quantitative determinations of amino acids can be performed in the presence of carbohydrates.

It was considered whether it would be possible to remove the carbohydrates from the ground tissues first, before liberating the combined amino acids by hydrolysis. As the insoluble carbohydrates in the powder could dissolve by a strong hydrolysis, this was not practical.

After this it was investigated if a satisfactory result could be obtained by dissolving the nitrogenous compounds first, separated from the carbohydrates.

For this it is recommended to dissolve the proteins either with Na_2SO_3-solution, or with NaCl-solution or with a solution of urea. We have investigated the usefulness of these methods further.

According to BERKHOUT (1948) reliable results for the isolation of the protein from peas and beans are obtained by application of the method of KREUGER (see Berkhout). We have adapted this method also to the powder of the seeds of *Lupinus*, by which the free amino acids were not removed by an alcoholic extraction. 15 g

powder was shaken with 150 ml 1.4 % Na_2SO_3 for 3 hours, centrifuged, the precipitate still shaken with 225 ml 2 % Na_2SO_3 for 2 hours twice and centrifuged. After evaporating the collected centrifugates to 225 ml on a boiling waterbath, the proteins precipitated almost completely after addition of 22.5 g trichloroacetic acid. After shaking for 20 minutes they were centrifuged and washed out with 375 ml 1 % trichloroacetic acid once and then with 96 % alcohol twice. In a Soxhlet apparatus the last traces of trichloroacetic acid were extracted by a continuous percolation with petroleum ether for 24 hours. No more than 70 % of the total-N could be dissolved as protein-N from the powder of *Lupinus* , which had not been treated with alcohol. From the powder, which had been extracted with 70 % alcohol first, 30 % of the total-N however could be isolated as the protein-N with the method of Kreuger. From these experiments it follows that the isolation of the protein according to the method of Kreuger does not yield good results, especially when plant material, treated with 70 % alcohol, is used.

In experiments, concerning dissolving of animal proteins hardly soluble, such as collagen, various investigators use 30–40 % solutions of urea.

From a number of experiments it appeared that the proteins, denaturated by 70 % alcohol, dissolve in this solvent however for 30 %; the urea was removed from the extract and the residue by dialysing for 3 days, changing the water twice a day. This result shows that this method could not be used.

As contrasted with the extraction methods, just mentioned, the procedure, described under (7.4) gave reliable results. The HCl-extract of seeds contained 94 % of the total-N. This method had the advantage that the insoluble carbohydrates and the proteins, hydrolizing easily, were removed at room temperature and that during the elution at heating the products of the hydrolysis were exposed to this higher temperature for a short time and in a low concentration.

8. The determination of nitrogen according to Kjeldahl

The total-N of the samples and fractions were determined according to the method of Kjeldahl. We applied the procedure of Reith and Wansink (1947).

A sample, containing about 6.5 mg N, was digested with 233 mg oxyde of mercury, 5 g sodium sulfate (a.r.) and 9 ml concentrated sulfuric acid (a.r.) for 3 hours at least, after which this mixture, diluted with an about equal volume of water, was transferred quantitatively into the distillation apparatus according to Parnas–Wagner (1938). 30 ml 50 % NaOH, and 10 ml 20 % $Na_2S_2O_3$ were added and the ammonia distilled by steam-distillation in 10 ml 0.1 n HCl in half an hour. The distillate was titrated with 0.1 n NaOH to the indicator of Tashiro (200 ml 0.1 % alcoholic methylred and 50 ml 0.1 % alcoholic methyleneblue).

The average fresh-weight of 100 seeds was 9.698 g, which contained 871 mg N (Table 3).

9. Results

The method, described in (7.), was applied to different samples. The amounts of total-N in the extracts of seeds or seedlings have been summarized in Table 3.

TABLE 3

Total-N in the alcoholic extract, in the HCl-hydrolysate and in the residue of seeds or seedlings of different age

Mean values of 100 seeds or seedlings. — Plants grown in the dark on water without nutritive salts. — Temperature 20 °C — Total-N of 100 seeds and seedlings (7, 14 and 21 days old): 876 mg (mean of A, B, C and D, columns a and e).

Total-N in seeds or seedlings	Total-N in alcoholic extract (Extract I)	Total-N in HCl-hydrolysate (Extract II)	Total-N in residue	Total-N in seeds or seedlings (b + c + d)	Deviation in % of the average value (deviations due to sampling) $\frac{A-876}{876} \times 100$ A = a or e
mg N a	mg N b	mg N c	mg N d	mg N e	f
A. Seeds:					
867					— 1.0
886					+ 1.1
859					— 1.9
	47	794	5	846	— 3.4
	48	817	4	869	— 0.8
	47	834	3	884	+ 0.9
	48	840	4	892	+ 1.8
	46	820	5	871	— 0.6
B. Seedlings 7 days old:					
871					— 0.6
869					— 0.8
897					+ 2.4
	291	655	0	946	+ 8.0
	280	628	1	909	+ 3.8
	273	600	0	873	— 0.3
	279	565	0	844	— 3.7
	259	615	1	875	— 0.1
C. Seedlings 14 days old:					
847					— 3.3
870					— 0.7
	526	368	1	895	+ 2.2
	540	385	1	926	+ 5.7
	543	344	2	889	+ 1.5
	564	372	1	937	+ 7.0
D. Seedlings 21 days old:					
832					— 5.0
	688	133	1	822	— 6.2
	696	127	1	824	— 5.9
	711	172	0	883	+ 0.8
Mean:					
866 ± 6[1])				881 ± 9[1])	
Standard deviation per 100 specimens:					
19[1])				36[1])	

[1]) For the calculation of the standard deviation see FISHER (1948) page 46.

It is evident that the extraction has been practically complete, as no more than 0–0.6 % of the total-N, originally present, remained in the residue.

The standard deviation for the variability of the total-N in the samples of 100 seeds or seedlings was calculated; the σ of each series of samples (A, B, C or D) amounted to 4.1 % of the total-N value.

CHAPTER III

THE PURIFICATION OF THE EXTRACTS

10. Introduction

The extracts of the plants, which we obtained according to the method, described in the previous chapter, must undergo further treatments, before the amino acids could be isolated.

With the paper chromatographical analysis of the extracts, obtained by hydrolysing the plant residues with 20 % hydrochloric acid (7.4), it appeared that peptides were still present. Consequently the extracts had to be hydrolyzed further.

Moreover it was necessary to purify the extracts, before applying a chromatographical separation. With this purification we had in view to remove the salts and the carbohydrates. Large amounts of salts and carbohydrates may appreciably affect the results of the quantitative estimation of amino acids by the method of Stein and Moore.

Also this purification was necessary, because the carbohydrates react with the amino acids during the hydrolysis. The carbohydrates and salts may be removed by means of various artificial resins (ion-exchangers). The solutions are percolated through a column of a cation-exchanger; usually these are used in the H^+-form and exchange H^+-ions to cations. The positive amino acid-ions of the extracts are retained, whereas the carbohydrates and the anions pass the column uneffected.

The purification succeeds within certain pH limits. The ionisation of the exchanger depends on the concentration of the hydrogen-ions and attains a minimum in a strong acid solution. Under these conditions the resin retains few ions; the capacity of the column is small. This is especially the case with a weak exchanger. Moreover as the amino acids will be weakly dissociated, they may partly pass the column together with the carbohydrates and will be lost. At the same time the adsorption of the amino acids to the exchanger is still reduced by the hydrolysis of the salt resin-electrolyte (Partridge and Swain, 1953). That is why it is necessary to purify the extracts in a weak acid solution, where these errors do not occur.

The adsorption of an amino acid to the ion-exchanger leads to an equilibrium, which depends on the relative amounts present.

The amino acids are displaced by diluted NaOH-solutions from the column. The adsorption of the different amino acid cations to the

resin diverges, as the affinity to the column depends on the pK-value; glutamic acid has a weaker adsorption than leucine and this has a weaker adsorption than arginine. This causes that the least strongly combined acids appear soon after the displacement of the amino acids by sodium ions, e.g. aspartic and glutamic acid. As the adsorption is partly due to an attraction of the compounds by London–v.d. Waals forces, phenylalanine and tyrosine appear later on during the displacement than we would expect on the basis of their pK-values (PARTRIDGE and BRIMLEY, 1952).

After the carbohydrates and anions have been removed by a cation-exchanger, the cations may be separated by running through an anion-exchanger. Under the experimental conditions the amino acids are retained and the cations pass the column. Diluted mineral acids displace the amino acids.

The adsorption on the anion-exchangers is influenced by the same factors as the adsorption on the cation-exchangers, however some of these factors are in an opposite direction.

Moreover some anion-exchangers have the peculiarity, that they react with positive ions at a high pH (PARTRIDGE and BRIMLEY, 1949). This occurs by weak anion-exchangers, prepared from phenol-derivatives. At a high pH-value the still existing acid-groups are ionized and exchange positive ions. As this has a disturbing influence on the purification, alcoholic extracts must be fractionated by a strong anion-exchanger.

11. THE PROCEDURE OF THE PURIFICATION

At present we give the description of the treatments, which the extracts must undergo, before they were suitable for the chromatographical separation of the amino acids. As the procedure varies for the extracts of the free amino acids and the hydrolysates of proteins, both methods will be discussed separately.

11.1. *The Hydrolysates of the Protein*

11.1.1. *Pretreatment of the* HCl-*Extracts.* 1 ml of the HCl-extract was dried at room temperature and the residue dissolved in 35 ml water. 0.2 ml of this solution was treated with the reagent of Stein and Moore (19.5 and 19.6). The optical density was estimated; if this $E_{1\,cm}$ (λ 570 mμ) was about 0.750, 10 ml of the original HCl-extract was dried by distillation in vacuo at 50 °C. A corresponding amount of the original extract was dried in vacuo, when the amino acid content of the extract differed from the value just mentioned.

After addition of about 10 ml water the solution was dried in vacuo; this procedure was repeated once again, after which the residue was dissolved in 10 ml water; the pH of this solution was 1.7.

11.1.2. *Fractionation by Imac C 12.* During the chromatographical analysis of these extracts the carbohydrates run through the column, whereas the amino acids were retained quantitatively. After this they were eluted from the column.

For the first phase of the purification a strong cation-exchanger was used. The extracts were percolated through a column of Imac C 12 in the H^+-form; the column was obtained as described under (11.1.2b) and had a diameter of 12 mm; the dry-weight of the Imac C 12 was 30 grams. When the liquid was drawn in the column, 0.2 n NaOH was poured on the column and the effluent collected in 40 fractions of 15 ml. It appeared that the amino acids were present in the fractions 26–36; for this reason the fractions 26–40 were combined.

(a) For the purification and isolation of amino acids from protein hydrolysates different artificial resins have been used. PARTRIDGE (1949) has investigated Zeo-Karb-215, De-Acidite-FF and Dowex-2. After a solution of egg albumine had been hydrolyzed by hydrochloric acid and the excess of acid removed by distillation in vacuo, the solution was percolated through a cation-exchanger; half the column was used. The amino acids were displaced by 0.15 n ammonia. Partridge worked with strongly concentrated solutions and collected the effluent in fractions; the amino acids appeared in a definite sequence. Each fraction however contained one to three amino acids, whereas the solvent ammonia occurred in the last one.

For our purpose the use of ammonia as solvent had two difficulties: the arginine is not displaced and the ammonia previously present in the extracts of the plants, cannot be estimated separately.

Arginine may be removed by diluted hydrochloric acid (PARTRIDGE, BRIMLEY and PEPPER, 1950); the acid decreases the ionisation of the resin and of the amino acid. The exchanger comes in the non-ionized H-form and cannot retain cations. If we should have applied this elution method, large amounts of hydrochloric acid would have been necessary.

The displacement by sodium ions gave a complete removal of the amino acids from the column; these ions have a greater affinity to the exchanger than the amino acids. That is why excellent results were obtained.

(b) Before we start with the purification of the extracts, Imac C 12 ("Activit" Amsterdam) must undergo a special treatment. This resin was ground, sieved and dried at 70 °C; the fraction between 70–90 mesh/inch was used. 30 g of this powder was suspended in 1.5 n HCl; poured into a tube and washed out with water till the effluent was neutral (controled by lyphan-paper). Now the resin was treated with 0.2 n NaOH, until the effluent was alkaline. The excess of sodium ions was removed by water. The treatments with HCl, NaOH and water were repeated twice and finally the resin was brought into the H^+-form with HCl and washed with water, before the column was ready for use. In all cases the liquid passed the columns with a velocity of about one drop per three seconds.

In a blank determination with the ninhydrin-reagent it appeared that the resin did not give off substances, which disturb the estimations of amino acids.

(c) We investigated, whether the cation-exchanger was strong enough to exchange cations in a sufficient amount in a pH-range of 1 to 11. For this purpose 10 ml of a buffersolution was percolated through a column of Imac C 12 in the H^+-form (500 mg dry-weight, diameter 7 mm). This solution was obtained by adding 85 % phosphoric acid to 50 ml 0.2 n NaOH until the desired pH was reached after diluting to 100 ml with water. The column was washed with water, until the pH of the effluent was about 7 (control with lyphan-paper). 10 ml 1 n HCl removed the Na^+-ions completely; the column was washed again with water. By titrating this effluent with 1 n NaOH the amount of sodium ions, previously combined, could be determined. Averagely 1 m. eq. Na^+-ions was combined. Buffersolutions, with a pH varying from 0.9 to 11, were tested and gave the same result. From this it was deduced that Imac C 12 has been ionized in this pH range. Each column has a limited capacity, so that the maximum capacity had to be determined. A column of Imac C 12 in the H^+-form (500 mg dry-weight, diameter 7 mm) needed 20 ml 0.1 n NaOH at least to be transformed into the Na^+-form. At first the effluent had a high hydrogen-ion concentration, as these ions had been exchanged, but later on sodium ions were not adsorbed and the concentration of NaOH of the solution did not alter. Regeneration to the H-form was performed

with 4 ml 1 n HCl. In both cases, during the transformation of the cation-exchanger from the H^+-form into the Na^+-form, the amounts of alkaline, were measured by titration with the Tashiro-indicator (8.). The maximum capacity measured was about 2 m. equiv.

(d) As the amounts of the amino acids however, which are adsorbed on the column, do not depend on the capacity alone, but also on their concentration, the combining-capacity under the experimental circumstances must be estimated.

This test was performed with a caseine hydrolysate, after removing the excess of hydrochloric acid by distillation in vacuo. The residue was dissolved in water and diluted until the solution in a 1:50 dilution had an optical density $E_{1\ cm}$ (λ 570 mμ) of 0.750 with the reagent of Stein and Moore (19.5 and 19.6). From this stock solution a series of solutions was prepared from an undiluted solution to a 1:100 dilution by addition of diluted HCl (pH 1. 7).

Each solution was percolated through a column of Imac C 12 (500 mg dry-weight, diameter 7 mm); the effluent was collected in fractions of 1 ml, until the first positive ninhydrin reaction was obtained. From the quantity of hydrolysate, which had already passed the column, the amount of adsorbed amino acids was calculated. In Fig. 2 the retention of the hydrolysate has been plotted against the relative concentration. It was observed that the adsorption of the amino acids depends on the concentration of the amino acids in the solution. The experiment was repeated; the solutions had a pH of 1.0. It appears that the adsorption capacity was greater at a pH of 1.7 than at 1.0.

In connection with this result it is important to point out here that the purification of the protein hydrolysates, mentioned in (11.1.2), was performed at a pH 1.8.

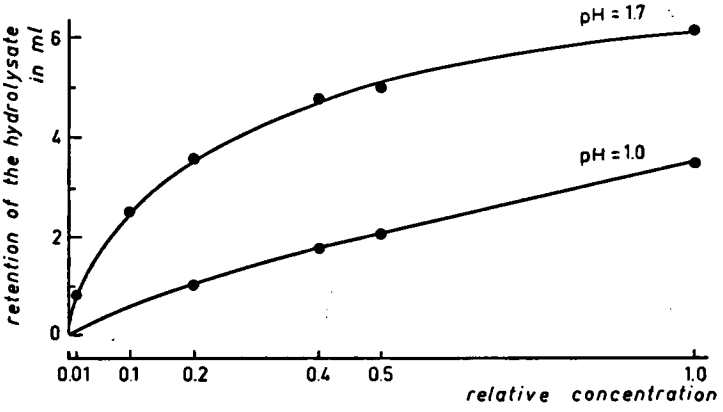


Fig. 2. The influence of the concentration and of the pH upon the adsorption of amino acids on a column of Imac C 12. Column: Imac C 12 (dry-weight 500 mg, size of the grains 70–90 mesh/inch, diameter 7 mm). Stock solution: caseine hydrolysate (diluted 1:50, 0.2 ml with the reagent of Stein and Moore $E_{1\ cm}$ (λ 570 mμ) = 0.750.) Dilutions from an undiluted solution to a 1:100 by addition of diluted HCl (pH 1.7 respectively 1.0).

It appeared from further experiments, that a proportionally larger amount of amino acids is adsorbed by larger quantities of Imac C 12.

PARTRIDGE (1952) recommends to percolate an amount of amino acids that only half of the column is saturated. We have followed this advice. Then a column of Imac C 12 with a dry-weight of 30 grams has a sufficient capacity to adsorb the amino acids of 10 ml HCl-extract, which meets the requirements, mentioned. The amino acids of these diluted plant extracts however does not pass the column in the definite sequence, such as Partridge had observed; the sodium ions leave the column at about the same time as the amino acids.

(e) In order to show that the carbohydrates were completely separated from the amino acids, the effluent of the chromatographical analysis of 10 ml tissue

hydrolysate was collected in 40 fractions of 15 ml. It appeared that soluble carbohydrates were present in the fractions 3 to 8 (the 8th fraction contained about 15 mg sugars) and the amino acids in the fractions 26 to 36 (the 36th fraction contained about 8 mg amino acids).

The presence of carbohydrates was demonstrated by spraying the spots of 2μl hydrolysate on a filter paper with 0.93 % aniline in 50 % ethylalcohol, containing 1.26 g oxalic acid, and heating for 10 minutes at 100 °C; brown spots appear. Sensitivity: 1 g carbohydrates/l. The presence of amino acids was shown by spraying with 0.2 % ninhydrin in alcohol and heating for 5 min. at 100 °C. Sensitivity: 500 mg amino acids/l.

11.1.3. *Fractionation by Dowex-2 or De-Acidite-F.* Before determining the amino acids, qualitatively or quantitatively, the cations must be removed from the effluent.

For this purpose dry Imac C 12 was added to the solution till the pH, measured with a glass electrode (Electrofact), remained constant at 12 for 15 minutes at least. The suspension was filtered and the filter washed with 10 ml water. The washing was added to the filtrate.

The solution was percolated through a column of De-Acidite-F or Dowex-2 (length 40 cm, diameter 2.5 cm, size of grains 70–90 mesh/inch) in the OH^--form; immediately followed 0.2 n HCl. The effluent was collected in 20 fractions of 100 ml. Ammonia and amines were found in the first and the second fraction; the amino acids in the fractions 14 to 17. The fractions 14 to 20 inclusive were combined.

(a) The effluent from the column of Imac C 12 is too alkaline that strong anion exchangers, like Dowex-2 and De-Acidite-F can remove the cations. Neutralizing the effluent in advance by hydrochloric acid is not appropriate as the chloride ions would be retained by the anion exchanger. During the chromatographical analysis the pH would increase to the original value and this would bring about that the lower parts of the column were no longer ionized.

Consequently it was necessary to remove the excess of sodium ions without the addition of mineral acids. This may be achieved by shaking the effluent with a strong cation exchanger (for instance Imac C 12) in powder form. During this procedure no loss of amino acids occurred, because the affinity of the sodium ions to Imac C 12 is greater than that of the amino acids.

After this treatment the filtrate passed a column of an anion exchanger; the amino acids were completely adsorbed. The increase in the concentration of OH^--ions, caused by the exchange of OH^--ions, remained small and the dissociation equilibriums were not changed in an unfavourable direction.

(b) Different control experiments have demonstrated that no amino acids were adsorbed on Imac C 12 at a pH = 12. If the added dry cation exchanger lowered the pH further than 12, large losses occurred. (see Table 4).

(c) For the separation of the amino acids from the cations a strong anion exchanger was necessary, e.g. Dowex-2 or De-Acidite-F (PARTRIDGE and BRIMLEY, 1952). These resins were suspended in water and then ground. In all the experiments the grains with the size of 70–90 mesh/inch were used.

In contrast with the other exchangers the dry-weight of the columns of Dowex-2 cannot be indicated. This creamcoloured substance discoloured by drying. De-Acidite-F could be prepared as Imac C 12.

The procedure of PARTRIDGE and BRIMLEY (1951) was followed; the column was converted in the OH^--form by percolating a solution of 2 n NaOH, containing 10 g $Ba(OH)_2$ per liter, and filtrated before use. This was followed by washing with CO_2-free water. After this we percolated 0.2 n HCl in order to convert the column in the Cl^--form and washed out with water. This procedure was repeated twice. Finally the anion-exchanger was brought into the OH^--form and washed again with CO_2-free water. Now the column was ready for use. The column did not give off material, which reacts with ninhydrin.

(d) In the plant extracts, purified in this way, the amount of the cations has

been reduced strongly. This could be demonstrated by determining the dry-weight of the ash. 100 ml plant extract had a residue of 13.4 mg; after it had been purified only 6.0 mg[1]).

The ammonium ions eluates with the metal ions simultaneously. These fractions were used for the estimation of the amount of ammonia in the extracts. In Ch. V this will be discussed more in detail.

TABLE 4

Influence of a cation exchanger (Imac C 12 in powdered form) on the pH of a solution of amino acids and the adsorption of the amino acids

100 ml 0.2 n NaOH contained 1 mg of each of the following amino acids: leucine, phenylalanine, valine, tyrosine, proline, glutamic acid, alanine, threonine, aspartic acid, serine, glycine, ammonium chloride, arginine, lysine, histidine, cystine. Dry Imac C 12 (size of grains 70–90 mesh/inch) was added to 100 ml of the amino acid solution until the pH remained constant at the desired value during 15 minutes at least. The suspension was shaken for 1 hour and filtrated. Determination of the concentration of the amino acids in the filtrate with the reagent of Stein and Moore. pH determined electrometrically with a glass electrode.

pH solution before addition cation exchanger Imac C 12	pH solution after addition cation exchanger Imac C 12	decrease of the concentration of amino acids (% initial concentration)
13–14	7	53
13–14	9	33
13–14	10	19
13–14	12	0

11.1.4. *Hydrolysis of the Polypeptides.* The combined fractions 14 to 20, which contained soluble polypeptides were dried at 60 °C. They were dissolved in 10 ml 6 n HCl and boiled under reflux for 16 hours. After drying in vacuo this hydrolysate was dissolved in 25 ml water. 3 Samples, each of 8 ml, were dried at 60 °C and used for the further quantitative determinations (Ch. V). The remaining solution was dried and used for the qualitative investigation (Ch. IV).

11.2. *The Free Amino Acids*

11.2.1. *Pretreatment of the Alcoholic Extracts of the Free Amino Acids.* 1 ml of the alcoholic extract was dried at room temperature and the residue dissolved in 2 ml water. In 0.2 ml of this solution the amino acid content was determined with the method of Stein and Moore (19.5 and 19.6). When the optical density $E_{1\,cm}$ (λ 570 mμ) was approximately 0.750, 200 ml of the original alcoholic extract was dried at 40 °C. A corresponding amount of the original extract was dried in vacuo, when the amino acid content of the extract differed from the value, just mentioned. The residue was dissolved in 10 ml water. The solution was percolated through a column of Asmit 173 (length 7 cm, diameter 8 mm); the column was washed with water until the combined effluents had a volume of 25 ml.

[1]) I am indebted to Drs H. L. Golterman, Laboratory for General Botany, Plant physiology and Pharmacognosy, Amsterdam, for the performance of this determination.

(a) It was not possible to chromatograph the alcoholic extracts through a column of Imac C 12, as these columns were choked quickly probably by the presence of certain dyes. A percolation through a column of a discolouring resin, e.g. Asmit 173 ("Activit", Amsterdam) solved this difficulty. Solutions of various concentrations, percolated through a column of Asmit 173 (length 7 cm, diameter 8 mm), gave no loss. Perhaps it might be expected that tyrosine and phenylalanine would be adsorbed to the resin by London-v.d.Waals forces; we could demonstrate however that less than 3 % of the amino acids were lost by a passage through a column of Asmit.

(b) The grains of Asmit 173, had a diameter of about 1 mm. They were suspended in water and poured into the column. Before use the column was treated with 200 ml of a hot solution (100 °C) of 1 n NaOH and then washed with water until the effluent had a pH of about 7; the dropping speed was one drop per 3 seconds. The effluent did not react with ninhydrin.

11.2.2. *Fractionation by Imac C 12.* This fractionation was performed as indicated in the procedure of the protein hydrolysates. The pH of the solution however was about 7; that is more favourable for the separation.

11.2.3. *Fractionation by Dowex-2 or De-Acidite-F.* The procedure of this fractionation was similar to that of protein hydrolysates.

11.2.4. *After-Treatment.* The fractions 14 to 20 inclusive, which had passed through the column of Dowex-2, were combined and dried at 60 °C. The residue was dissolved in 25 ml water; 4 samples each of 6 ml, were dried at 60 °C and used for the further quantitative investigations (Ch. V). The remaining solution was dried at 60 °C and used for the qualitative investigation.

12. Control

In order to investigate, whether losses of amino acids occur during the method of purification, described above, the following experiment was performed.

(a) The total amount of ammonia and amino acids was determined in an aliquot of the HCl-extract (25 ml),—obtained from seedlings of 7 days old—, before and after the purification.

An aliquot of the unpurified extract (25 ml) was dried in vacuo at 60 °C and the residue dissolved in 1 l water. The total amount of ammonia and amino acids was determined in 0.2 ml colorimetrically after addition of the reagent of Stein and Moore.

A second aliquot of the extract (25 ml) was purified and the fractions, which contained ammonia, and those, in which the amino acids were present, were collected separately and diluted with water to 1 l, after which these substances were determined separately in 0.2 ml with the same method.

(b) Also the optical density of a solution, containing various amino acids, was determined after the reaction of Stein and Moore; at the same time this was repeated after this mixture had been treated as indicated in 11.

100 ml 6 n HCl contained 5 mg of each of the following amino acids: leucine, isoleucine, phenylalanine, valine, methionine, tyrosine, γ-aminobutyric acid [1]),

[1]) I am indebted to Dr W. Brackman, Royal Shell Laboratories, Amsterdam, for supplying γ-aminobutyric acid.

proline, glutamic acid, alanine, threonine, aspartic acid, serine, glycine, arginine, lysine, histidine and cystine. For the experiment 25 ml of this solution was dried in vacuo at 60 °C and dissolved in 1 l water and the amount of amino acids was determined in 0.2 ml, as mentioned above.

(c) Finally we determined the amount of ammonia and amino acids, present in a mixture of 25 ml of the HCl-extract (*a*), and 25 ml of the mixture of amino acids (*b*) before and after the purification.

TABLE 5

The amount of ammonia and amino acids in a HCl-hydrolysate of seedlings of Lupinus luteus. Recovery of the quantity of amino acids, added, after the chromatographical analysis.

Age of the seedlings 7 days. — HCl-extract prepared according to the method, described in (7.4) — composition of the mixture of amino acids, see (12.b) — 25 ml of the solution dried in vacuo at 60 °C and then dissolved in 1 l water. — figures in the table are the optical densities $E_{1\,cm}$ (λ 570 mμ) of 0.2 ml after the reaction with the ninhydrin reagent of Stein and Moore (19.5 and 19.6)

	amino acid solution	plant-extract	plant-extract + amino acid solution	
			found	calculated (a + b)
	a	b	c	d
Unpurified	0.134	0.163	0.298	0.297
Purified (11.1)				
Ammonia. . . .	—	0.030	0.033	0.030
Amino acids . .	0.132	0.130	0.254	0.262
Total	0.132	0.160	0.287	0.292

It appears from Table 5 that a loss of about 4 % may occur during the procedure of the purification. It must be noticed that the concentrations of the amino acids and the relative amounts, in which they are present in the reaction mixture, are similar. This was necessary, as equimolar solutions of different amino acids have not the same optical density with the method of Stein and Moore.

The differences of the values of the optical density of the extracts before and after the purification was always determined. The greatest difference, found, amounted to 5 % of the measured value.

CHAPTER IV

THE QUALITATIVE ANALYSIS

13. Introduction

The general investigation into the constituents of a mixture of amino acids, is considerably simplified by the classical work of Consden, Gordon and Martin (1944). They succeeded in separating and identifying amino acids quickly by paper chromatography. The separation is based on a different solubility of the various components in a stationary phase, rich in water, and in a mobile phase, which contains substantially an organic liquid. The solvents, mostly used, are phenol,

cresol, collidine, lutidine and n-butanol, saturated with water. The greater the solubility of an amino acid in the mobile phase, the faster the substance is transferred, which finds expression in a higher R_F-value [1]).

The amount of water, which a solvent can take up, depends on the temperature. For this reason the R_F-values of the amino acids, obtained at various temperatures, show great differences.

The analyses were performed in closed cabinets with a great humidity in order to prevent an evaporation of the liquid. Moreover Consden and collaborators recommend to equilibrate the filter paper in the moist atmosphere for a few hours, before beginning with the analyses. But according to a personal communication of Dent this is not necessary. Probably the way, in which the analyses are performed, may be the cause of the difference. If a cabinet is left open after the experiments, it will take more time to equilibrate the filter paper. Equilibrating is superfluous, when the cabinet is kept closed.

The paper chromatography has simplified the experimental technique; however it is impossible to analyse every extract with this method. Salts, present in the extract, cause spots, long drawn out; no satisfactory results could be obtained. A desalting of the extract by an electrodialytical method however is desired. (see CONSDEN *et. al.* 1947).

The salt-effect disappears when the filter paper has been drenched in a buffersolution previously. Moreover this has the advantage that we can analyse at a different pH. For this purpose LEVY and CHUNG (1953) used a borate buffer with pH = 8.3 and as solvent a mixture of meta-cresol, phenol and borate buffer. Now a salt effect did not occur. Moreover the diffusion of the amino acids is more difficult. Consequently the spots are smaller and give a more intensive colour after spraying with ninhydrin.

According to PARTRIDGE (1952) the paper chromatographical analysis of a group of amino acids, may be disturbed by other substances, for instance sugars. That is why he fractionated the crude extracts with ion-exchangers. His method has been described in Ch. III in detail.

Chromatographing the extract of the free amino acids, we did not establish any disturbance. This agrees with the results, obtained by DENT *et. al.* (1947) and by ALLSOPP (1948). This was of great importance, as the amides were deamidated into the corresponding acids during the purification process, under these conditions their presence could not be demonstrated in the purified extracts. The presence of asparagine and glutamine could be identified on the paper chromatograms of the crude alcoholic extracts of the free amino acids.

14. CHOICE OF THE SOLVENTS FOR THE PAPER CHROMATOGRAPHICAL METHODS

Using the method, described by CONSDEN *et. al.* (1944), some difficulties were experienced.

[1]) The R_F-value is the proportion of the distance which the centre of a spot has run to the displacement of the solvent.

Firstly the spots of the amino acids spread out considerably. This concerned those substances especially, which were displaced by phenol-water or collidine-water to a lower level. The result was that small amounts of amino acids could not be detected.

Secondly in agreement with the results of Dent the R_F-values, obtained with collidine-water, were much smaller than those found by Consden (Table 6).

TABLE 6

The R_F-values for the solvent collidine-water at 15 °C

Amino acids	R_F-values		
	observation by myself	according to	
		CONSDEN (1944)	DENT (1947)
Alanine	0.22	0.32	0.25
Arginine	0.16	0.16	0.13
Aspartic acid	0.14	0.22	0.27
Cystine	0.13	0.14	0.11
Glutamic acid	0.16	0.25	0.29
Glycine	0.17	0.25	0.22
Histidine	0.25	0.28	0.24
Isoleucine	0.41	0.54	0.43
Leucine	0.45	0.58	
Lysine	0.05	0.14	0.11
Methionine	0.52	0.57	0.42
Ornithine	0.04	0.13	
Phenylalanine	0.51	0.59	0.44
Proline	0.37	0.35	0.24
Serine	0.21	0.28	0.28
Threonine	0.27	0.32	0.30
Tryptophane	0.53	0.62	0.47
Tyrosine	0.58	0.64	0.53
Valine	0.34	0.45	0.34

The collidine had been purified by adding 1–2 ml bromine to 1 l collidine. About 20 hours later the solution was shaken with a few ml 40 % NaOH and distilled in vacuo over solid $Na_2S_2O_3$ (see CONSDEN *et. al.* 1947). It is not surprising that different results were obtained as collidine consists of various isomers.

Using a mixture of n-butanol-acetic acid-water as the first and a mixture of phenol-meta-cresol-borate buffer (pH = 8.3) as the second solvent LEVY and CHUNG (1953) succeeded in distributing the amino acids almost over the whole filter paper. Moreover the spots were more compact by spraying the filter sheet between both runs with borate buffer (pH = 8.3). We applied this method for our investigation too.

15. PROCEDURE

n-Butanol was distilled and the fraction which boils at 117–118 °C was used. A mixture, containing 4 volumes of n-butanol, 1 volume of acetic acid and 5 volumes of distilled water was prepared and shaken

in a separatory funnel. After separating into two layers the upper layer was used as a solvent and the lower one for equilibrating.

The borate buffer was prepared by adding 0.1 n NaOH to 300 ml 0.1 Mol. boric acid (a.r.) until the pH was 8.3.

Phenol and m-cresol were purified by distillation. The phenol-fraction, which boiled at 178 °C, and the m-cresol-fraction, which distilled at 198–202 °C, were used. The second solvent was prepared by mixing 30 grams meta-cresol with 15 grams phenol and 7.5 ml borate buffer.

The filter paper, which we used for the analyses, was Whatman 1. On this (Fig. 3) one or more drops of the plant extract was fitted in point 0 in the right top corner, after which n-butanol-acetic acid-water as the first solvent was used. After 16 hours the sheet was taken from the trough and dried at 50 °C during 2 or 3 hours. After cutting the paper along the upper folding lines it was turned a quarter turn and sprayed with borate buffer from 3 cm below the second starting line. The sheet was dried again at 50 °C for half an hour. Now the amino acids were separated with the second solvent (meta-cresol-phenol-borate buffer). After 16 hours the paper was dried again at 50 °C for 3 hours.

The spots of the amino acids were made visible by spraying the filter paper with a mixture of 50 ml of a 1 $^0/_{00}$ ninhydrin solution in 96 % alcohol, 2 ml collidine and 15 ml acetic acid and heating the paper at 100 °C for 5 minutes. The visible spots were outlined with a pencil.

16. The identification of the amino acids

If a paper chromatogram is sprayed with an alcoholic ninhydrin solution, purple coloured spots appear; proline and oxyproline however form respectively a yellow and an orange spot. Levy and Chung (1953) found that various amino acids gave colours, clearly diverging after spraying with an alcoholic ninhydrin solution, mixed with collidine and acetic acid.

Though we did not obtain similar results, the differences of these colours enabled us to identify the amino acids.

The distribution of the amino acids over the paper chromatogram has been investigated with synthetic mixtures. Twodimensional chromatograms were analyzed and the R_F-values of each amino acid for both solvents were determined (Table 7). Fig. 4 gives a general view of the distribution over the chromatogram.

Aspartic acid, glutamic acid, serine, glycine, threonine, α-alanine, valine and leucine can be easily distinguished on the chromatograms. The six, first mentioned, occur in about a straight line below each other. At the right lower part of the paper valine, methionine, leucine and phenylalanine can be found. Phenylalanine is striking by the turquoise colour and asparagine by the brown-orange tint, whereas tyrosine is present in the middle of the chromatogram. The other amino acids can be determined by the place, which they occupy with respect to the substances, already mentioned.

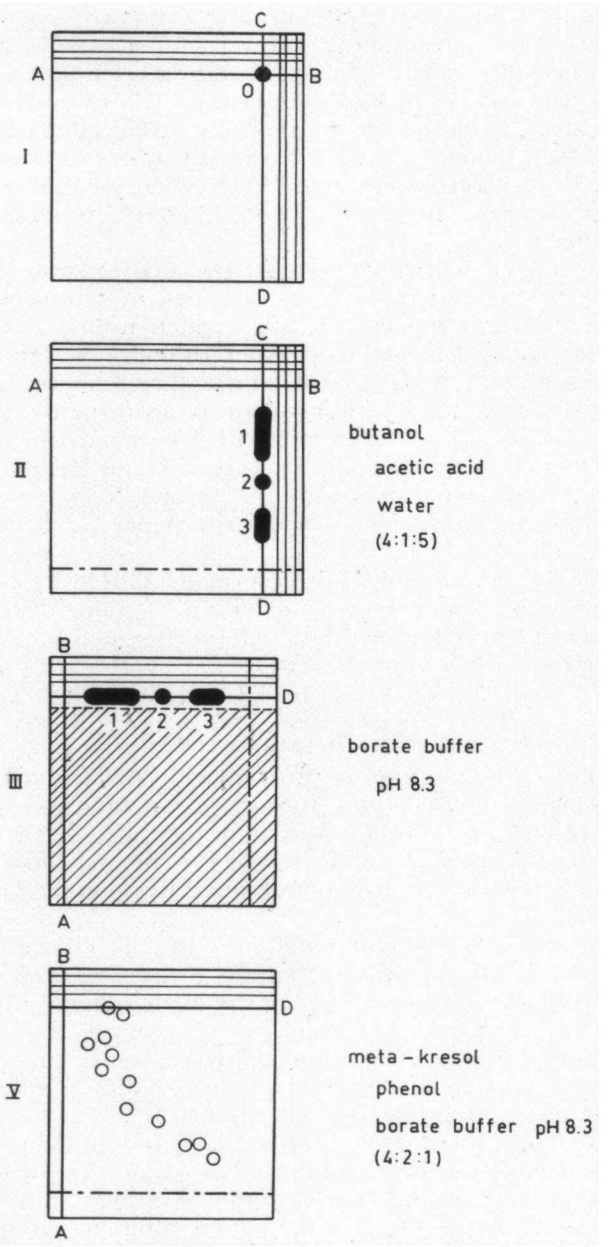


Fig. 3. The various steps of the paper chromatographical analysis. I 0 is the original spot of the extract; AB the first starting line. II The distribution of the amino acids with the first solvent. III Paper attached with the right side up and sprayed with borate buffer (pH = 8.3). IV The distribution of the amino acids with the second solvent.

TABLE 7

The R_F-values of the amino acids for n-butanol-acetic acid-water and meta-cresol-phenol-borate buffer by the twodimensional paper chromatography with spot-colours after fixation with the ninhydrin reagent of Levy and Chung.

Temperature 20° C

amino acid	colour		R_F-values	
	observed	according to Levy and Chung	Butanol-acetic acid-water	m-cresol-phenol-borate buffer
α-Alanine	purple-blue	purple-blue	0.30	0.32
β-Alanine	blue	purple-blue	0.35	0.38
α-Aminobutyric acid	purple-blue	purple-blue	0.42	0.46
γ-Aminobutyric acid	purple-blue	purple-blue	0.38	0.49
Arginine	purple-blue	purple-blue	0.18	0.53
Asparagine	brown-orange	brown-orange	0.21	0.21
Aspartic acid	blue	blue	0.24	0.01
Cysteine	pink	purple-blue	0.36	0.05
Cystine	pink	brown	0.14	0.04
Citrulline	purple-blue	purple-blue	0.27	0.45
Glutamine	purple-blue	purple-blue	0.20	0.33
Glutamic acid	purple-blue	purple-blue	0.27	0.04
Glutathione	pink	purple-blue	0.13	0.01
Glycine	grey-brown	red-purple	0.26	0.19
Histidine	purple-blue	turquoise-blue	0.15	0.29
Isoleucine	purple-blue	purple-blue	0.64	0.81
Leucine	purple-blue	purple-blue	0.72	0.79
Lysine	grey	purple-blue	0.15	0.31
Methionine	purple-blue	grey	0.56	0.71
Ornithine	grey-purple	purple-blue	0.14	0.17
Phenylalanine	turquoise-blue	turquoise-blue	0.75	0.85
Proline	yellow	yellow	0.37	0.81
Serine	purple-blue	sallow	0.25	0.15
Threonine	purple-blue	grey	0.27	0.26
Tryptophane	brown	yellow-brown	0.68	0.80
Tyrosine	pink	turquoise-blue	0.56	0.46
Valine	purple-blue	purple-blue	0.55	0.63

For the qualitative estimation of the amino acids it is of importance to know the minimum amount of each amino acid, which gives still a spot on the chromatogram, clearly observable. For this purpose decreasing amounts (10 μl, 8 μl etc.) of an amino acid solution (2.5 mg/10 ml) were fitted on the filter paper (Whatman 1) and after chromatographing and spraying the minimum amount of amino acid, which gave a spot, just visible, was determined. The result has been mentioned in Tabel 8. For purposes of comparison the corresponding values, which PRATT and AUCLAIR (1948) found by chromatographing with phenol-water and collidine-lutidine-water, have been indicated too.

17. AMINO ACIDS PRESENT IN *Lupinus*, AS FREE AMINO ACIDS OR AS COMPONENTS OF PROTEIN

It is evident from Table 8 that 5–10 μg at least of each amino acid is necessary in order to get a distinct spot on a paper chromatogram.

As the amounts of the amino acids in the extracts diverge considerably, it was necessary to estimate how much of the extract must be fitted on the paper.

The analysis of the protein hydrolysates could be performed, when the dried residue of the purified extract (11.1.4) was dissolved in 0.5 ml water; 5 drops of this solution were sufficient.

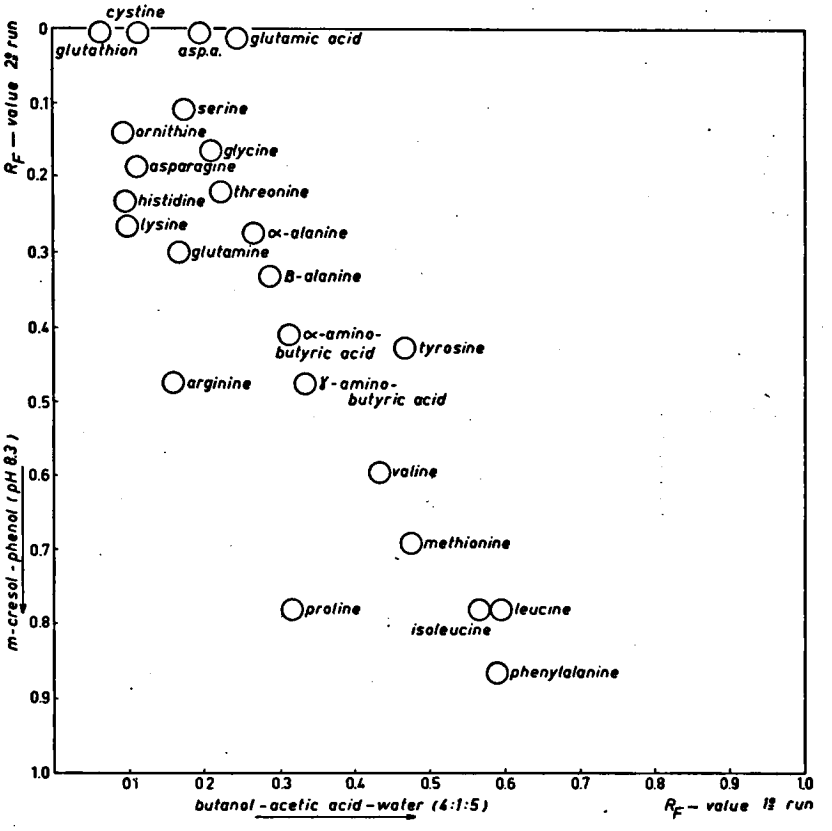


Fig. 4. Scheme of a twodimensional paper chromatogram. Solvents: n-butanol-acetic acid-water (4:1:5) and meta-cresol-phenol-borate buffer (pH = 8.3).

The concentration of the free amino acids in the extracts varied widely; especially asparagine was most noticeable. Good results were obtained by evaporating 8 ml of the crude alcoholic extract in vacuo and dissolving the residue in 0.5 ml water. From this solution 10–20 μl must be taken for a chromatogram. The concentration of the amino acids in the purified extract (11.2.4) was also sufficient.

The results of these analyses have been given in Table 9 and 10.

On the chromatograms of the crude alcoholic extracts of the free amino acids the spot of aspartic acid was hardly visible; on the other hand the spot of asparagine was most noticeable. By the process of the purification this amide was split in the corresponding acid and

TABLE 8

The minimum amount of amino acids, which may be determined on a twodimensional paper chromatogram after spraying with ninhydrin.

Amino acid	Amount in μg, hardly visible	
	Observed	According to Pratt and Auclair
α-Alanine	1	0.2
β-Alanine	< 0.5	0.2
α-Aminobutyric acid	5	0.2
Asparagine	5	1
Aspartic acid	< 0.5	0.4
Cysteine	6	8
Cystine	< 0.5	
Glutamic acid	< 0.5	0.1
Glutamine	5	2
Glutathione	< 3	10
Glycine	< 0.5	0.1
Histidine	4	25
Leucine	3.5	0.5
Lysine	2.5	3
Methionine	5	1
Ornithine	1	3
Oxyproline	5	1
Proline	3.5	1
Serine	< 0.5	0.3
Threonine	3	2
Tryptophane	5	2
Tyrosine	1.5	3
Valine	4	0.2

TABLE 9

Fraction of the free amino acids. The amino acids present in the purified alcoholic extracts of seeds and seedlings ± spots hardly visible; + spots with a diameter of 1 cm; ++ spots with a diameter of 2 cm; etc.

Alcoholic extract of seeds and seedlings of	0 days		7 days		14 days		21 days	
Volume of extract per spot in μl	10	20	10	20	10	20	10	20
Amino acids								
Glutathione?	+	+						
Cystine					+	+	+	+
Aspartic acid	+++	+++	++++	++++	++++	++++	++	++++
Glutamic acid	++	++	+	+	+	+	±	±
Serine	±	+	+	+	++	++	++	++
Glycine	±	±	±	±	±	+	±	+
Threonine	±	±	±	+	±	+	±	+
α-Alanine	±	±	+	+	+	+	+	+
Lysine			+	+	+	+	+	+
Arginine	++	++	+	++	+	++	+	++
Valine			±		±			±
Methionine			+	+	±	+	+	+
Leucine /isoleucine						±		+
Phenylalanine			±	±	±	±	±	+

in ammonia. Rather large amounts of arginine also occurred, whereas the other amino acids however were present in small concentrations.

The proteins contained large amounts of aspartic acid and glutamic acid; especially in the seeds and young seedlings. During the development of the seedlings this quantity decreased considerably. The relative amounts of the other amino acids did not vary much during the germination.

TABLE 10

Protein fraction. The amino acids present in the HCl-extracts of seeds and seedlings. Indications see Table 9.

Purified and hydrolyzed HCl-extract of seeds and seedlings of	0 days		7 days		14 days		21 days	
Volume of extract per spot in μl	10	20	10	20	10	20	10	20
Amino acids								
Cystine	±	+	+	+				+
Aspartic acid . .	++	++	+++	+++	++	++	+	+
Glutamic acid .	+++	+++	++	+++	++	++	+	+
Serine	+	+	+	+	±	+	+	+
Glycine	+	+	+	+	±	±	+	±
Threonine . . .	±	±	+	+	+	+	+	±
α-Alanine . . .	+	+	++	++	±	+	±	+
Histidine . . .		+	±	+		±		±
Lysine		+		+		+		+
Arginine . . .	±	±	+	+	+	+	+	+
Proline	±	±	+	+	+	+	±	±
Valine		+	+	++	+	+	+	+
Leucine/ isoleucine	±	+	++	++	++	++	+	+

CHAPTER V

THE QUANTITATIVE ANALYSIS

18. Introduction

Many investigators tried to modify the paper chromatographical method in order to make this method suitable for quantitative determination of amino acids. They have tried to achieve this in many different ways.

According to Fisher and collaborators (1948) the concentration of the amino acids may be determined from the size of the spots on the paper. The area of a spot is proportional to the logarithm of the amino acid concentration.

Helen Kirby Berry and Cain (1949) estimated the amount of amino acids by comparing the colour-intensity of the spots after spraying with ninhydrin with the intensity of the spots of a known amino acid concentration.

Other investigators separated the amino acids on paper first, cut out the paper where the amino acids occurred, extracted these separately and determined the amino acids in the solutions quantitatively.

However the techniques used differ on matters of minor importance. NAFTALIN (1948) sprayed the paper with 0.025–0.05 % ninhydrin in dry n-butanol, cut out the spots, and extracted the amino acids with a solution of 5 % ninhydrin in n-butanol, saturated with water and adjusted at a pH = 7 with 0.1 % phosphate buffer. After heating from 55 °C to 80 °C, the concentrations were determined photometrically. POLSON and collaborators (1947) extracted the spots, stained with ninhydrin, with acetone. PEREIRA and SERRA (1951) localized the amino acids in ultraviolet light.

WOIWOD (1948, 1949) based the determinations on the titration method of Pope and Stevens. He separated the amino acids paper chromatographically. By adding an alkaline suspension of copper phosphate, a soluble copper-amino acid complex was formed, which could be removed from the mixture by filtration. The amino acid concentration was estimated by treating the complex with sodium diethyldithiocarbaminate and measuring the optical density of the coloured reaction product, formed. MARTIN and MITTELMANN (1948) estimated the copper of the formed complex polarographically, but these results were less satisfactory and not suitable for a quantitative determination.

BLOCK (1948) determined the amino acids in the stained paper chromatograms directly with a special photometer.

However it is evident that these paper chromatographical methods are not satisfactory entirely for the quantitative determination of amino acids. One of the most important errors is that the spots merge sometimes so that no complete separation is achieved.

The best paper chromatographical separations are obtained with phenol or with mixtures wich contain phenol. The use of these solvents in a quantitative analysis however has the disadvantage that the intensity of the staining with ninhydrin diminishes after drying at a higher temperature, necessary for the removal of phenol.

A difficulty is also that the area of the spots cannot be estimated accurately and necessitates the taking of a large margin for the cutting out of the spots.

Moreover the amounts, which can be separated on paper, are small. This requires the determination of the minute amounts of amino acids but the staining with the ninhydrin reagent is not very intensive.

Moreover the comparison of the staining of a spot of an unknown amount of an amino acid with those of known quantities is limited by the concentration intervals used.

All these disadvantages obliged us to apply the separation technique of STEIN and MOORE (1948, 1949a, 1949b).

These investigators used starch columns, obtained by suspending the dried starch in n-butanol. The plant-extracts were percolated through the column and the chromatograms were developped by percolating mixtures of various isomers of butanol. The effluent was collected in small fractions to separate the amino acids. The amount of the amino acids was determined with ninhydrin colorimetrically.

Every analysis took at least five days. Using different columns and solvents a complete analysis may be achieved. Most amino acids can be separated when a starch column is eluated by a mixture of 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl first, followed by an elution with a solution, containing 2 vol. n-propanol and 1 vol. 0.5 n HCl. The separation of leucine, isoleucine, phenylalanine, valine, methionine and tyrosine is incomplete, but these separations

can be achieved with a mixture of 1 vol. n-butanol, 1 vol. benzylalcohol and 0.288 vol. water or with n-butanol containing 15 % water. We did not succeed in getting satisfactory results. Glutamic acid and α-alanine can be separated with a mixture of 2 vol. tertiary butanol, 1 vol. secondary butanol and 1 vol. 0.1 n HCl.

For this technique it is necessary that one fraction at least, completely free of amino acids, is collected between the fractions containing different amino acids. When this does not happen, sometimes the concentration of both amino acids can be estimated with sufficient accuracy. The diagram, representing the concentration-distribution of an amino acid is practically a Gauss curve (Fig. 8). Two distribution-curves partially overlapping each other, can be solved when the height of the point of intersection is less than approximately 0.7 of the maximum value.

This technique of Stein and Moore has been developed further by the use of a column of Dowex-50 with a length of 1 meter instead of a starch column (STEIN and MOORE 1951). A solution, containing 30 substances at least, reacting with ninhydrin, could be separated very well. The performance of this analysis is rather complicated, as the amino acids are eluated by various buffer solutions. Moreover the elution had to occur at different temperatures. The technique with a Dowex-50 column requires about 3.5 times as many fractions as with a starch column. The amino acids with a basic group must be investigated separately with a shorter column of Dowex-50.

According to Stein and Moore both methods have the disadvantage that many solvents are necessary for a complete separation. Moreover using starch columns, extracts with a high salt content must previously be desalted and freed from carbohydrates, as these disturb the isolation of the components.

With columns of Dowex-50 serine and asparagine, as well as α-alanine and α-aminoadipic acid are not separated. This may be performed however by a starch column using two solvents: a solution, containing 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl and a mixture of 2 vol. n-propanol with 1 vol. 0.5 n HCl (WINDSOR 1951). Also the analysis of tyrosine and phenylalanine is performed easier with a starch column than with a Dowex-50 column (SCHROEDER, 1954). Moreover the separation by the ion exchanger is very sensitive to fluctuations of temperature, in contrast with the other adsorbents. SCHROEDER (1954) prefers besides the more modern method with Dowex-50 the use of starch columns.

In this investigation we used starch columns for the separation of the amino acids; the plant extracts, which must be investigated, are free from inorganic ions and carbohydrates.

19. THE QUANTITATIVE ANALYSIS OF THE AMINO ACIDS WITH THE METHOD OF STEIN AND MOORE

19.1. *Columns*

268 g potato-starch, dried at 100 °C for 24 hours, was suspended

in a mixture of 500 ml dry n-butanol and 80 ml water and shaken for 1 hour. A starch-column (diameter 2 cm; length 30 cm after 1 hour settling; Fig. 5) was made and the excess of butanol sucked off. When the liquid had just passed the surface about 5 ml of a mixture of 1 vol. n-propanol and 1 vol. 0.5 n HCl was poured

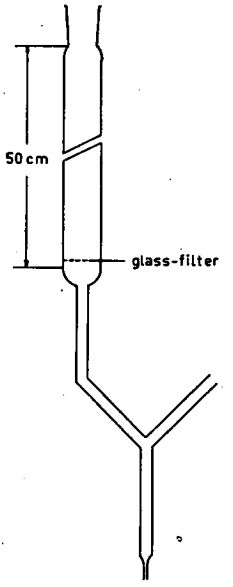


Fig. 5. Glass filtertube for a starch column

carefully into the tube. A separatory funnel was placed on the column filled with the same solution; the percolation was continued, whether under pressure or not until the effluent no longer reacted with ninhydrin.

Then the solvent, which was used for the analysis, was percolated through the column for 2 days, when the percolation was performed without pressure; under a pressure of 0.1 atm. it took 24 hours.

The mixture of amino acids, obtained according to the method described in 11. was dissolved in 2.5 ml of the solvent which was used. When the solvent just passed the surface of the column, the solution was poured into the tube carefully. When the surface was dry again, the tube was washed with 2.5 ml of the solvent. This was repeated once again, after which the column was placed on the automatic fraction collector and the separatory funnel filled with the solvent. Now the fractionation process started.

19.2. *Solvents*

a. The first analysis was performed with a mixture of 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl as solvent. With this solution the following compounds or mixtures of compounds were separated from the plant extracts or hydrolysates: *a:* leucine, isoleucine, phenylalanine; *b:*valine, methionine and tyrosine; *c:*α-aminoadipic acid; *d:*γ-

aminobutyric acid; *e:*proline; *f:*glutamic acid and alanine; *g:*threonine; *h:*aspartic acid.

When the fraction with the maximum amount of proline, which could be detected easily by the yellow colour with ninhydrin, had been collected, the solvent was changed at the fraction number, which corresponded to the fraction number of the aforesaid maximum, multiplied by 1.6. The separatory funnel was removed, the solvent sucked off from the column. When the remaining liquid just passed the surface of the column, 2 vol. n-propanol and 1 vol. 0.5 n HCl was poured into the tube and a separatory funnel, filled with the same solvent, was placed on the column. The fractionation was continued with this second solvent. Serine; glycine; a solution containing ammonia, glutamine and asparagine; arginine; lysine; histidine; cystine were collected separately. Approximately 10 to 15 fractions after changing the solvent a small peak was observed, in consequence of this change in solvents.

b. Using a new starch-column (length 30 cm; diameter 2 cm) glutamic acid and alanine could be separated by a mixture of 2 vol. tertiary butanol, 1 vol. secondary butanol and 1 vol. 0.1 n HCl.

c. Leucine, isoleucine and phenylalanine were collected separately with a starch column (length 50 cm; diameter 2 cm) and a mixture of 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl as solvent. The same procedure was applied for the separation of valine, methionine and tyrosine.

d. The separation of glutamine, asparagine and ammonia is discussed in 19.

19.3. *Automatic Fraction Collector*

The fraction collector, used in this investigation, is based on a fractionation after equal volumes and was made after the model of the collector which had been designed at the Laboratory of "Organon" at Oss. LENS and EVERTZEN (1952a) had constructed this collector after the model of RANDALL and MARTIN (1949).

The apparatus, consists of a circular rack (1), in which test tubes are placed (Fig. 6). Above the tubes there is the outlet of the tube (2) of the siphon (3) which hangs on the balance (5) by a twisted thread (4). By the contra-weight (6) the balance is kept in equilibrium. The weight (7) controlls the hanging of the funnel with the wide tube of the siphon vertically under the point of suspension of the balance. Through the tube (8) the drops from the column fall on the funnel of the siphon. The siphon can move in a horizontal plane by turning back of the thread (4). When the level of the liquid in the siphon is as high as the turn of the capillary tube (9), the siphon empties itself quickly; the balance pulls the siphon up; the outlet moves over the pen (10) and is checked by the pen (11), bent twice. The weight is greater by the drops which come into the siphon, so that the balance is no longer in equilibrium. The siphon comes down slowly; the outlet passes the horizontal part of pen (11), and now stops by the next pen (10). Then the process starts again.

In order to prevent the siphon moving in the opposite direction during siphoning, the ring (12) has been fitted, which is wider than the tube. During emptying a slight friction arises, which prevents the balance to swing.

The tube between the points (13) and (14) is a capillary. The diameter may not be wider than 3.2 mm, otherwise the solvents used cannot be siphoned; when the diameter is too small, the emptying of the siphon is too slow.

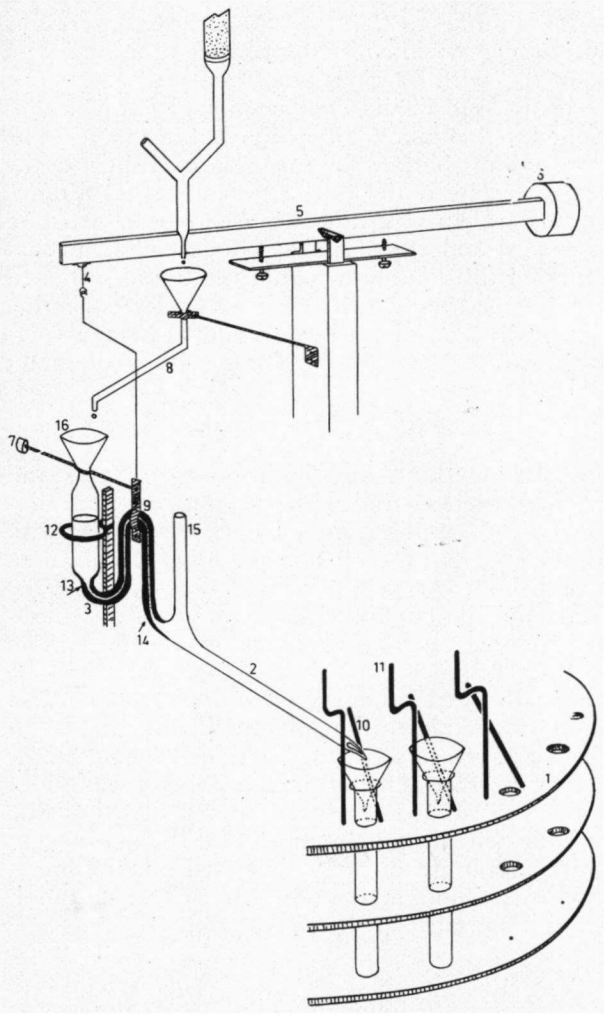


Fig. 6.
The automatic fraction collector.

It is necessary that the emptying proceeds so quickly that the next drop falls, when the tube has been emptied entirely. In order to shorten the time of emptying, the tube (15) has been fitted. If this drop falls, when the siphon was not entirely empty, a small air-bubble was locked

up, by which the following fraction will be siphoned too soon and its volume too small.

After emptying of the siphon, a small drop of liquid remained in the lower turn. This had no influence on the accuracy of the analysis. In order to prevent a drop, falling just in the capillary, from creating an air-bubble, we let the drops fall on the rim of the funnel (16), by which they flowed down along the glass.

Following the procedure of LENS and EVERTZEN (1952a, b) starch columns of 30 × 2 cm were used; to achieve better separations, we reduced the fraction-volume to 2.60 ml. As satisfactory results were obtained as those which Stein and Moore had obtained with columns of 30 × 0.9 cm and with fractions of 0.5 ml.

In order to be able to use one fraction collector with two columns a second tube was fastened to the siphon, following its movements. The drops, which passed the second column, fell through this tube into the test-tubes of the inner row. The outlet of this tube and the distance of the centre of the circular rack to the inner row had been fitted in such a way that the drops were collected in a tube from the moment that the siphon had just emptied till the moment that the liquid siphoned again. This could be performed, as the time, necessary for collecting each fraction, was practically constant. The second apparatus permitted to collect fractions of 0.2 ml with a column with a diameter of 0.5 cm.

19.4. *The Fractionation*

The samples of the plant extracts and hydrolysates (11.1.4 and 11.2.4) were fractionated. Fractionating with columns of 2 cm diameter the dried extracts, dissolved in 2.5 ml solvent, were used; with columns of 0.5 cm diameter 1/5–1/10 of this amount, dissolved in 0.5 ml solvent, was sufficient.

When the amino acid solutions contained large amounts of asparagine, glutamine and ammonia (e.g. the extracts of the free amino acids), then no more than 25 ml of the alcoholic extract could be chromatographed. Under the experimental conditions glycine and arginine, which eluated respectively immediately before and after these amides, could be collected in separate fractions. The solutions passed the columns under pressure of 0.1 atm.; the velocity was about 5–7 ml per hour. If the velocity was reduced to 2/3 of this value the amino acids were collected in later fractions and the peaks were irregular. Normally with a column of 30 × 2 cm the maximum amount of glutamic acid was found in fraction 106, with n-butanol, n-propanol, 0.1 n HCl as a solvent.

19.5. *The Ninhydrin Reagent*

For the analysis of 10 fractions the following solutions are necessary:

(a) 100 mg ninhydrin dissolved in 5 ml of a mixture of equal volumes of methylcellosolve and water;

(b) 8 mg stannous chloride (a.r.) dissolved in 5 ml citrate buffer (pH = 5.0);

(c) 4.2 g citric acid-H_2O dissolved in about 50 ml water, with n NaOH adjusted at a pH = 5.0 and filled up with water to 100 ml; a few drops of amylalcohol stabilized the solution for a long time. It was necessary to adjust the pH again the next day; then it remained constant.

Equal parts of the ninhydrin and the stannous chloride solutions were mixed. Each day new reagent had to be prepared.

19.6. *The Colorimetric Determination of the Amino Acids with the Ninhydrin Reagent*

When a mixture of n-propanol and 0.5 n HCl had been used, 0.2 ml from each fraction, 0.5 ml citrate buffer and 1 ml of the ninhydrin reagent were added together. The samples were heated on a boiling waterbath for 20 minutes. The solution was cooled and diluted with 4.5 ml of mixture of equal volumes of n-propanol and water. The concentration of the amino acids was measured spectrophotometrically at λ 570 mμ (Beckman spectrophotometer).

Using the other solvents 0.2 ml of a fraction and 1 ml ninhydrin reagent were added together, heated, diluted after cooling with 5 ml of the mixture of n-propanol and water and the concentration of the amino acids was measured. The colorimetric determination of proline however was performed at λ 440 mμ.

19.7. *Remarks*

(a) Columns with a length of 30 cm were too short for the fractionation with the solvents n-butanol, n-propanol and 0.1 n HCl and n-propanol, 0.5 n HCl, because the crude extracts of the free amino acids (7.3) contained chiefly asparagine; a sufficient separation of serine, glycine, asparagine and arginine could not be obtained. With columns of 40 cm length however these amino acids, were separated completely. The peaks of the amino acids were lower and broader than with shorter columns but they could be identified easily.

For the purified extracts (11.1.4 and 11.2.4) columns of a length of 30 cm were sufficient.

The separations with a mixture of 2 vol. tertiary butanol, 1 vol. secondary butanol and 1 vol. 0.1 n HCl, (column 30 cm) or with a mixture of 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl (column 50 cm) were only performed with the crude alcoholic extracts (7.3) and with the purified HCl-extracts (11.1.4).

(b) Stein and Moore pointed out that 20 mg ninhydrin would be necessary for each amino acid determination. Some preliminary experiments showed however that the same results could be obtained with 10 mg ninhydrin. This is in agreement with the results of VAN DER SCHAAF and HUISMANS (1954).

The optical densities of a series of measurings (λ 570 mμ) of 0.2 ml, solution, containing 0.6 μg threonine-N, were 0.164 $\pm$ 0.015 with 20 mg ninhydrin and 0.162 $\pm$ 0.008 with 10 mg ninhydrin.

(c) A dilution of the methylcellosolve with an equal volume of water had no influence on the reaction.

(d) Stein and Moore analysed the fractions obtained with n-propanol, and 0.5 n HCl as solvent after neutralizing with 0.1 n NaOH. We could demonstrate that addition of 0.5 ml citrate buffer gave the same results.

Aspartic acid, dissolved in 0.2 ml of a mixture of 1 vol. n-butanol, 2 vol. n-propanol, and 1 vol. 0.1 n HCl had the same optical density after reacting with ninhydrin and diluting with 5 ml n-propanol-water, as dissolved in 0.2 ml of a solution of 2 vol. n-propanol and 1 vol. 0.5 n HCl, provided that the last mentioned samples were neutralized with 0.5 ml citrate buffer before the ninhydrin reaction and diluted with 4.5 ml n-propanol-water.

(e) The concentration of the citrate buffer was twice the concentration of the buffer used by Stein and Moore.

(f) In the extract of the free amino acids of seeds an unknown compound was found; it might have been glutathione.

19.8. *Calculations*

Stein and Moore calculated the concentrations of the amino acids, present in the various fractions, from the values of the optical densities, measured. For this purpose they determined the optical densities of a series of leucine solutions, diluted with water, after addition of the ninhydrin reagent. In the same way they determined the optical densities of equimolecular amounts of the other amino acids. As they neutralized the fractions with diluted NaOH and as the organic solvents evaporated partly during heating on the waterbath, they had to introduce correction factors.

Unlike Stein and Moore we have determined for each amino acid in series of dilutions under the same experimental conditions as the plant extracts were investigated, the corresponding extinction-value for various amounts of the amino acids in 0.2 ml. For each amino acid the optical densities of these solutions after reacting with ninhydrin plotted against the concentrations, gave straight lines, as is shown in the following table.

All the density measurings were performed against distilled water. For the blank determination fractions, containing the solvent which had passed the column and free from amino acids, were taken from the fraction collector every day; ninhydrin was added in the usual way. The mean of the optical densities of 10 fractions was used as the value of the blank determination for the intermediate fractions, containing amino acids. The variations in the optical densities of the blank determinations were small; the mean value, measured against distilled water was 0.058 ± 0.010 (mean of 100 determinations) and did not differ for the solvents, used.

The amount of each amino acid present in 0.2 ml of all the fractions (n), in which the amino acid was present was calculated by adding the measured optical densities and subtracting $n \times 0.058$, as the optical densities had been measured against water. This result was divided by the corresponding value of $E\ \frac{1\ \mu g}{1\ cm}$. The amount of

TABLE 11

The optical densities of amino acid solutions after the ninhydrin reaction.

The optical density $E\,\frac{1\ \mu g\ N}{1\ cm}$ has been measured in 6.2 ml reaction mixture (λ 570 mμ; for proline λ 440 mμ) . For each amino acid a series of solutions was prepared, rising from 0.4 μg, 0.8 μg to 4 μg amino-N in 0.2 ml of the solvent, used for the fractionation through the starch column. $E\,\frac{1\ \mu g}{1\ cm}$ was calculated from the measured optical densities as a mean of $E_1 = \frac{\Sigma (c-\bar{c})\ (E-\bar{E})}{\Sigma (c-\bar{c})^2}$ and $E_2 = \frac{\Sigma (E-\bar{E})^2}{\Sigma (E-\bar{E})\ (c-\bar{c})}$. The correlation coefficient was calculated from $R = \frac{\Sigma (E-\bar{E})(c-\bar{c})}{n\ \sigma_E\ \sigma_c}$ and the standard deviation of R from $\sigma_R = \frac{(1+R)\ (1-R)}{\sqrt{n}}$. (c is concentration of amino acid-N in μg/0.2 ml and E is the corresponding extinction-value, measured; $\bar{c}$ and $\bar{E}$ are the corresponding mean values). (FISHER 1948, page 183). Solvent 1: 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl; Solvent 2: 2 vol. n-propanol and 1 vol. 0.5 n HCl. Solvent 3: 2 vol. tertiary butanol, 1 vol. secondary butanol and 1 vol. 0.1 n HCl.

Amino acid	Solvent	$E\,\frac{1\ \mu g}{1\ cm}$	R	$\sigma_R \times 10^3$
Glutathione	1	0.069	0.999	0.4
Leucine	1	0.234	0.970	11
Isoleucine	1	0.231	0.997	1.1
Phenylalanine	1	0.205	0.999	0.4
Valine	1	0.239	1.000	0
Methionine	1	0.236	0.999	0.4
Tyrosine	1	0.221	0.999	0.4
γ-Aminobutyric acid	1	0.204	0.998	0.7
Proline	1	0.067	0.999	0.4
Glutamic acid	1	0.255	0.992	2.9
α-Alanine	1	0.233	0.997	1.1
Threonine	1	0.204	0.998	0.7
Aspartic acid	1	0.210	0.997	1.1
Aspartic acid	2	0.209	0.999	0.4
Serine	2	0.246	0.998	0.7
Glycine	2	0.214	0.999	0.4
Ammonium chloride	2	0.195	0.999	0.4
Asparagine	2	0.109	0.998	0.7
Glutamine	2	0.117	0.998	0.7
Arginine	2	0.056	0.998	0.7
Lysine	2	0.116	0.998	0.7
Histidine	2	0.074	0.999	0.4
Cystine	2	0.123	0.871	44.1
Leucine	3	0.236	0.998	0.7
Isoleucine	3	0.238	0.997	1.1
Phenylalanine	3	0.205	0.997	1.1
Valine	3	0.235	0.998	0.7
Methionine	3	0.212	0.998	0.7
Tyrosine	3	0.209	0.998	0.7
Glutamic acid	3	0.240	0.998	0.7
α-Alanine	3	0.209	0.996	1.1

We assumed that $E\,\frac{1\ \mu g}{1\ cm}$ of α-aminoadipic acid is 0.255. The determination of this value could not be performed, as we did not dispose of a sufficient amount of this compound.

[1]) My thanks are due to Drs P. E. Venekamp, Municipal Bureau of Statistics of Amsterdam, for his advice in the statistical calculations.

amino acid-N, which had passed the column, was calculated by multiplying this quotient, by 13, as the fraction-volume of the fraction collector, was 2.60 ml. From this the total quantity of each amino acid in 100 seeds or seedlings could be easily deduced.

For the columns with a diameter of 0.5 cm the multiplication by 13 must be omitted, because the fractions, collected, had a volume of 0.2 ml.

The results of the quantitative determination of ammonia, present in the fractions 180–205, obtained by the chromatographical separation of the amino acids in the protein hydrolysate of seeds, have been given in Fig. 7. Also a Gauss curve, calculated by the method of least squares from the extinction values, measured, has been plotted. It is evident that both curves agree very closely. Similar results were obtained with the other amino acids.

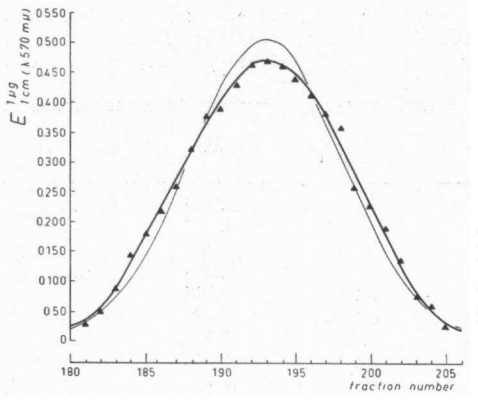


Fig. 7. The graph of the extinction values of the fractions, containing ammonia, compared with the Gauss curve, calculated from these values by the method of least squares. This graph shows a part of Fig. 8., enlarged.

Sometimes a partial overlapping of two peaks occurred. If this is the case, the amount of the amino acids could be easily determined by considering both peaks as Gauss curves. A small number of fractions was concerned by the overlapping; the distance between the tops of both peaks being larger than the sum of 1.5 σ_1 and 1.5 σ_2 (σ_1 and σ_2 were the standard deviations of both peaks.)

During the purification the amides are split in the corresponding amino acids and ammonia. The difference of the amount of aspartic acid-N, respectively glutamic acid-N of a crude and of a purified alcoholic extract indicates the amount of asparagine-N, respectively of glutamine-N present; the same result was obtained from the difference of NH_3-N between both extracts.

20. Control

It was still necessary to investigate if a loss of amino acids occurred during the treatments (fractionation and assessment). For this reason the amounts of amino acids present in a HCl-extract of seeds were determined, also when known amounts of amino acids were added. Moreover the synthetic mixture was analysed separately.

Table 12 shows that the methods, used for the determinations of the amino acids, gave reliable results.

To check the method further the sum of the optical densities of all the fractions was calculated for the crude alcoholic extracts separated

TABLE 12

The amino acids in the protein-hydrolysate of seeds of Lupinus luteus; recovery of the amino acids added to the hydrolysate.

Starch column: height 30 cm, diameter 2 cm. Solvents: 1 vol. n-butanol; 2 vol. n-propanol and 1 vol. 0.1 n HCl; 2 vol. n-propanol and 1 vol. 0.5 n HCl; 2 vol. tertiary butanol, 1 vol. secundary butanol and 1 vol. 0.1 n HCl (for the separation of glutamic acid and alanine).

A: 10 ml solvent, acidified with hydrochloric acid, containing 2 mg of each of the following amino acids: leucine, phenylalanine, tyrosine, valine, proline, alanine, threonine, aspartic acid, serine, glycine, arginine, lysine, histidine, cystine, and 2 mg ammoniumchloride.

B: HCl-extract from seeds (11.1.4).

Amino acid	µg N in 2.5 ml solution A	recovery after chromatographical separation: µg N in 2.5 ml solution A	% recovery	µg N in 1.5 ml solution B	µg N in 1.5 ml solution B and 2.5 ml solution A	difference 5–4	% recovery
	1	2	3	4	5	6	7
Leucine/isoleucine	53.5	51.9	97	125.3	177.4	52.1	97
Phenylalanine	42.4	42.8	101	39.1	80.9	41.8	99
Valine	59.8	61.0	102	45.1	105.6	60.5	101
Tyrosine	38.7	37.9	98	24.1	61.6	37.5	97
Proline	60.9	63.3	104	21.3	81.1	59.8	98
Glutamic acid	—	—	—	238.8	237.9	—	—
Alanine	78.7	77.1	98	73.1	150.1	77.0	98
Threonine	58.8	58.2	99	25.5	84.6	59.1	101
Aspartic acid	52.6	50.5	96	175.8	228.8	53.0	101
Serine	66.7	66.7	100	59.5	125.0	65.5	98
Glycine	93.3	94.2	101	135.7	228.6	92.9	100
Ammonia	130.9	137.4	105	137.7	270.5	132.8	101
Arginine	160.9	162.5	101	229.3	387.3	158.0	98
Lysine	76.6	78.1	102	119.8	195.4	75.6	99
Histidine	100.2	104.2	104	75.3	176.3	101.0	101
Cystine	58.3	60.0	103	42.3	98.4	56.1	96

with the solvents n-butanol, n-propanol and 0.1 n HCl and with n-propanol and 0.5 n HCl. Also the total optical density of the extract had been determined before the chromatographical analysis. Both values did not differ more than 4 %.

21. The determination of ammonia

During the purification of the extracts with the anion exchangers De-Acidite-F and Dowex-2 the ammonium ions pass the column first and are separated from the amino acids (11.1.3d).

Besides ammonia another substance, reacting with ninhydrin, appeared to occur in this fraction. After drying a crystalline residue was obtained. Dissolved in 1 ml water, the alkaline solution was chromatographed on paper with a solvent, containing 4 vol. m-cresol, 2 vol. phenol and 1 vol. borate buffer; a distinct spot was visible. It may be considered, whether perhaps the most strongly basic amino

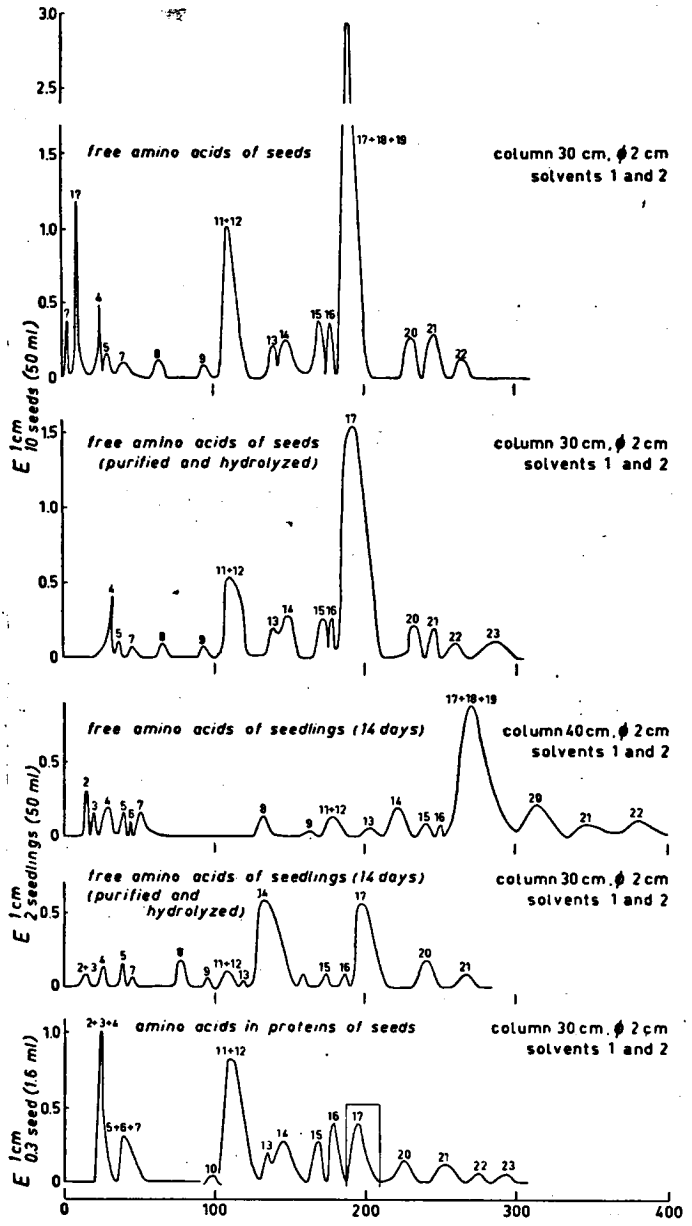
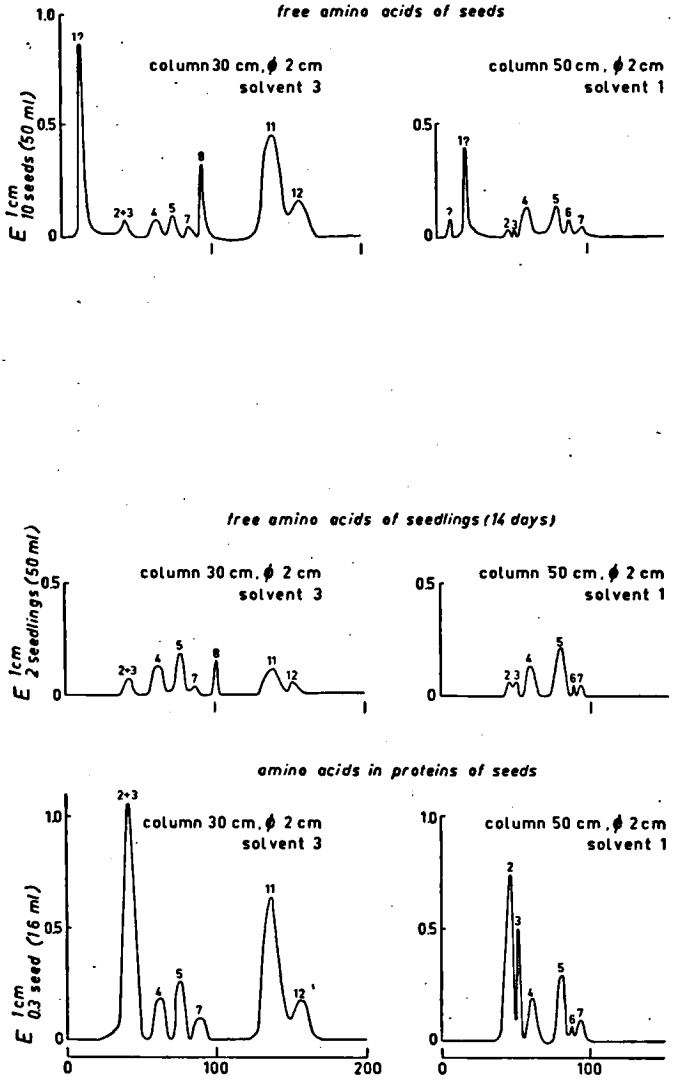


Fig. 8. The fractionation by starch columns. Abscissa: fraction number; ordinate: optical density. Volume of each fraction: 2.60 ml, Solvent 1: 1 vol. n-butanol, 2 vol. n-propanol and 1 vol. 0.1 n HCl; Solvent 2: 2 vol. n-propanol and 1 vol. 0.5 n HCl; Solvent 3: 2 vol. tertiary butanol, 1 vol. secondary butanol and 1 vol. 0.1 n HCl. The figures of the framed part of the curve (protein hydrolysate of seeds) has been given in detail in Table 14.

acid, arginine, would be adsorbed by the anion exchanger incompletely. This is not the case. On the one hand arginine was recovered completely in the control experiment (Table 12), on the other arginine had a R_F -value of 0.53 and the unknown compound had a R_F -value of 0.40. We have assumed arbitrarily that the unknown substance had



Amino acids: 1 Peptides; 2 Leucine; 3 Isoleucine; 4 Phenylalanine; 5 Valine; 6. Methionine; 7. Tyrosine; 8. α-Aminoadipic acid; 9. γ-Aminobutyric acid; 10. Proline; 11. Glutamic acid; 12. Alanine; 13. Threonine; 14. Aspartic acid; 15. Serine; 16. Glycine; 17. Ammonia; 18. Asparagine; 19. Glutamine; 20.Arginine; 21. Lysine; 22. Histidine; 23. Cystine.

the same optical density as ammonia after reacting with ninhydrin.

The amount of ammonia was determined by comparing the optical densities of the fractions with and without this substance after reacting with ninhydrin according to Stein and Moore. The extinction of 1 μg NH_3-N in 0.2 ml was 0.227.

22. Results

The amounts of amino acids present in the purified HCl-extracts (the protein-hydrolysates) and in the crude and purified alcoholic extracts (the free amino acids) were determined quantitatively by the method, described in this Chapter. The results, summarized in Table 13, refer to 100 seeds or seedlings of respectively 7, 14 and 21 days old.

TABLE 13

The amino acids in 100 *seeds or seedlings of* 7, 14 *and* 21 *days old in mg nitrogen*
The amino acids and amides in mg nitrogen

	Free amino acids				Amino acids in protein-hydrolysates				Total			
Age in days	0	7	14	21	0	7	14	21	0	7	14	21
Glutathione	3	—	—	—	—	—	—	—	3	—	—	—
Leucine	0	0	1	1	36	27	23	5	36	27	24	6
Isoleucine	0	1	1	1	12	11	14	5	12	12	15	6
Phenylalanine	0	2	4	6	15	13	12	5	15	15	16	11
Valine	0	2	3	4	16	13	13	7	16	15	16	11
Methionine	0	0	0	0	2	1	1	1	2	1	1	1
Tyrosine	0	2	3	1	8	4	4	0	8	6	7	1
α-Aminoadipic acid	0	0	1	1	3	4	—	—	3	4	1	1
γ-Aminobutyric acid	0	1	1	1	—	—	—	—	0	1	1	1
Proline	—	—	—	—	6	9	9	2	6	9	9	2
Glutamic acid	1	3	4	1	93	59	40	7	94	62	44	8
Alanine	1	2	—	—	28	10	9	7	29	12	9	7
Threonine	0	1	2	2	12	8	7	6	12	9	9	8
Aspartic acid	1	2	3	5	64	58	45	6	65	60	48	11
Serine	1	4	5	6	24	15	11	8	25	19	16	14
Glycine	0	1	3	4	55	25	20	13	55	26	23	17
Ammonia	9[1]	13[1]	3[1]	—	—	—	—	—	58	87	86	14
	—	—	—	—	49[2]	74[2]	83[2]	14[2]				
Asparagine[3]	0	98	169	351	—	—	—	—	0	98	169	351
Glutamine[3]	0	2	3	5	—	—	—	—	0	2	3	5
Arginine	3	37	65	102	85	64	26	29	88	101	91	131
Lysine	1	5	9	10	51	28	10	5	52	33	19	15
Histidine	1	9	14	17	28	19	15	3	29	28	29	20
Cystine	1	2	9	8	18	12	—	3	19	14	9	11
Unknown	—	—	—	—	19	5	4	1	19	5	4	1
Total	22	187	303	526	624	459	346	127	646	646	649	653

[1]) free ammonia in plant extracts (alcoholic extract)
[2]) ammonia present in the protein hydrolysate
[3]) absent in the protein fraction by hydrolysis of the amides in ammonia and the corresponding acids.

Fig. 8 indicates the distribution of the various amino acids over the 600–700 fractions as well as the total amount of the amino acids in

0.2 ml of each fraction. It is demonstrated that the amides have been hydrolyzed by the purification. In order to show the course of the analysis in detail, the results of the colorimetric determination of ammonia has been given in Table 14. It is to be noted that two substances, soluble in alcohol and reacting with ninhydrin, occur in the seeds; they eluate before leucine. By the purification they disappeared and more cystine occurred. Within the limits of errors of the analysis an increase of other amino acids did not occur.

TABLE 14

The optical densities of the ammonia peak of the protein hydrolysate of seeds

These figures belong to the framed part of Fig. 8. The optical densities $E_{1\ cm}$ were determined at λ 570 mμ in 0.2 ml of each fraction with the ninhydrin reagent.

Fraction number	$E_{1\ cm}$	Fraction number	$E_{1\ cm}$
180	0.005	194	0.460
181	0.031	195	0.441
182	0.050	196	0.414
183	0.090	197	0.380
184	0.144	198	0.357
185	0.183	199	0.255
186	0.217	200	0.225
187	0.255	201	0.191
188	0.316	202	0.135
189	0.378	203	0.074
190	0.391	204	0.059
191	0.430	205	0.024
192	0.466	206	0.008
193	0.471	207	0.001

23. Remarks

From Table 13 it is evident that the seed proteins contained 624 mg N or about 71 % of the total N, present in the seed. This percentage is in agreement with the results, described in (7.4). After dissolving the proteins in a solution, containing 1.4–2 % Na_2SO_3 or 30 % urea, 70 % of the total-N was precipitated by trichloroacetic acid. When the denaturated proteins were centrifuged, no more than 20 mg amino-N ($2^1/_2$% of the total-N) could be found in the supernatant. By dialysing against water these amino acids were removed. It appeared that proteins were absent; this means that the total amount of the proteins in the seeds or seedlings had been analyzed. The same protein content had been obtained by Kostytschew (1926) and Heinrich (1941). They reported that the protein-N content of lupin seeds was 70–72 %.

CHAPTER VI

DISCUSSION

24. Introduction

During the catabolic changes, which occur in etiolated seedlings of *Lupinus luteus* under aerobic conditions, a striking accumulation

of amides takes place. SCHULZE (1892) supposed, following the suggestion of BORODIN, that this accumulation is the result of the starvation. Then the proteins are hydrolyzed and the resulting amino acids are used as substrates for the respiration. He demonstrated that in lupins, grown in the light, no accumulation of asparagine, such as found in the darkness, occurs.

In etiolated seedlings, transferred to light, the accumulated asparagine gradually disappears.

When we enter into this problem in detail, we have to discuss the following questions: (a) which are the sources for the amino-groups of the accumulated amides?; (b) from where do their amide-groups originate?; (c) what is the origin of the nitrogen-free precursor of these amides?; (d) which amino acids serve in the respiratory process as the source of energy for the essential functions of the living matter?

The last two problems are of special importance. We have to assume that etiolated seedlings do not dispose of other sources of the nitrogen-free precursors, necessary for the formation of the amides, than the carbon-skeletons of amino acids; the amino acid transformation as well as the respiration depends on the breakdown of certain amino acids. This is in contrast with the respiration processes of the normal seedlings, when a sufficient supply of sugars is available.

Moreover the problem arises whether any relation exists between the processes of amino acid transformation and the respiration in the etiolated seedling.

Our knowledge of both processes is limited. Certain facts however are known; now we have succeeded to give a rather complete balance of the nitrogen metabolism, as far as the amino acids are concerned, there are perhaps sufficient facts available to permit convincing speculation on the processes involved.

25. THE FORMATION OF GLUTAMINE AND ASPARAGINE

25.1. *Glutamine*

The formation of glutamine from glutamic acid and ammonia is the well-known reaction (1).

[1] glutamic acid (I) + ammonia → glutamine (II)

SPECK (1949) succeeded in obtaining an active enzyme preparation from pigeon liver, catalyzing this reaction. The synthesis of the amide was accelerated by phosphate, magnesium ions and adenosine triphosphate. With A.T.P. a stoichiometric relationship was established between the ammonia utilized, the glutamine formed and the inorganic phosphorus liberated. KREBS (1935) had already shown that glutamine synthesis in kidney and brain tissue proceeds only aerobically; the role of respiration in amide formation is a production of energy-rich phosphate, necessary to drive the reaction.

A few years later DENÉS and GAZDA (1953) could demonstrate the presence of this enzyme system in germinating *Lupinus alba*; according to WEBSTER and VARNER (1954) it is also present in peas.

$COOH$ $\vert$ CH_2 $\vert$ CH_2 $\vert$ $CHNH_2$ $\vert$ $COOH$	$CONH_2$ $\vert$ CH_2 $\vert$ CH_2 $\vert$ $CHNH_2$ $\vert$ $COOH$	$COOH$ $\vert$ CH_2 $\vert$ $CHNH_2$ $\vert$ $COOH$	$CONH_2$ $\vert$ CH_2 $\vert$ $CHNH_2$ $\vert$ $COOH$
I	II	III	IV
glutamic acid	glutamine	aspartic acid	asparagine

25.2. *Asparagine*

Till now no evidence has been obtained that in biological systems asparagine can be formed in an analogous way from aspartic acid and ammonia [1]).

(a) MARDASHEV and LESTROVAJA (1951) demonstrated that asparagine can be synthesized in the presence of rat liver tissue according to the following reaction:

[2] aspartic acid (III) + glutamine (II) → asparagine (IV) + glutamic acid (I)

Probably this transamidation may also occur in *Saccharomyces cerevisiae*, as SHAFFNER and GRABOW (1953) could demonstrate that glutamine is synthesized, when asparagine and glutamic acid were added to the culture medium.

As aspartic acid can be formed by a transamination, involving glutamic acid and oxaloacetic acid [6a] and glutamine reacts with aspartic acid [2], we expect that asparagine can be synthesized from oxaloacetic acid and glutamine:

[6a] oxaloacetic acid (V) + glutamic acid (I) → aspartic acid (III) + α-keto glutaric acid (VI)

[2] aspartic acid (III) + glutamine (II) → asparagine (IV) + glutamic acid (I)

[2a] oxaloacetic acid + glutamine → asparagine + α ketoglutaric acid

According to this scheme the first reaction would be a transamination, the second a transamidation.

$COOH$ $\vert$ CH_2 $\vert$ CO $\vert$ $COOH$	$COOH$ $\vert$ CH_2 $\vert$ CH_2 $\vert$ CO $\vert$ $COOH$	$CONH_2$ $\vert$ CH_2 $\vert$ CO $\vert$ $COOH$	$CONH_2$ $\vert$ CH_2 $\vert$ CH_2 $\vert$ CO $\vert$ $COOH$
V	VI	VII	VIII
oxaloacetic acid	α-ketoglutaric acid	α-keto-succinamic acid	α-keto-glutaramic acid

[1]) In a recent publication WEBSTER and VARNER (1955) described the formation of asparagine in *Lupinus* from aspartic acid and ammonia.

(b) GREENSTEIN and CARTER (1947) found that in liver an enzyme is present, which deamidates glutamine in the presence of an α-keto-acid [2a]. Later on this was confirmed by the work of SHEPHERD and KALNITSKY (1951). MEISTER and TICE (1950) studied this amidation in detail. Their data indicated that transamidation occurred without an antecedent hydrolysis of the glutamine, as the transamination from glutamate to α-keto acid [6a] occurred at a much slower rate than the deamidation reaction [2a].

MEISTER (1954c) succeeded in catalysing the conversion of α-keto succinamic acid (VII) and glutamine by a liver preparation into asparagine and α-ketoglutaramic acid (VIII).

[3] α-ketosuccinamic acid (VII) + glutamine (II) → asparagine (IV) + α-ketoglutaramic acid (VIII)

In this transamination reaction glutamine may be replaced by other amino acids: in the sequence of their activity these are: α-aminobutyric acid, alanine, norvaline, methionine, histidine, norleucine, leucine, tyrosine, aethionine, phenylalanine and serine. Valine, isoleucine, glutamic acid and glycine were inactive (MEISTER and FRASER, 1954).

Now it is possible that reaction [2a] may be brought about by the following processes:

[4] oxaloacetic acid (V) + NH_3 → α-ketosuccinamic acid (VII)
[3] α-ketosuccinamic acid (VII) + glutamine (II) → asparagine (IV) + α-ketoglutaramic acid (VIII)
[5] α-ketoglutaramic acid (VIII) + NH_3 → α-ketoglutaric acid (VI)

[2a] oxaloacetic acid + glutamine → asparagine + α-ketoglutaric acid

This scheme differs from the preceding one; the proceeding transamidation is followed by a transamination.

26. THE SYNTHESIS OF GLUTAMIC ACID BY A TRANSAMINATION OF VARIOUS AMINO ACIDS

It follows from (25) that the sources for the synthesis of the amides are glutamic acid and aspartic acid. Which are the precursors of these amino acids?

In 1937 BRAUNSTEIN and KRITZMANN described that in the muscles of pigeon breast an enzyme is present, which converts glutamic acid and pyruvic acid to α-ketoglutaric acid and alanine [6b]; a second enzyme gives a transamination between glutamic acid and oxaloacetic acid [6a].

[6a] aspartic acid (III) + α-ketoglutaric acid (VI) → oxaloacetic acid (V) + glutamic acid (I)
b] alanine (IX) + α-ketoglutaric acid (VI) → pyruvic acid (X) + glutamic acid (I)

They showed that an equilibrium was established between these substances. These enzyme systems require a coenzyme, pyridoxal-

phosphate or pyridoxaminephosphate which, combined with the enzyme, act as intermediate carriers of the amino group. Generally large quantities of combined pyridoxal have been found in higher plant tissues. (RABINOWITZ and SNELL, 1948).

The transaminases are widely distributed in higher plant tissues, such as has already been shown by BRAUNSTEIN (1947) and KRITZMANN (1947). A comprehensive survey of the transaminases in a wide variety of plant tissues has been given by LEONARD and BURRIS (1947); see also ADLER (1938), VIRTANEN (1939, 1941), ALBAUM and COHEN (1943), SMITH and WILLIAMS (1951) and RAUTANEN (1948).

Glutamic acid however is able to participate in the transamination reactions with a variety of other amino acids; transfer of amino groups from one amino acid to another can be achieved through the mediation of glutamic acid as a carrier. Reversely, glutamic acid may be formed

CH_3	CH_3
$CHNH_2$	CO
$COOH$	$COOH$
IX	X
alanine	pyruvic acid

from different amino acids; so it becomes more and more evident that glutamic acid constitues an important link in the transamination reactions.

For instance this could be demonstrated for the transamination of phenylalanine (LERNER, 1953; ALTENBERN and HOUSEWRIGHT, 1953); tyrosine (KNOX, 1951; LA DU and GREENBERG, 1951; SCHEPARTZ, 1951); valine (UMBARGER and MAGASANCK, 1952; MEISTER, 1954); isoleucine (ALTENBERN and HOUSEWRIGHT, 1953); leucine (MEISTER, 1954b; ALTENBERN and HOUSEWRIGHT, 1953) to glutamic acid.

CAMMARATA and COHEN (1950) studied the transamination activity in extracts of tissues (muscle of heart, liver and kidney of the pig). The reaction medium contained α-ketoglutaric acid, pyridoxalphosphate, the amino acid, which was investigated, and a dialyzed extract of the tissue. They measured the CO_2, liberated, after decarboxylation of the glutamic acid, formed. The amino acids, which were transaminated for more than about 10 % of the quantity, added, were aspartic acid, alanine, valine, leucine, isoleucine, tyrosine, phenylalanine, methionine, tryptophane and arginine. The other amino acids were transaminated very slowly.

FELDMAN and GUNSALUS (1950) demonstrated a series of transaminases in dried cells of *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis*. Aspartate, alanine, valine, leucine, tryptophane, tyrosine, phenylalanine, methionine and to a lesser degree isoleucine, histidine, lysine, glycine and threonine served as amino donors to α-ketoglutaric acid.

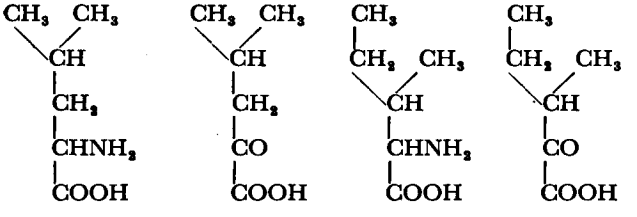
WILSON, KING and BURRIS (1954) investigated the activity of

transaminases in particulate and soluble preparations from etiolated white lupins and illuminated barley seedlings. They found that aspartic acid, alanine, arginine, leucine, isoleucine, asparagine, methionine, cysteine and valine could transfer their amino group to α-ketoglutaric acid. Phenylalanine, lysine, histidine, tryptophane and tyrosine were transaminated also, though to a lesser degree.

Summarizing, we can conclude that the following transamination reactions may occur:

[6] aspartic acid (III), alanine (IX), leucine (XI), isoleucine (XIII), valine (XV), phenylalanine (XVII), tyrosine (XIX) + α-ketoglutaric acid (VI) ⇌

oxaloacetic acid (V), pyruvic acid (X), α-keto-γ-methylvaleric acid (XII), α-keto-β-methylvaleric acid (XIV), α-keto-β-methylbutyric acid (XVI), phenylpyruvic acid (XVIII), p-hydroxyphenylpyruvic acid (XX) + glutamic acid (I)

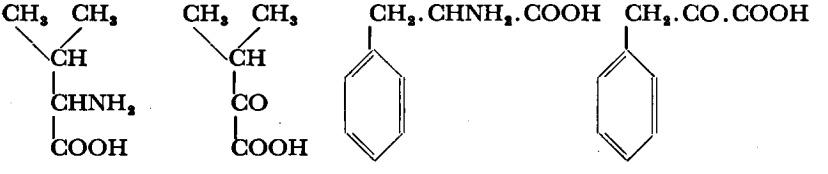


XI leucine

XII α-keto-γ-methylvaleric acid

XIII isoleucine

XIV α-keto-β-methyl valeric acid

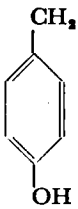


XV valine

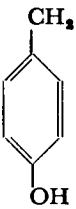
XVI α-keto-β-methyl butyric acid

XVII phenylalanine

XVIII phenylpyruvic acid



XIX
tyrosine

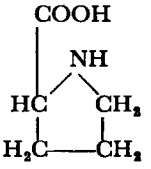


XX
p-hydroxyphenylpuryvic acid

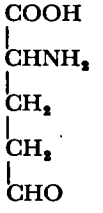
27. The synthesis of glutamic acid by a non-transaminating interaction of amino acids

In the preceding paragraph it was shown that glutamic acid may be synthesized from various amino acids by a transamination reaction. We have to discuss now that heterocyclic acids, such as proline and histidine, can also be converted to glutamic acid.

Tatum (1945) found that a prolineless mutant of *Escherichia coli* could grow without proline (XXI), if it received glutamic acid. In agreement with the result, Vogel (1951) and Vogel and Davis (1952) succeeded also in demonstrating a replacement of proline by glutamic acid for growth of proline requiring mutants of *Escherichia coli*. They synthetized glutamic-γ-semialdehyde (XXII) and found this com-



XXI
proline



XXII
glutamic-γ-semialdehyde

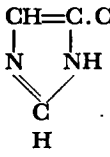
pound to be as active as proline in the growth experiments of the mutants. The glutamic semialdehyde formed under physiological conditions spontaneously an intramolecular cyclized compound, which was reducible to proline in vitro. Forbes and Sevag (1951) could demonstrate that the reverse reaction from proline to glutamic acid occurs too.

[7] proline (XXI) → glutamic-γ-semialdehyde (XXII) → glutamic acid (I)

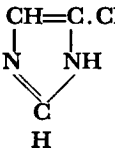
The conversion of histidine (XXIII) to glutamic acid was demonstrated by Abrams and Borsook (1952). Presenting animals carboxyl-C^{14}-histidine, they could isolate radioactive glutamic acid from the liver.

Wickremasinghe and Fry (1954) investigated the fermentation of histidine by *Clostridium tetanomorphum*. They were able to demonstrate

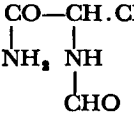
that the first reaction was a deamination of histidine to urocanic acid (XXIV). This compound was isolated and fermented; glutamic acid was also found to be an intermediate. TABOR and MEHLER (1954) isolated another intermediate as a crystalline compound, after incubating urocanic acid with an extract of a guinea pig liver. This compound consisted of glutamic acid, formic acid and ammonia. Extracts of histidine-adapted *Pseudomonas fluorescens* converted this compound to formylisoglutamine (XXV) and this is the immediate precursor of glutamic acid.



XXIII
histidine



XXIV
urocanic acid



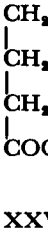
XXV
formyl-isoglutamine

According to these data 1 Mol. histidine produces 2 Mol. NH_3, 1 Mol. glutamic acid and 1 Mol. formic acid.

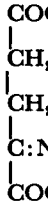
[8] histidine (XXIII) $\rightarrow$ urocanic acid (XXIV) $\xrightarrow{\nearrow NH_3}$ formyl-isoglutamine (XXV) $\rightarrow$ glutamic acid (I) + formic acid + NH_3

Glutamic acid can be decarboxylated by an enzyme, present in mouse brain, yielding γ-aminobutyric acid (ROBERTS and FRANKEL, 1950, 1951) [9]. A similar enzyme preparation had been obtained by NAJJAR and FISHER (1954) from *Escherichia coli*. γ-Aminobutyric acid was demonstrated by HULME and ARTHINGTON (1950) in extracts of Bramley's Seedling apples and by WESTALL (1950) in extracts of beetroots. This may suggest the presence of this enzyme in higher plants.

[9] glutamic acid (I) $\rightarrow$ γ-aminobutyric acid (XXVI) + CO_2



XXVI
γ-aminobutyric acid



XXVII
α-imino-glutaric acid

XXVIII
glycine

XXIX
glyoxylic acid

28. The deamination of amino acids

According to the scheme [1] of (25) we may derive the amide-N from ammonia. A number of processes, by which amino acids are deaminated in nature, are known. A conversion of the amino acid to the corresponding hydroxy acid and ammonia (hydrolytic deamination) and a conversion of the amino acid to the corresponding substituted fatty acid and ammonia (reductive deamination) have not been found in the higher plant, so far as it is known.

The deamination of amino acids in the plants takes place primarily by oxidative deaminations of the general type shown in reaction [10]. The amino acid is oxidized to the corresponding α-keto acid and ammonia.

[10] glutamic acid + oxidized glutamic dehydrogenase $\leftrightarrows$ α-iminoglutaric acid (XXVII) + reduced glutamic dehydrogenase
α-iminoglutaric acid (XXVII) + H_2O $\rightleftarrows$ α-ketoglutaric acid (VI) + NH_3

Glutamic dehydrogenase and other oxidases for the l-isomers of the amino acids have been found in a wide variety of higher plant tissues and they may be distributed universally. A purified flavoprotein enzyme, which oxidizes a variety of monoamino-monocarboxylic acids is also known in animals; the enzyme oxidizes the l-form of leucine, methionine, proline, norleucine, norvaline, phenylalanine, tryptophane, isoleucine, tyrosine, valine, histidine, cystine and alanine (Blanchard, 1944, 1945; Ratner, 1944).

Thayer and Horowitz (1951) demonstrated the deamination of a great number of amino acids by l-amino acid oxidase from *Neurospora crassa.*

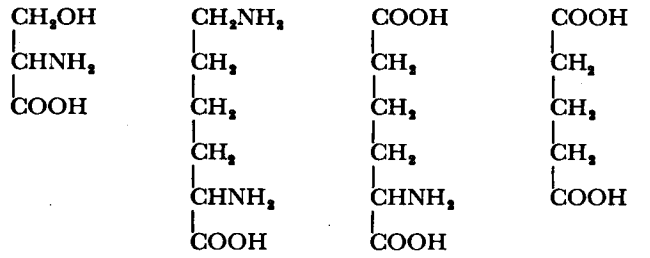
Handler *et. al.* (1949) and Kamin and Handler (1951) investigated the ammonia and urea-excretion of dogs after infusing of various amino acids at a constant rate. Glutamine, asparagine, alanine, histidine and glycine caused the highest rates of ammonia-formation, whereas aspartic acid, leucine, methionine and cysteine occupied an intermediate position.

The question is, if all the conversions have to be explained by a deamination. The possibility is not excluded that the amino groups of the amino acids, which can participate in a transamination, are incorporated into glyoxylic acid (XXIX), forming glycine (XXVIII), which is deaminated. In this process the amino group is converted into ammonia.

[11] alanine (IX) + glyoxylic acid (XXIX) $\rightarrow$ pyruvic acid (X) + glycine (XXVIII)

According to the experiments of Shemin and Russell (1953) glycine is deaminated in the presence of succinate. After the condensation of the two compounds ammonia is split off and α-ketoglutaric acid is produced. The deamination of serine has been studied by Blakley (1954). He could demonstrate the conversion of serine (XXX) to glycine (XXVIII) and formaldehyde by an enzyme from the liver of pigeon and chicken (reaction [12]).

[12] serine (XXX) ⇆ glycine (XXVIII) + formaldehyde



XXX	XXXI	XXXII	XXXIII
serine	lysine	α-amino-adipic acid	glutaric acid

According to CLARK and RITTENBERG (1951) lysine differs from the other acids in so far that replacement of the α-amino group by a keto group is not utilizable for growth of many organisms. Moreover it is not involved in the transaminase system. This was also found by ROBERTS (1954). ROTHSTEIN and MILLER (1954), who used an isotope-trapping technique in rats, identified the specific catabolites from radio active precursors and found evidence for the conversion of lysine by the following way:

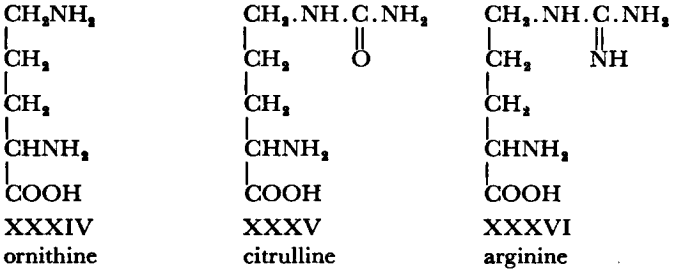
[13] lysine (XXXI) → α-aminoadipic acid (XXXII)
→ glutarate (XXXIII)
→ α-ketoglutarate (VI)

29. THE SYNTHESIS OF ARGININE

During the germination of the seeds of lupins and the growth of the seedlings in the dark a rather considerable increase of the amount of arginine was observed. The problem is, which amino acids are the precursors for the synthesis of this compound.

The synthesis of arginine is known to take place from ornithine, according to the steps, first proposed by KREBS and HENSELEIT (1932):

[14] ornithine (XXXIV) → citrulline (XXXV) → Arginine (XXXVI)

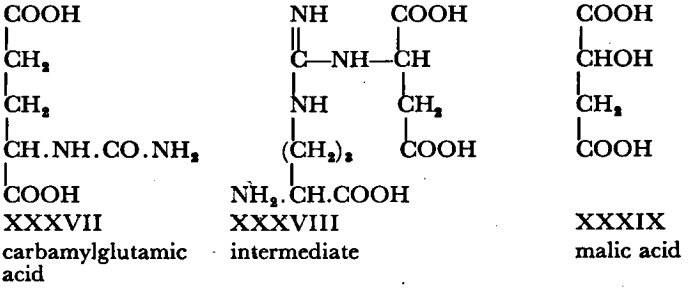


XXXIV	XXXV	XXXVI
ornithine	citrulline	arginine

VOGEL (1953) studied the ornithine-synthesis in *Escherichia coli*. Mutant strains accumulated metabolites as a result of a block in the ornithine formation. One of these metabolites, which he isolated, was glutamic-γ-semialdehyde (XXII). FINCHAM (1953) could demonstrate the presence of an enzyme in extracts of *Neurospora crassa*, transferring

the δ-amino group of ornithine (XXXIV) to α-ketoglutaric acid (VI) (reaction [15b]). This reaction is reversible, so that ornithine may be synthetised by a transamination from glutamic acid to glutamic-γ-semialdehyde. MEISTER (1954a) found many amino acids capable of transaminating with glutamic-γ-semialdehyde.

An enzyme, which catalyzed the synthesis of citrulline (XXXV) in the presence of ornithine (XXXIV), A.T.P., Mg^{++}, NH_3, CO_2 and carbamylglutamic acid (XXXVII), was isolated by GRISOLIA and COHEN (1951) (reaction [15c]). The carbamylglutamic acid cannot be replaced by glutamic acid. Studies of GRISOLIA, BURRIS and COHEN (1951) with C^{14} -labeled carbamylglutamic acid revealed that the carbon and nitrogen of the carbamyl group of this acid do not appear in the carbamyl group of citrulline. This group is derived from NH_3 and CO_2 directly. The carbamylglutamic acid acts as a catalyst.



RATNER and collaborators (1949, 1951) isolated an enzyme system from mammalian liver, catalyzing the conversion of citrulline and aspartic acid to arginine and malic acid. This reaction was established consisting of two different steps ([15d]; [15e]).

In accordance with the experiments, mentioned above, we can give the following scheme:

[15a] glutamic acid (I) ⟶
glutamic-γ-semialdehyde (XXII)

[15b] glutamic-γ-semialdehyde (XXII) + glutamic acid (I) ⟶
ornithine (XXXIV) + α-ketoglutaric acid (VI)

[15c] ornithine (XXXIV) + CO_2 + NH_3 + A.T.P. $\xrightarrow[\text{carbamyl glutamic acid}]{Mg^{++}}$
citrulline (XXXV) + A.D.P. + H_3PO_4

[15d] citrulline (XXXV) + aspartic acid (III) + A.T.P. ⟶
intermediate (XXXVIII) + A.D.P. + H_3PO_4

[15e] intermediate (XXXVIII) ⟶
arginine (XXXVI) + malic acid (XXXIX)

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[15] 2 glutamic acid + aspartic acid + NH_3 ⟶
arginine + α-ketoglutaric acid + malic acid

The arginine breaks down in the presence of the enzyme arginase; urea and ornithine are liberated. From this compound glutamic acid may be formed by a transamination reaction (RATNER 1954). During this conversion 2 Mol. glutamic acid are equivalent to 1 Mol. arginine.

[16] arginine → ornithine + urea

30. Discussion of the interconversions of the amides and amino acids

30.1. *Introductory Remarks*

In the germinating seed of *Lupinus luteus* the reserve protein is hydrolyzed, the resulting amino acids translocated, transformed partially and finally resynthesized to the protein of the seedling.

The seedlings, grown for this experiment may be divided into two groups. Those of 7 and 14 days old were respectively 2 and 4 cm long, whereas the seedlings of 21 days old had a length of 10 cm. These were spindly. We may assume that the physiological conditions of the seedlings, mentioned first, were normal in contrast to the latter, which had been starved.

Our analyses indicate that the amounts of methionine and γ-aminobutyric acid per 100 seeds or seedlings hardly alter; cysteine was not demonstrated by the method of paper chromatography; cystine has been measured by the quantitative analysis and this compound is inactive in the transamination.

Lugg and Clowes (1945) analysed some amino acids in the protein of *Lupinus luteus*. They found about 5 mg tryptophane-N per 100 specimens. The participation of this substance may be negligible for our investigation.

30.2. *The Transfer of the Amino Group*

The dominating process during the germination of lupin seeds in the dark is the production of asparagine and arginine; in addition negligible amounts of glutamine and γ-aminobutyric acid are formed.

Now we have to discuss the problem: what are the sources, from which the amino groups of these compounds are accumulated?

As in the etiolated seedlings of lupins the supply of carbohydrates gives out, an active amination of keto acids into the corresponding amino acids may not occur. This had already been demonstrated by Prianischnikow (1922). It is true that ammonia, supplied to these plants, poor in carbohydrates, is assimilated, but it is accumulated as free ammonia; a synthesis of asparagine however was encouraged by carbohydrates, supplied.

Only one source is available for the formation of the amino groups of the accumulated compounds. They originate from the aspartic acid, present in the protein, or synthesized as an intermediate, either by a transamination reaction from glutamic acid, alanine, leucine, isoleucine, phenylalanine, tyrosine and valine or by a non-transamination reaction from proline and histidine.

In Table 15 the changes in the amounts of the amino groups of the amino acids, just mentioned, are summarized.

It has already been pointed out that during the synthesis of arginine 3/4 of the total-N of this compound originates from the amino groups of glutamic acid and aspartic acid (reaction [15]), whereas during the dissimilation of arginine only $\frac{1}{2}$ of the total-N gives rise to the formation of an amino group (reaction [16], [15b] and [15a]). Furthermore an

TABLE 15

The transfer of the amino groups between amino acids and amides in seedlings of Lupinus luteus in the dark

The figures have been calculated from Table 13 and represent the changes of each amino acid during the experimental interval. Data in m.equiv. amino-N per 100 seedlings.

Amino acids	period of growth					
	0–7 days		7–14 days		14–21 days	
	amino groups					
	increase	decrease	increase	decrease	increase	decrease
Asparagine	3.50		2.54		6.50	
Glutamine	0.07		0.04		0.07	
γ-Aminobutyric acid	0.07					
Arginine	0.69[1])			0.36[2])	2.13[1])	
Glutamic acid		2.29		1.29		2.57
Aspartic acid		0.36		0.86		2.64
Alanine		1.21		0.21		0.14
Leucine		0.64		0.21		1.29
Isoleucine			0.21			0.64
Valine		0.07	0.07			0.36
Phenylalanine			0.07			0.36
Tyrosine		0.14	0.07			0.43
Proline	0.21					0.50
Histidine		0.02	0.02			0.21
	4.54	4.73	3.02	2.93	8.70	9.14

[1]) 3 × the increase in the amount of arginine (reaction [15])
[2]) 2 × the decrease in the amount of arginine (reaction [16])

amino group of glutamic acid may also be derived from one of the three nitrogen atoms of histidine (reaction [8]).

These figures agree entirely with the results of the enzymological investigations. During the first two weeks of the germination the increase of the amino groups of asparagine, glutamine and γ-aminobutyric acid and of 3/4 of the increase of total-N of arginine may be attributed completely to a decrease of amino groups of glutamic acid, aspartic acid, alanine and leucine; however the other amino acids, mentioned above, change in negligible amounts.

During the third week of the germination in the dark the amounts of asparagine and arginine still increased further and now also isoleucine, phenylalanine, valine and tyrosine have been transaminated under these unphysiological conditions, though the transaminases for these reactions however are present in a low concentration. Moreover proline and histidine have been deaminated.

30.3. *The Transfer of Ammonia*

The second question, which we have to discuss, concerns the sources of the amide groups of the accumulated compounds.

In Table 16 the changes in the amounts of the amino- and amide-groups of those amino acids, which give rise to the synthesis of the

TABLE 16

The transfer of ammonia and its precursors in seedlings of Lupinus luteus in the dark

The figures have been calculated from Table 13 and represent the changes of each amino acid during the experimental interval. Data in m.equiv. amide group-N in 100 seedlings.

Amino acids	period of growth					
	0–7 days		7–14 days		14–21 days	
	amide groups, ammonia and amide group percursors					
	increase	decrease	increase	decrease	increase	decrease
Asparagine	3.50		2.54		6.50	
Glutamine	0.07		0.04		0.07	
Arginine	0.23			0.36[1])	0.71	
Ammonia	2.07			0.07		5.14
Glycine		2.07		0.21		0.43
Serine		0.43		0.21		0.14
Cystine		0.36		0.36	0.14	
Lysine		1.36		1.00		0.28
α-aminoadipic acid	0.07			0.21		
Threonine		0.21				0.07
Peptide		0.21				
Histidine		0.04	0.04			0.42[2])
unknown compound		1.00		0.07		0.21
	5.94	5.68	2.62	2.49	7.42	6.69

[1]) 2 × the decrease in the amount of arginine (reaction [16]).
[2]) 2 × the decrease in the amount of histidine (reaction [8]).

amide group of asparagine and of 1/4 of the total-N of arginine (reaction [15]) have been summarized.

Though all the amino acids may undergo an oxidative deamination, a few amino acids however are deaminated.

During the first week of the germination a rather sharp decrease in the amounts of glycine, lysine, serine and of an unknown compound could be observed; to a lesser degree also of cystine. At the same time a considerable quantity of ammonia was produced.

During the second week the situation changes: all ammonia produced is immediately converted in asparagine. A marked decrease in the amounts of lysine and cystine however was observed, whereas the variations of the other amino acids are of little importance.

In the last of our experiments (14–21 days) the situation changes again: a small breakdown of the amino acids remained, but now a remarkable fall in the amount of ammonia can be observed.

30.4. *The Possible Physiological Significance of the Formation of Asparagine in the Starved Lupin Seedlings*

According to the view of PRIANISCHNIKOW (1895, 1899) the free ammonia would be toxic. He suggested that this toxic substance is removed by the formation of the relatively harmless asparagine. The result is an accumulation of nitrogen in a form, in which it may be subsequently utilized.

We will not deny the detoxicating effect involved in the formation

of asparagine, though normal growth of plants, fed with ammonia, may be achieved easily. We are convinced that this cannot be the ultimate interpretation of the observed amide accumulation.

During the first week of the germination, when a difference between the seedlings, grown in darkness and in light does not exist and when the development proceeds quite normally, a relatively large amount of ammonia was accumulated. Any pathological effect, resulting from this accumulation, had never been observed.

In the third period (14–21 days), when we might expect a pathological effect of the presence of ammonia, stimulated by extremely abnormal conditions, practically no decrease of the content of the amino acids, which act as precursors of ammonia, occurred and subsequently there was no production of free ammonia. On the contrary a definite fall of the ammonia content far beyond the amount, originally present in the seeds, could be observed. There is no reason to suggest that in this period the synthesis of large amounts of asparagine has the tendency to detoxicate, as no production of ammonia occurred at all.

30.5. *The Possible Origin of the Carbon-Chain of Asparagine*

In the foregoing we paid special attention to the origin of the amino- and amide-N of the amides accumulated. The question is, which amino acids may contribute to the carbon-chain of the produced amide. In (25) we saw that we may derive asparagine from oxaloacetic acid.

We can divide the amino acids into two groups. Those of the first group transform to products, closely related to pyruvic acid, such as leucine, alanine, serine, glycine and cystine. These products are oxidized and release the energy for the development of the seedling. The second group may be transformed by a transamination or non-transamination reaction to oxaloacetic acid or α-ketoglutaric acid and can give rise to the synthesis of asparagine. These amino acids are aspartic acid, glutamic acid, arginine, proline, histidine, α-amino-adipic acid, lysine and threonine (?). Tyrosine and phenylalanine are split in acetoacetate and fumaric acid. The first compound is oxidized, yielding CO_2 and H_2O; the second product is transferred to oxaloacetic acid.

The oxidation of isoleucine and valine has not been studied, but according to their chemical structure they belong to the first group.

Now the carbon-chain of the increasing amides and amino acids has to be derived from the compounds of the second group, because no other C-source is available.

In the following Table we compared the increase in the amounts of asparagine, glutamine, γ-aminobutyric acid and arginine with the decrease in the amounts of the amino acids, which can easily be converted in the compounds, just mentioned; the amounts have been given in m.equiv. oxaloacetic acid or α-ketoglutaric acid.

It may be seen from Table 17 that the carbon-skeletons of asparagine, glutamine, γ-aminobutyric acid and arginine originate from the,

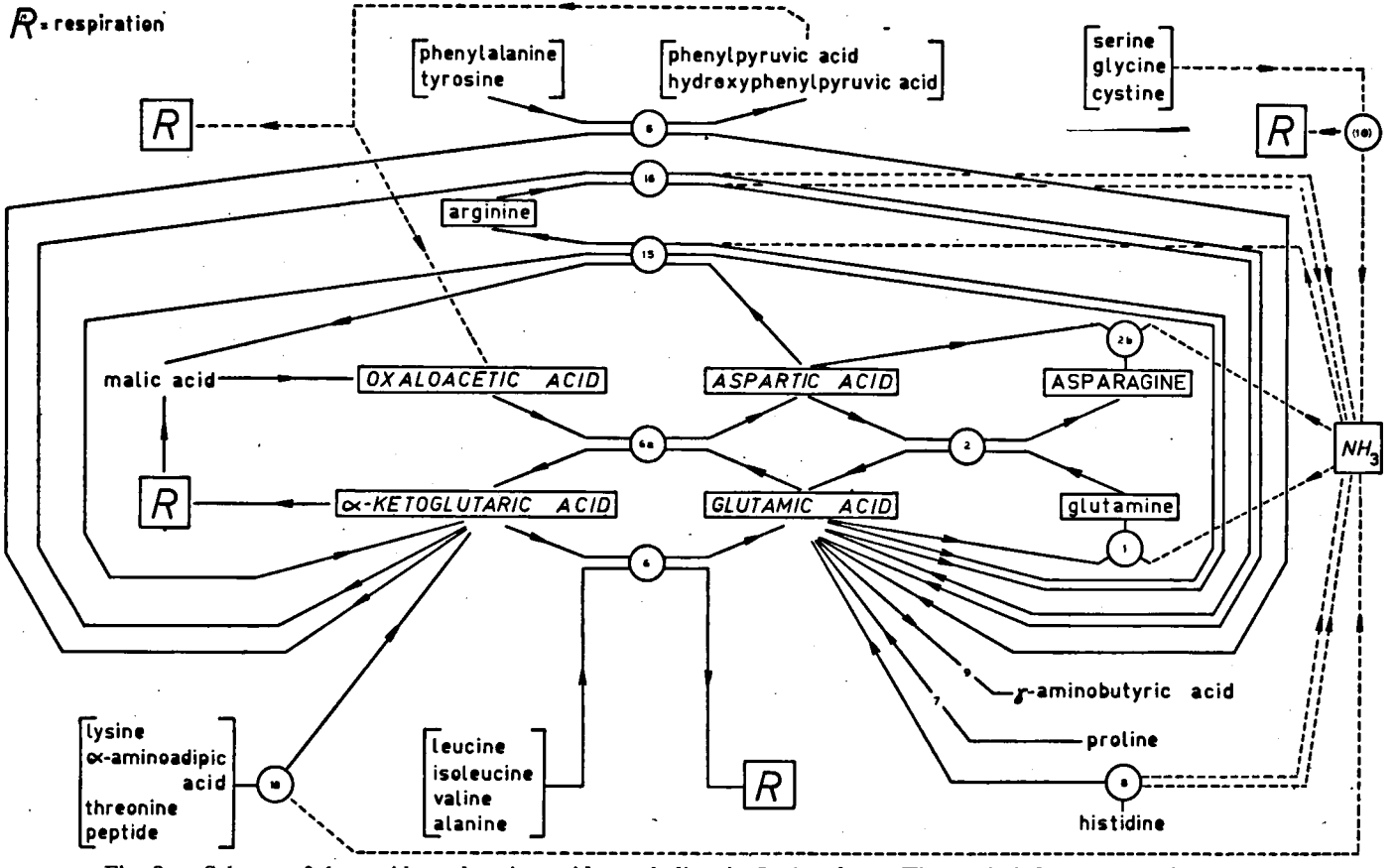

Fig. 9. Scheme of the amide and amino acid metabolism in *Lupinus luteus*. The encircled numbers refer to the reactions, mentioned in this Chapter.

TABLE 17

Changes in the N-free components of the amides and related amino acids

The figures, derived from Table 13, in m.equiv. oxaloacetic acid or α-ketoglutaric acid.

Amino acids	period of growth					
	0–7 days		7–14 days		14–21 days	
	N-free components of amides and amino acids					
	increase	decrease	increase	decrease	increase	decrease
Asparagine	3.50		2.54		6.50	
Glutamine	0.07		0.04		0.07	
γ-aminobutyric acid	0.07					
Arginine	0.23			0.18	0.71	
Glutamic acid		2.29		1.29		2.57
Aspartic acid		0.36		0.86		2.64
Lysine		0.68		0.50		0.14
α-Aminoadipic acid	0.07			0.21		
Threonine		0.21				0.07
Peptide		0.21				
Phenylalanine			0.07			0.36
Tyrosine		0.14	0.07			0.43
Proline	0.21					0.50
Histidine		0.02	0.02			0.21
	4.15	3.91	2.74	3.04	7.28	6.92

TABLE 18 A

The metabolism of aspartic acid during the germination of Lupinus luteus

Increase: m.equiv. aspartic acid, synthesized from the compounds mentioned in column A.

Decrease: m.equiv. aspartic acid, converted into the compounds mentioned in column A.

A	period of growth					
	0–7 days		7–14 days		14–21 days	
	increase	decrease	increase	decrease	increase	decrease
Aspartic acid	0.36		0.86		2.64	
Asparagine		3.50		2.54		6.50
Arginine		0.23	0.18			0.71
Oxaloacetic acid	3.37		1.50		4.57	
	3.73	3.73	2.54	2.54	7.21	7.21

amino acids, decreased, as for the three growth-periods the total increase of the N-free compounds is equivalent to the total decrease.

The conversions concerning the formation of asparagine, glutamine, γ-aminobutyric acid and arginine have been summarized in the following scheme. This scheme has been based on the reactions, given in (25), (26), (27), (28) and (29).

Now we consider the metabolism of aspartic acid. This acid can be converted in asparagine and is concerned in the synthesis of arginine. It is synthesized from oxaloacetic acid by transamination;

moreover the amount of aspartic acid decreases during the germination. The quantity of oxaloacetic acid, transaminated in aspartic acid, may be derived from the figures, given in Table 18A.

TABLE 18 B

The metabolism of oxaloacetic acid during the germination of Lupinus luteus

Increase: m.equiv. oxaloacetic acid, synthesized from the compounds mentioned in column A.

Decrease: m.equiv. oxaloacetic acid, converted into the compounds mentioned in column A.

A	period of growth					
	0–7 days		7–14 days		14–21 days	
	increase	decrease	increase	decrease	increase	decrease
Aspartic acid [1]		3.37		1.50		4.57
Arginine	0.23				0.71	
Phenylalanine				0.07	0.36	
Tyrosine	0.14			0.07	0.43	
α-ketoglutaric acid	3.00		1.64		3.07	
	3.37	3.37	1.64	1.64	4.57	4.57

[1] Compare with Table 18A under oxaloacetic acid.

TABLE 18 C

The metabolism of α-ketoglutaric acid during the germination of Lupinus luteus.

Increase: m.equiv. α-ketoglutaric acid, synthesized from the compounds mentioned in column A.

Decrease: m.equiv. α-ketoglutaric acid, converted into the compounds given in column A.

A	period of growth					
	0–7 days		7–14 days		14–21 days	
	increase	decrease	increase	decrease	increase	decrease
Glutamic acid[1]	3.37		1.50		4.57	
Arginine	0.23			0.18	0.71	
Lysine	0.68		0.50		0.14	
α-Aminoadipic acid		0.07	0.21			
Threonine	0.21				0.07	
Peptide	0.21					
Leucine		0.64		0.21		1.29
Isoleucine			0.21			0.64
Valine		0.07	0.07			0.36
Alanine		1.21		0.21		0.14
Phenylalanine			0.07			0.36
Tyrosine		0.14	0.07			0.43
Oxaloacetic acid[2]		3.00		1.64		3.07
	4.70	5.13	2.63	2.24	5.49	6.29

[1] Compare with Table 18A under oxaloacetic acid. By the transamination reaction the decrease in the amount of oxaloacetic acid equals to the quantity of α-ketoglutaric acid formed.

[2] Compare with Table 18B under α-ketoglutaric acid. By the transamination reaction the increase in the amount of oxaloacetic acid equals to the decrease in the quantity of α-ketoglutaric acid.

The amount of oxaloacetic acid, transaminated into aspartic acid, being known, we can calculate the amount of α-ketoglutaric acid, which has been converted into oxaloacetic acid via the Krebs-cycle. The latter product is derived from phenylalanine, tyrosine, α-ketoglutaric acid and from malic acid. The oxaloacetic acid metabolism has been given in Table 18B.

Now we can determine the conversions of α-ketoglutaric acid. This compound can be formed from glutamic acid, lysine, α-aminoadipic acid, threonine, peptide and arginine; it can disappear by a transamination reaction with leucine, isoleucine, valine, alanine, phenylalanine and tyrosine or it can be converted into oxaloacetic acid. The metabolism of α-ketoglutaric acid has been summarized in Table 18 C. The result of this investigation is that during the germination in the dark the increase of the quantity of α-ketoglutaric acid is in fairly agreement with the decrease, as we had assumed on page 530.

We need not give a survey of the metabolism of glutamic acid. These conversions have been given in Table 15.

According to the citric acid cycle of Krebs oxaloacetic acid has a catalytic function for the oxidation of pyruvic acid, but it is not oxidized.

These conversions give another aspect of the function of asparagine. Etiolated seedlings need energy in order to remain alive. The supply of carbohydrates and lipids gives out and proteins are respired. Many amino acids are deaminated and oxidized. The results, given in this paragraph indicate that the respiration proceeds with the following amino acids: leucine, isoleucine, valine, alanine, serine, glycine and cystine and with the fraction of α-ketoglutaric acid, converting in oxaloacetic acid. Ammonia is produced and this inhibits the Krebs-cycle. Other amino acids must be broken down, in order to give more oxaloacetic acid. These are aspartic acid, glutamic acid, lysine, threonine, α-aminoadipic acid and arginine. A part of phenylalanine and tyrosine is respired and a part of them is converted to oxaloacetic acid. As oxaloacetic acid may be the only compound, which cannot be oxidized further, it combines with ammonia, yielding asparagine, after transamination. The formation of asparagine and arginine may be considered as the final product of the respiration at the expense of proteins.

SUMMARY

(1) It is well-known that etiolated seedlings of *Lupinus luteus* produce considerable amounts of asparagine. Many investigations demonstrated a stimulating effect of ammonia and of glucose on the amide-synthesis. In seedlings, grown on distilled water in darkness, asparagine is synthetized at the expense of protein. Moreover the etiolated seedlings have not a sufficient amount of carbohydrates for the respiration, so that the protein is an important source for these functions. The problem is, which amino acids are concerned in the asparagine synthesis and which are broken down in the respiration processes. In order to solve this problem, it was necessary to determine the total increase and decrease in the amounts of the various amino acids in the seeds and seedlings. To simplify the experimental conditions, the seedlings were cultivated on distilled water in the dark at a constant temperature.

(2) 100 seeds or seedlings of 7, 14 or 21 days old were ground and suspended

in 96 % alcohol. The free amino acids were extracted by 70 % alcohol and the amino acids of the proteins brought into solution by a partial hydrolysis of the proteins with 20 % HCl.

(3) The HCl-extracts were purified by ion-exchange, using a cation-exchanger first and then an anion-exchanger. The alcohol-extracts were treated similarly. The purified HCl-extracts were hydrolyzed completely. During the purification the amides were split in the corresponding acids and ammonia. Asparagine and glutamine were determined by comparing the analysis of crude and of purified extracts.

(4) Using paper chromatography, the amino acids, present in the extracts, were identified. The solvents, used, were a mixture of 4 vol. n-butanol, 1 vol. acetic acid and 5 vol. water or a mixture of 4 vol. m-cresol, 2 vol. phenol and 1 vol. borate buffer (pH = 8.3).

(5) The quantitative determination of the amino acids was performed by use of starch chromatography on a fraction-collector, according to the method of Stein and Moore, slightly modified. A considerable increase of asparagine was observed, whereas glutamine, γ-aminobutyric acid and arginine increased less strikingly. The amounts of isoleucine, phenylalanine, valine, tyrosine, proline and histidine however decreased after 14 days. The content of methionine remained practically constant. The other amino acids decreased during the whole experiment.

(6) The amino-groups of asparagine and glutamine and of 3/4 of the arginine-N have been derived from aspartic acid and glutamic acid directly and by a conversion of leucine, isoleucine, phenylalanine, valine, tyrosine, proline, alanine and histidine in glutamic acid. The amide groups and 1/4 of the arginine-N may be derived from the remaining amino acids and ammonia. The carbon-chains of the amides and arginine originate from aspartic acid, glutamic acid, phenylalanine, tyrosine, α-aminoadipic acid, proline, threonine, lysine and histidine. The carbon-skeletons of leucine, isoleucine, valine, alanine, serine, glycine and cystine are respired. The metabolism of the amino acids during the development of the etiolated seedlings has been discussed in detail in (30) and summarized in Fig. 9 and in Table 15, 16, 17 and 18.

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