ALKALOID AND NITROGEN METABOLISM IN THE GERMINATION OF LUPINUS LUTEUS

by

J. C. J. WALLEBROEK.

I. Introduction.

At the end of his comprehensive monograph on the alkaloids TRIER (116) has put forward a suggestion as to the significance of these compounds for the vegetable organism in the following terms:

"Man wird also die Alkaloide als Nebenprodukte des Stoffwechsels der Eiweiszstoffe oder der ihnen physiologisch vergleichbaren Plasmastoffe betrachten, die da und dort in den Entwicklungsreihen der Pflanzengruppen und Familien ihre gesetzmäszige Ausbildung erfuhren und sie erbbiologisch festhielten, auch wenn die Ursachen, die zu dieser Bildung führen konnten, heute nicht mehr vorhanden sind oder von uns nicht mehr erkannt werden können".

This not very encouraging dictum would tend to make the chemicophysiological investigation of alkaloids of small attraction, if this "gesetzmäszige Ausbildung" did not imply a number of very important problems. The question which rôle the alkaloids play in metabolism, from which substances they are formed, to which changes they may be subject and in what manner these transformations take place, is of primary importance for a better conception of these compounds. Without this fundamental knowledge an extension of the problem is premature.

A warning must be uttered against generalisation in this sphere. Only a great number of facts covering a great number of alkaloids will enable us to establish, what these compounds have in common in physiological and phytochemical behaviour.

Above all, an exact method for the determination of the alkaloids is required. It will be necessary to investigate how far existing methods answer the purpose.

Of prime importance also is the arrangement of the physiological tests and the interpretation of the phenomena observed.

WEEVERS (123) has subjected the physiological methods concerning alkaloid investigation to a careful criticism.

GOUWENTAK (31) pointed out, that the results of an observation unless all factors have been sufficiently taken into account in the investigation, may lead to conclusions quite contrary to reality.

In the following chapters the metabolism of the alkaloids in the germination of the yellow lupin in connection with the nitrogen metabolism will be investigated.

The behaviour of the alkaloids of Lupinus luteus seeds when germinating has been already studied by VAN DIJK (16), who limited himself chiefly to micro-chemical observations and by SABALITSCHKA and JUNGERMANN (96; 97) who determined quantitatively the concentration changes in germination both in darkness and in light.

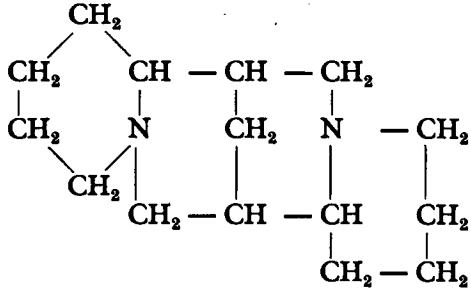
The work of the two last mentioned investigators gives rise to some remarks which will also be discussed in the following pages.

II. THE METHODS.

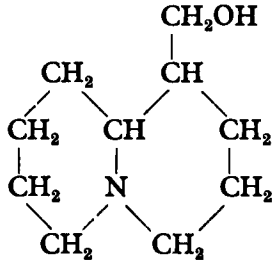
A. THE DETERMINATION OF THE ALKALOIDS.

The alkaloids of the yellow lupin are sparteine and lupinine.

Sparteine $C_{15}H_{26}N_2$ is an oily fluid. Bp. 311° (723 mm). Specif. weight 1.099 (20°). $[\alpha]_D = -14.6°$ (in ethyl alcohol). Slightly soluble in water, readily in alcohol, ether and chloroform. According to WINTERFELD and HOFFMANN (126) the most likely structure is



Lupinine $C_{10}H_{19}NO$ is a crystalline compound. Mp. 69°. Readily soluble in water, alcohol and ether. Volatile with water vapour, although less than sparteine. KARRER (47-48) states as the most probable structure of this compound



As in the germination of Lupinus luteus only slight changes occur in the alkaloid content, it is desirable for the study of the behaviour of these compounds, to raise the accuracy of the alkaloid determination as high as possible.

The requirements necessary for a suitable method for this pur-

pose may be summarised as follows: Alkaloids exclusively must be determined and these as completely as possible.

On a critical consideration, not one of the existing methods fulfils these conditions. Titration methods give too high a value, precipitation methods one which is too low.

With all these methods, extraction of the alkaloids takes place in the same manner. After making the lupin flour alkaline with sodium hydroxide, the alkaloids are extracted with a mixture of chloroform and ether. This extraction proceeds quantitatively, provided the duration of shaking is sufficiently long.

MACH and LEDERLE (68) precipitate the alkaloids with silico-tungstic acid, ignite the precipitate at white heat and calculate the alkaloid content from the weight of the $SiO_2 . 12WO_3$, in which connection it is accepted that 1 mol of $SiO_2 . 12WO_3$ binds 2 mol of sparteine or 4 mol of lupinine. The reagent, however, does not precipitate the alkaloids quantitatively. As appears from a subsequent investigation by MACH (69), especially the solubility of the combination of lupinine with silico-tungstic acid is not to be neglected. The alkaloid content determined by this method becomes thereby too low.

Titration methods on the other hand give too high a value, because by these methods it is not exclusively alkaloids which are determined. MACH (ibid.) has already pointed out that in a titrimetric alkaloid determination according to the method of THOMS (114), substances are passed into the chloroform-ether mixture which bind acids but which are not alkaloids. What substances these are, MACH did not investigate. The same objections are valid against the method of SABALITSCHKA and ZAHER (95), of BRAHM and ANDRESEN (5) and of GSTIRNER (37), which in essence are only variations of the above mentioned method of THOMS.

It is necessary to determine the nature and origin of the substances which have a disturbing effect on the alkaloid titration. A quantitative determination of these compounds in addition to the alkaloids, will considerably improve the existing methods.

Before coming to this point it will be proved, that there are three factors which influence the alkaloid figure:

1. the preliminary treatment of plant material.
2. the fatty oil content.
3. the strongly alkaline medium during the extraction.

The influence of each of these factors will be traced. Extraction with petroleumether will remove the disturbing influence of the fatty oil. The alkaloid determinations in the following investigation

were always carried out in material freed from fat. A method will be designed whereby an estimation of the effect of the third disturbing factor also becomes possible.

Initially for the determination of alkaloids in outline the SABALITSCHKA and ZAHER method was applied. The material, however, was defatted beforehand. The determination was carried out as follows (for similarity and difference from the method mentioned, the original description is referred to):

5 grams of the material reduced to fine powder (sieve opening 0.150 mm) were defatted by percolation with 100 cc of petroleum ether (Bp. 40—60°). The defatted powder was dried below 40° and mixed with 5 cc of 10% sodium hydroxide. 10 grams of calcium sulfate was added to obtain a nearly dry powder. This was transferred to a high cylindrical stoppered flask in which subsequently 125 grams of a mixture of equal parts of chloroform and ether were exactly weighed off. The mixture was shaken during three hours in an automatic shaking device. Part of the chloroform-ether mixture is decanted in the manner shown in figure 1, filtered through a dry filter of 11 cm diameter into a

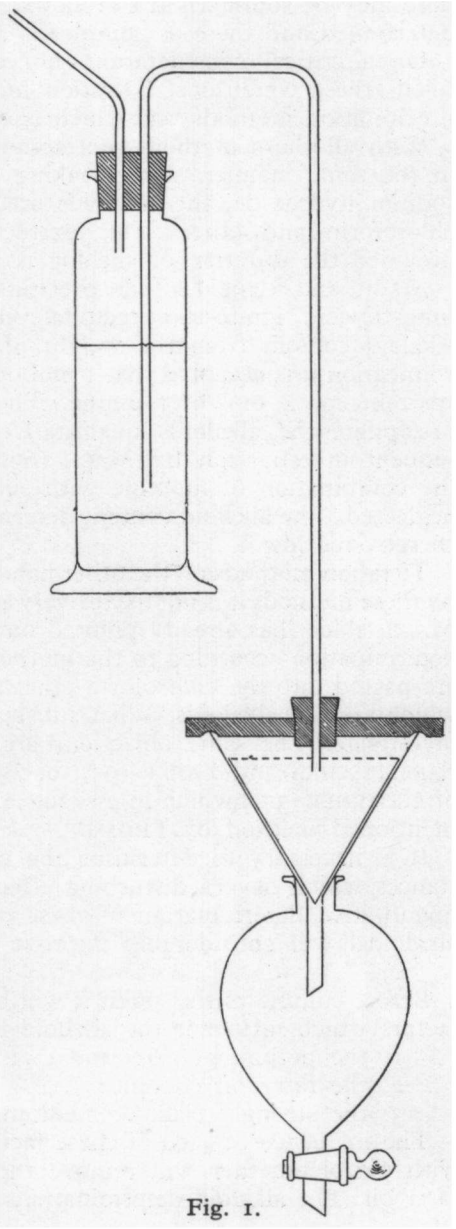


Fig. 1.

separating funnel, which has been previously weighed. The separating funnel is glass-stoppered. The weight of the chloroform-ether mixture in the funnel is now carefully determined. By this arrangement filtering and decanting can be done in one operation which limits evaporation to a minimum.

The chloroform-ether mixture was shaken with 10 cc 0.04 N sulfuric acid + 10 cc water and thereafter 3 times washed with distilled water. From the combined liquids, after adding 3 drops of a watery methylred solution ($\frac{1}{2}$ = 1000) (59), the excess acid was titrated with 0.04 N sodium hydroxide from a semi-microburette (10 cc divided into 0.02 cc divisions).

In the same manner a blank determination was made without lupin flour.

The alkaloids were calculated in terms of lupinine (mol. weight 169).

As the semi-microburette can be read to 0.004 cc, a delivery of 0.8 cc can be measured accurate to within 1%. 0.8 cc bound 0.04 N acid is equivalent to $0.8 \times 0.00676 = 0.0054$ gram of alkaloid calculated as lupinine. The titration therefore allows to determine 5 mg of alkaloid with an error not exceeding 1 per cent.

The various factors which might influence the alkaloid determination will now be discussed.

Effect of the preliminary treatment of the material on the alkaloid value and on the nitrogen fractions.

Before the alkaloids and the nitrogen fractions of the material (seeds and seedlings) can be determined, this material has first to be reduced to a suitable form. The seedlings are dried and pulverized. This procedure has to be considered somewhat closely.

If the seedlings are dried in a heating-chamber at a temperature of 72° C over lime, the tissues are apt to tear causing liquids to be lost. This may entail loss of alkaloids. Moreover, the alkaloids of the yellow lupin are volatile with water vapour, so that the possibility of loss in this manner has to be taken into account.

There is another important drawback attached to the above manner of drying. The procedure takes about 10 hours. Enzyme effects therein are not excluded. Not all enzymes are ineffective at 72° C. Furthermore it must not be assumed that this temperature occurs in all parts of the tissues as soon as the seedlings are placed in the heating-chamber. The more thermostable enzymes may play a part when the protoplasts are slowly dying off and thereby become permeable.

Also in connection with the determination of the nitrogen frac-

tions, it is of importance to obviate these objections as fully as possible. Attempts were made in order to exclude loss of liquids and to shorten as much as possible the drying process.

The following solution was arrived at. A small, electrically heated stove was constructed in such a way that by the aid of a fan a current of hot air of about 70° could be introduced through an opening in one of the side walls, while the moist air was able to escape through a number of openings in the opposite wall. The seedlings were placed in a small basket of metal gauze, to prevent them from being carried off by the draught. In this manner no liquid is separated and the drying only takes from 1 to 1½ hour.

In the course of this investigation on a few occasions a slightly different method was followed in the determination of the alkaloid content of young seedlings. The fresh seedlings were ground as finely as possible in a mortar with the aid of coarse sand at a low temperature. After adding a sufficiently quantity of calcium sulfate to obtain a crumbling powder the alkaloids were liberated by sodium hyroxide and extracted with a carefully weighed mixture of equal parts chloroform and ether. In a measured quantity of this chloroform-ether mixture, the alkaloid content was determined with due observation of the corrections to be discussed later.

For the sake of comparison the results of the alkaloid determination and of the nitrogen fractions in green seedlings after 12 days' germination in the light follow here. The material of every time 100 plants was prepared in three different ways.

		Alkaloids	Protein-N	Soluble-N
		in mgr per 100 seedlings		
I	Drying over lime at a temperature of 72° for 10 hours, thereafter pulverizing.	124	157	681
II	Drying for one hour in a warm air current, thereafter pulverizing.	130	218	633
III	Pulverizing by rubbing with quartz sand.	133	—	—

The results of the alkaloid determinations II and III are in close agreement. In determination I alkaloids may have been lost during drying. The difference in the nitrogen fractions is of impor-

tance. The figures of determination I point to a proteolysis during the drying process.

The disturbing effect of the fatty oil content of the seeds on the determination of the alkaloids.

If in material rich in fatty oil the alkaloids are liberated by sodium hydroxide, saponification occurs. When extracted by means of a chloroform-ether mixture, small quantities of the soap are transferred to the extract together with the alkaloids. This may considerably hamper the filtration of such a solution and furthermore, causes too high an alkaloid value. For if the chloroform-ether mixture is shaken out with a known quantity of acid, the alkaloids as well as the alkali of the soap are bound by the acid while the free fatty acids remain in the mixture. The alkali bound in this manner by the acid is counted as alkaloid.

This disturbing factor has been first pointed out by CORDES (14). FROMME (28) noticed a considerable increase of the alkaloid figure when sodium hydroxide was used for the determination of alkaloids in seeds of Strychnos Nux vomica not defatted. SABALITSCHKA and JUNGERMANN (98) also demonstrated the necessity of defatting if sodium hydroxide is used for the determination of alkaloids in the oil-containing seeds both of Strychnos and Datura. In Lupinus luteus, however, they noticed no disturbing effect of the fatty oil content of the seeds on the determination. This result is rather unexpected as the seeds of Lupinus luteus are as rich in fatty oil as these of Strychnos.

According to GRÜN and HALDEN (36) the fatty oil content of Strychnos seed is 4—4.2% and that of lupin seed 4.2%. From our own observation, when extracting with petroleum ether, we obtained for the seeds of Lupinus luteus a fatty oil content of 4.8%.

It seemed therefore desirable to trace the effect of the fatty oil content on the determination of alkaloids in the seeds of Lupinus luteus. For this purpose the alkaloid content of 5 grams of pulverized seed was determined thrice directly and thrice after extracting the fatty oil with 100 cc of petroleum ether. The following results were obtained:

Alkaloid content	
determined in not defatted powder	determined in powder defatted by 100 cc petroleum ether
0.98%	0.923%
0.97%	0.924%
0.98%	0.920%

With not defatted powder a fully 6% higher alkaloid content was obtained.

It was of importance to trace whether the petroleum ether extracts alkaloids from the seeds. The petroleum ether extract was shaken in a separating funnel with 10 cc of 0.04 N sulfuric acid + 10 cc of water and thereupon with three successive 20 cc portions of water. In the combined liquids the excess acid was titrated with 0.04 N sodium hydroxide (indicator methyl red). By the petroleum-ether-fat-mixture were bound respectively:

0.14 cc of 0.04 N sulfuric acid
0.15 cc „ „ „ „
0.13 cc „ „ „ „

In a blank determination where 100 cc of petroleum ether were directly shaken with 10 cc of 0.04 N acid, 0.15 cc were bound by the petroleum ether. No measurable quantity of alkaloids therefore is transferred to the petroleum ether by the procedure of defatting. The higher alkaloid value of not defatted material must therefore be attributed to saponification.

With lupin seeds of another source the same result was obtained.

Alkaloid content determined in 5 g of powder	
not defatted	defatted with 100 cc of petroleum ether
1.08%	1.00%
1.07%	0.99%

That also after three days' germination the fatty oil content may have a disturbing influence on the determination of the alkaloids is evident from the following observation, whereby the cotyledons of 210 seeds (germinating for 3 days in the dark on moist filter paper) were examined.

Alkaloid content of the cotyledons of 100 seedlings	
determined in not defatted powder	determined in powder defatted with petroleum ether
0.1024 g	0.1005 g
	0.1007 g

Here also the result in case of no defatting is an alkaloid figure of about 2% higher value.

The opinion of SABALITSCHKA and JUNGERMANN, that for a determination of the alkaloids in the seeds of Lupinus luteus previous defatting is superfluous, is therefore not correct. The above observations prove the necessity of making the determination in defatted material.

Influence of the duration of the extraction on the alkaloid figure.

The alkaloids of the yellow lupin after being liberated by sodium hydroxide are extracted with a mixture of chloroform and ether.

The question how long the extraction process should be carried on is answered variously by the different investigators. The time for extraction varies from ½—24 hours.

According to the method of the "*Reichsgesundheitsambt*-THOMS" (114) the mixture is extracted by shaking at intervals during 24 hours. Thereafter an extra amount of ether is added and the whole shaken again. After the chloroform-ether-layer has been clearly separated, the alkaloid content is determined in part of the liquid.

SABALITSCHKA and ZAHER (95) after adding the chloroform ether mixture, shake vigorously and repeat this 5 or 6 times whenever the powder has been settled. After complete sedimentation, they pipet part of the clear liquid. A definite duration for extracting is not indicated, but a procedure of this kind can be carried through in one hour.

GSTIRNER (37) is of the opinion "dasz eine halbstündige Mazeration unter häufigem kräftigem Schütteln genügt und eine Mazeration durch 24 Stunden die Bestimmungsdauer unnütz verlängert".

As there are so many different views, it seemed desirable to go more deeply into the question. The following observations were made to ascertain the influence of the duration of the extraction on the alkaloid figure.

300 seeds were pulverized. After mixing carefully, the weight of the powder was determined and six samples each of 5 g weighed off. Defatted with petroleum ether, the alkaloid content of each sample was determined with this difference that sample I was shaken for one hour with the chloroform-ether-mixture, sample II for two hours, sample III for three hours etc. In order to eliminate all subjective factors, the shaking was done in an automatic shaking device. Immediately after the shaking, decanting took place with exception of the last determination (VI) where decanting took place only after 18 hours. The alkaloid content was calculated per 100 seeds. The result was as follows:

	Time of shaking	Quantity of alkaloids per 100 seeds
Determination I	1 hour	0.1115 gram
„ II	2 hours	0.1209 „
„ III	3 „	0.1251 „
„ IV	4 „	0.1300 „
„ V	5 „	0.1354 „
„ VI	3 „ (decanted after 18 hours)	0.1283 „

The relationships are given by figure 2.

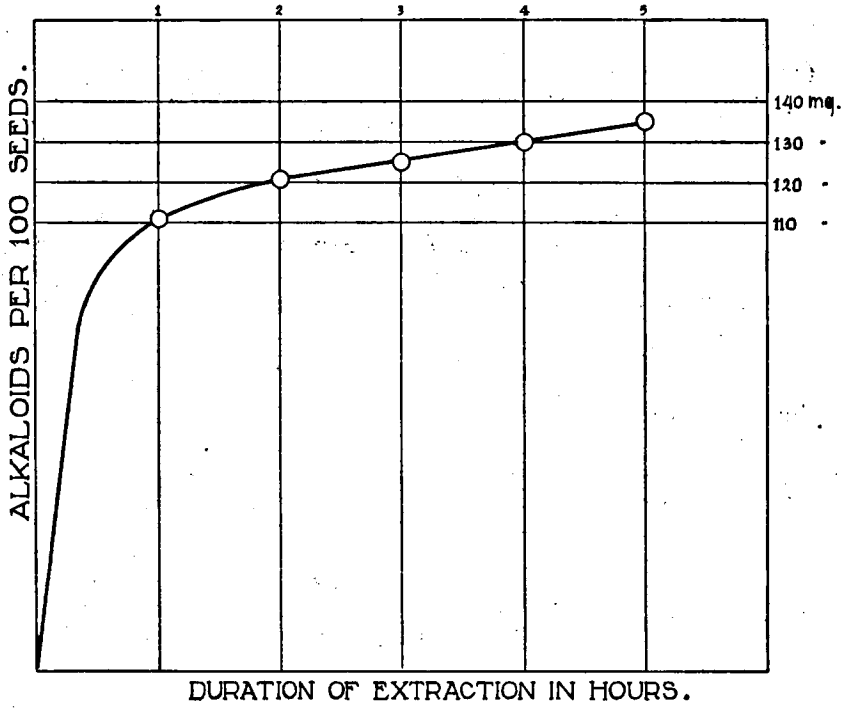


Fig. 2.

The curve proceeds quite differently than would be expected in a normal extraction. The slope of the line which decreases during the first two hours remains thereafter constant. A constant ultimate value for the alkaloid figure is not obtained. After two hours there

is a linear relation between the alkaloid value and the time used for extraction.

The course of the curve shows that during the extraction an alkaline compound is formed, soluble in the chloroform-ether-mixture, which in the titration is reckoned as an alkaloid.

In the following chapters it will be proved that this alkaline substance is ammonia, which is formed during the extraction from the amide groups which are present in the material under the influence of the strongly alkaline medium.

The extraction curve must be interpreted as the resultant of two components of which one is linear and must be ascribed to the occurrence of the disturbing substance. The point of the curve where the rise becomes constant denotes the time at which the alkaloid extraction is finished. This is the case after two hours. It goes without saying that the time required for a total extraction of the alkaloids will depend on the degree of fineness of the material examined.

It will be clear from the foregoing argument that for a complete extraction of alkaloids, an extraction time of 2—3 hours is required. If this should be shorter (GSTIRNER, SABALITSCHKA and ZAHER), there is no certainty that all alkaloids have been extracted from the lupin flour, whereas if the time is longer (THOMS) the correction is unnecessarily enlarged. The latter circumstance makes it also desirable to decant the chloroform-ether-mixture immediately after extraction (see determination VI).

In the alkaloid determinations carried out for this investigation, the time taken for extraction was always three hours, and immediately after the extraction liquid had become clear, part was decanted.

Ammonia as a disturbing substance in alkaloid determination.

MACH and LEDERLE (68) have already signalised the presence of a disturbing substance in the alkaloid determination according to the method of THOMS: "Es werden nämlich von den organischen Lösungsmitteln auch Stoffe ausgezogen, die wohl Salzsäure neutralisieren, nicht aber eigentliche Alkaloide sind". They were able to demonstrate that this substance was not precipitated by silicotungstic acid. As for their investigations they used the precipitation method, they did not trouble any further about this disturbing substance.

For an accurate alkaloid determination, however, it is inevitable to establish this disturbing factor. It was obvious to assume that

ammonia would be formed by the sodium hydroxide in the lupin flour from the amide groups and that this compound, which on extraction will partially pass into the chloroform-ether-mixture, will influence the alkaloid value. That the development of ammonia in etiolated seedlings with their high asparagine content may be considerable, is evident. But in the seed also, ammonia will be formed from the amide-nitrogen present in the protein and also from the amide groups of the soluble nitrogen fraction which amounts to 16% of the total-N. According to SCHULZE and FLECHSIG (101) the content of the amide-nitrogen in the seeds of Lupinus luteus amounts to 14.89% of the total-N. With a total-N content of 7% the content of the amide-N will be 1%.

For purposes of information the following observation was made to get an idea of the quantity of ammonia which in an alkaloid determination may be passed into the chloroform-ether-mixture.

A quantity of asparagine containing the same amount of amide-nitrogen as 5 grams of lupin flour, was treated exactly in the same manner as used in the determination of alkaloids. 0.5 g of asparagine was ground with 1 cc of water and mixed with 5 g of calcium sulfate. 5 cc of 10% sodium hydroxide was added and again another 15 g of gypsum. The crumbling powder was shaken with 125 g of chloroform-ether-mixture. In the manner usual for alkaloid titration, the amount of ammonia was determined which had developed in these circumstances from the asparagine and had been passed into the chloroform-ether-mixture. In addition a blank determination was made. The result was as follows:

Time of extraction:

1 hour 0.0072 millimol NH_3 equivalent to 1.22 mgr lupinine
3 hours 0.0208 „ NH_3 „ „ 3.52 mgr „

The amount of ammonia formed is directly proportional to the duration of the extraction.

5 grams of lupin flour in the same time delivering the same quantity of ammonia calculated as lupinine, then with an alkaloid content of the seed of 1%, the alkaloid value after 1 and 3 hours extracting will be increased by respectively 2.4 and 7%.

Anticipating the investigation, the result is here given of an alkaloid determination in the ungerminated seed with the correction which may be applied to the alkaloid figure by a determination of ammonia. For this determination extraction was made for three hours.

Alkaloids in 5 grams of the seed	
Alkaloid value uncorrected	Alkaloid value corrected for ammonia
0.0495 g	0.0463 g
0.0500 g	0.0461 g

The uncorrected alkaloid values are respectively 6.9 and 8.4% too high. This is quantitatively in agreement with what might be expected according to the views developed above, i.e. a 7% too high value of the alkaloid figure with an extraction time of 3 hours if the amide-nitrogen content of the seed amounts to 1%.

An accurate estimation of the alkaloids will therefore only be possible by a correction of the alkaloid value. The manner in which this correction can be determined will now be discussed.

Quantitative determination of ammonia and alkaloids in the chloroform-ether-mixture.

As both alkaloids of Lupinus luteus are volatile with water vapour it is not possible to separate the ammonia from the alkaloids by steam distillation of the solution made alkaline.

If the pH however does not exceed 9.4, and carrying out the distillation under decreased pressure at a temperature of about 35° C, a quantitative separation of alkaloids appears to be possible.

For this purpose the main features of ammonia determination applied by PARNAS and KLISIECKY (77-78) in organic material was followed. The liquid was made alkaline with a saturated solution of borax which through boiling had been freed of ammonia (PARNAS and MOZOLOWSKI) (79). The distillation flask was placed in a water bath of 35° C and the water vapour introduced passed through a washing flask placed in a bath of 50° C.

A few preliminary observations were made to test the usefulness of the method for our purpose.

In the first place I traced how far the ammonia present was recovered by this manner of distilling. Ammonium chloride "Kahlbaum" was pulverized and dried at 103° C. 97 mgr was dissolved to one liter. Of this solution respectively 10, 4, 2 and 1 cc were analysed, diluted with resp. 10, 16, 18 and 19 cc of water so that the total volume in each case amounted to 20 cc. After a vacuum had been obtained, from a separating funnel 20 cc of the saturated borax solution was added. The ammonia was distilled at 35° C into 5 cc of 0.01 N sulfuric acid. The excess acid was titrated back with sodium hydroxide. In addition a blank determination was made,

in which 20 cc of aq.dist. were distilled in the same manner with 20 cc of the saturated borax solution.

The results of the vacuum method were compared with those obtained by steam distillation in which ammonium chloride solutions of the same composition had been made alkaline with 5 cc of 33% sodium hydroxide and the ammonia had been distilled with steam. The ammonia obtained was expressed in terms of NH_4Cl.

Composition of the solution	Amount of NH_4Cl present in this sol. in mgr	Found by steam dist.	Found by vacuum dist. Parnas
20 cc of NH_4Cl sol.	1.94	1.94	
10 cc „ + 10 cc of water	0.970	0.971	0.970
4 cc „ + 16 cc „	0.388	0.388	0.384
2 cc „ + 18 cc „	0.194	0.196	0.192
1 cc „ + 19 cc „	0.097	0.096	0.095

With distillation in vacuo the ammonia is therefore quantitatively recovered in the distillate.

Thereupon the volatility of the lupin alkaloids was determined with steam and under the conditions of distillation in vacuo.

Prof. P. KARRER at the request of Prof. TH. WEEVERS was kind enough to put a quantity of lupinine at my disposal for which I beg to express my thanks here. For the examination of sparteine a preparation of "Merck" was used, sparteinum sulfuricum of the composition $C_{15}H_{26}N_2 . H_2SO_4 + 5 H_2O$.

190 mgr of sparteinum sulfuricum were dissolved to 250 cc. 10 cc of this solution + 10 cc of aq.dist. + 5 cc of 33% sodium hydroxide were distilled with steam for 20 minutes. In the distillate 7.64 mgr of sparteine sulfate was recovered.

When 10 cc of the solution + 10 cc of aq.dest. + 20 cc of saturated borax solution were distilled for 30 minutes under decreased pressure at 35° C, no trace of sparteine was transferred into the distillate.

105 mgr of lupinine were dissolved to 250 cc. Of this solution 20 cc were distilled with steam for 30 minutes in the above manner. In the distillate resp. 7.8 and 7.4 mgr of lupinine were recovered.

At distillation for 30 minutes with borax solution under decreased pressure, no trace of the alkaloid was recovered in the distillate.

By steam distillation therefore spartein passes quantitatively into the distillate within 20 minutes. Lupinine is less volatile, after 30 minutes resp. 93 and 89% were recovered in the distillate.

Under the conditions of vacuum distillation the alkaloids of the yellow lupin appear to be non-volatile with water vapour.

Finally a check was made as to whether from mixtures of alkaloids and ammonia the latter compound was quantitatively transferred into the distillate by vacuum distillation.

8 cc of the NH_4Cl solution were mixed with 12 cc of the sparteine solution. This mixture was made alkaline with 20 cc of saturated borax solution and distilled under decreased pressure. In the same manner a mixture of 4 cc of NH_4Cl solution + 16 cc of sparteine solution was treated. Mixtures of the same composition were also alkalised with sodium hydroxide and distilled with steam.

The result was as follows. The first column gives the composition of the mixture examined and the method of distilling, the second column the amount of 0.0177 N acid bound after distillation, the third the calculated amount of bound acid, with the assumption that by distillation in vacuo only ammonia, by steam distillation ammonia + sparteine are transferred.

Composition of the mixture	Bound quantity of 0.0177 N acid	
	Found	Calculated
8 cc of NH_4Cl sol. + 12 cc of sparteine sol. (vacuum distillation)	0.825	0.816
4 cc of NH_4Cl sol. + 16 cc of sparteine sol. (vacuum distillation)	0.408	0.408
8 cc of NH_4Cl sol. + 12 cc of sparteine sol. (steam distillation)	2.025	2.040
4 cc of NH_4Cl sol. + 16 cc of sparteine sol. (steam distillation)	2.020	2.040

The method therefore answers the purpose in every respect.

The determination of alkaloids was finally carried out as follows: 5 gram of pulverized material was defatted with 100 cc of petroleum ether and mixed with sodium hydroxide and calcium sulfate in the manner described. After this mixture had been shaken for three hours with 125 gram of equal parts of chloroform and ether in an automatic shaking device, an accurately weighed quantity (a gram) of the liquid was extracted in a separating funnel with 10 cc of 0.04 N sulfuric acid + 20 cc aq.dist. and washed with three successive 10 cc portions of water. In the combined liquids the acid

bound (b cc) by alkaloids + ammonia was determined by back-titration of the excess with 0.04 N sodium hydroxide. The carefully neutralised solution was made up with water to 110 cc. 20 cc of this solution was mixed with 20 cc of saturated borax solution. The ammonia was distilled with water vapour under decreased pressure at 35° C within 20 minutes into 5 cc of 0.01 N sulfuric acid. The acid bound by the ammonia (c cc) was determined by titrating the excess acid with 0.01 N sodium hydroxide.

The alkaloid content in 5 gram of the powder is calculated as lupinine by the equation

$$X = \left(b - \frac{5.5 \times c}{4}\right) \times \frac{125 \times 0.04 \times 0.169}{a} \text{ gram}$$

In the distillate of the vacuum distillation the ammonia could be detected with Nessler's solution. The assumption that NH_3 is the disturbing factor in the determination of the alkaloids is thereby confirmed.

B. TOTAL NITROGEN.

The total-N was determined according to Kjeldahl by a semi-micro method. The devices used for this purpose are described in numerous variations, to the literature of which we refer the reader.

The determination was carried out in the following manner:

100 mg material were digested in a Kjeldahl flask of 100 cc with 2 cc of strong sulfuric acid + 0.5 gram of sodium sulfate + 2 drops of 10% cupric sulfate solution. After the digestion was finished the cooled liquid was diluted with 15 cc of water and the Kjeldahl flask was attached to the distilling apparatus. 10 cc of 33% sodium hydroxide were added, and the ammonia distilled into 10 cc of 0.1 N sulfuric acid + 2 drops of methyl red solution ($\frac{1}{2}$: 1000). The excess sulfuric acid was titrated with 0.1 N sodium hydroxide. By a blank determination the nitrogen content of the reagents used was determined.

The titration was carried out with a semi-micro-burette divided into 0.02 cc divisions. 0.8 cc of the bound sulfuric acid corresponding with $0.8 \times 1.4 = 1.12$ mg of nitrogen can be determined to 1% accuracy.

C. PROTEIN-NITROGEN AND SOLUBLE-NITROGEN.

When determining these two nitrogen fractions, one is faced with the difficulty of choosing a suitable protein precipitant.

Most of the reagents used for this purpose remove not only proteins, but also some non-protein nitrogeneus constituents. The result will therefore depend on the method used. This problem has been most thoroughly studied for the removal of proteins from blood, blood plasma and serum, but here also opinions differ. The data are incomplete and are partly contradictory. For a survey one is advised to consult HINSBERG (39) and PETERS and VAN SLIJKE (82).

When examining vegetable material the method according to STUTZER is frequently used, in which the protein is precipitated with cupric hydroxide mash. MOTHES (71) used initially a 4% tannin solution and later (72) a 1% silico-tungstic acid solution which reagent was also used by TRG. SCHULZE (106). GOUWENTAK (31) used the STUTZER method and a 4% tannin solution and obtained with both procedures identical results.

In the experiments described in the following chapters trichloracetic and tungstic acid were used. According to GREENWALD (34-35) the blood proteins are completely removed by a 5—10% solution of trichloracetic acid without precipitation of other non-protein compounds.

The results of the trichloracetic acid procedure were compared with those for which tungstic acid had been used as precipitant. Tungstic acid was used by FOLIN and WU (26) in the examination of blood. Non-protein nitrogen compounds are also eliminated by this reagent, as e.g. ergothioneine and according to HINSBERG also other amino-acids.

With regard to the material examined here the results of the two methods were not always identical. Several times the non-protein nitrogen fraction was definitely lower with the tungstic acid than with the trichloracetic acid procedure. Especially in etiolated seedlings the difference was sometimes considerable.

Alkaloids are precipitated both by trichloracetic acid and tungstic acid. Alkaloid nitrogen, however, will have little influence on the nitrogen value as with a total nitrogen content of 7% and an alkaloid content of 1% (expressed in terms of lupinine), the alkaloid nitrogen only amounts to about 1.2% of the total-N.

The determinations were carried out as follows:

1. *With trichloracetic acid.*

100 mg of material were heated in an ordinary conical centrifuge tube of about 10 cc with 6 cc of water for 1 hour in a beaker of boiling water. The mixture being repeatedly stirred with a small glass rod. After cooling water was added to 6 cc. Thereupon 2 cc of a trichloracetic acid solution was added (40 gram in 100 cc so-

lution) and after being well stirred the tube was centrifuged for 20 minutes. The supernatant liquid is poured off and the precipitate is stirred up with a mixture of 6 cc of water and 2 cc of trichloracetic acid solution and after 10 minutes again centrifuged. The liquid is again poured off and added to the first quantity in a micro-Kjeldahl flask of 100 cc. After adding 2 cc of strong sulfuric acid, 0.5 gram of sodium sulfate and 2 drops of a 10% cupric sulfate solution, careful evaporation of the liquid was applied and the residue digested. The soluble nitrogen content is determined as described for micro Kjeldahl analyses of total-N.

The precipitate in the centrifuge tube is transferred to a micro Kjeldahl flask, in which after digestion the protein nitrogen is determined.

2. *With tungstic acid.*

The FOLIN-WU tungstic acid precipitant yields a nearly neutral filtrate suitable for the determination of all further non-protein nitrogen fractions.

In the investigation here described the proportion sodium tungstate: sulfuric acid was also chosen in such a way that after precipitation of the proteins the pH of the solution amounted to about 6.

For the conditions which the sodium tungstate used should fulfil see PETERS and VAN SLIJKE (ibid).

The determination of protein and soluble nitrogen took place in the following manner.

100 mg of material were heated in a centrifuge tube with 6 cc of water for 1 hour in a beaker of boiling water. The mixture being repeatedly stirred with a glass rod. 0.8 cc of 10% sodium tungstate was then added and thereafter slowly, with stirring, 0,64 cc of $^2/_3$ N sulfuric acid. After a quarter of an hour the mixture was centrifuged. The clear liquid was decanted and the precipitate stirred up with a mixture of 6 cc of water, 0.8 cc of tungstate solution and 0.64 cc of sulfuric acid. After centrifuging the clear liquid was transferred to the first quantity in a Kjeldahl flask. In this liquid the soluble nitrogen was determined.

The precipitate was quantitatively washed with water into a Kjeldahl flask. In the latter the protein nitrogen was determined.

III. THE INVESTIGATION.

1. SELECTION OF THE SEEDS.

Special attention was given to the selection of the material. A large collection of seeds of Lupinus luteus was carefully selected for size. The mean weight of 100 seeds was determined by 50 observations. The standard deviation of a single determination was calculated from the formula

$$\sigma = \sqrt{\frac{\Sigma \delta^2}{n}}.$$

As the investigations extended over two years, seeds from the 1933 and 1934 crops were used for the observations.
The mean weight of 100 seeds was respectively

crop 1933: $M_1 = 13.15 \pm 0.17$ gram
crop 1934: $M_2 = 14.09 \pm 0.12$ gram

This mode of selection is based on the expectation that seeds of equal weight will approximately contain a similar amount of alkaloids and also a similar amount of the other nitrogen fractions.

2. ALKALOIDS, TOTAL NITROGEN, SOLUBLE AND PROTEIN NITROGEN IN 100 SEEDS.

At first the seeds were pulverized in a mortar and passed through a sieve with openings of 0.150 mm. Later on a small grinder was used which produced a higher degree of fineness. After pulverizing, the material was carefully mixed and weighed. One hundred seeds give a sufficient quantity of powder to make all determinations in duplo.
The result was as follows, the figures giving the number of milligrams per 100 seeds. (See table I).

The average alkaloid content per 100 seeds, calculated as lupinine amounts to

crop 1933: 117.6 ± 3.5 mgr
crop 1934: 127.8 ± 1.7 mgr

TABLE I.

	Alkaloids expressed in terms of lupinine	Total nitrogen	Protein nitrogen		Soluble nitrogen	
			trichlor-acetic acid	tungstic acid	trichlor-acetic acid	tungstic acid
Crop 1933						
Det. I	122	865	—	—	—	—
	125	867				
Det. II	119	859	—	—	—	—
	121	856				
Det. III	113	868	—	—	—	—
	115	873				
Det. IV	114	863	725	—	138	—
	113	855	728		140	
Det. V	117	865	720	753	133	110
	116	866	724	764	140	107
Crop 1934						
Det. I	129	919	—	—	—	—
	130	926				
Det. II	126	914	749	—	159	—
	125	905	751		159	
Det. III	129	910	752	—	147	—
	128	918	758		157	

and the total nitrogen

crop 1933: 863.6 ± 4.8 mgr
crop 1934: 915.3 ± 5.0 mgr

For the soluble nitrogen expressed in percentages of the total nitrogen (resp. determined with trichloracetic acid and with tungstic acid as protein precipitating agents) we found

	Soluble nitrogen in percentages of the total nitrogen	
	Trichloracetic acid	Tungstic acid
Crop 1933. . .	16%	12%
Crop 1934. . .	17%	—

3. THE DISTRIBUTION OF NITROGEN AND ALKALOIDS OVER THE DIFFERENT PARTS OF THE SEED.

The distribution of the alkaloids and nitrogen over the different parts of the seed was traced by splitting them up into testa, cotyledon and plumule + radicle. This requires some practice. Especially the free preparation of the plumule + radicle offers some difficulties, as this part of the seed is very brittle and is partly hidden in a fold of the testa.

Of the seeds of the 1933 crop there were used for this investigation

the testas of . . . . . 361 seeds
the cotyledons of. . . 200 seeds
plumules + radicles of. 435 seeds

The results were calculated per 100 seeds. The following quantities were found in the different parts of the seed.

TABLE II.

	Testa of 100 seeds	Cotyledons of 100 seeds	Plumule + radicle 100 seeds	Total in 100 seeds
Total N	0.0175 0.0178	0.832 0.835	0.0163 0.0162	0.867
Soluble N	—	0.125	—	—
Protein N	—	0.710	—	—
Alkaloids	trace	0.113 0.114	0.0011	0.114

The testa is very poor in alkaloids. The amount is less than 0.0005 gram per 100 testas. In an alkaloid determination the testa may therefore be neglected.

For the determination of the alkaloids in plumule + radicle 0.905 gram of material was used. Here also the fat was first removed with 20 cc of petroleum ether. The powder was mixed with 1 cc of 10% sodium hydroxide and 2 gram of calcium sulfate and subsequently extracted for three hours with 50 gram of a mixture of equal volumes of chloroform and ether.

The parts of the seeds of the 1934 crop were also analysed for alkaloids. Here we found:

TABLE IIa.

	Testa of 100 seeds	Cotyledons of 100 seeds	Plumule + radicle of 100 seeds
Alkaloids . . .	trace	0.126 0.126	0.0009

4. THE ALKALOID AND NITROGEN CONTENT OF COTYLEDONS AND PLUMULE + RADICLE AFTER THREE DAYS' GERMINATION IN THE DARK.

To obtain an insight into the distribution of the alkaloids and the nitrogen fractions in the seedlings in the very first stage, seeds were brought to the germinating stage in darkness on moist filter paper. After two or three days, the testa was torn and the radicle appeared. Cotyledons and plumule + radicle were separated, dried in a hot air current at 70° C and analysed separately.

Plumule + radicle are still very small in this phase. For analysis of this part of the seedling, 300 seeds were treated in the above manner.

The results are summarised in table III. The quantities are calculated per 100 seedlings.

TABLE III.

	Cotyledons of 100 seedlings	Plumule + radicle of 100 seedlings	Total
Crop 1933			
Alkaloids	0.0989 0.0993	0.0118	0.1109
Total nitrogen. . . .	0.818 0.814	0.0446	0.861
Protein nitrogen . . . (trichloracetic acid). .	0.661 0.655	—	—
Protein nitrogen . . . (tungstic acid). . . .	0.662 0.656	—	—
Soluble nitrogen. . . (trichloracetic acid). .	0.152 0.158	—	—
Soluble nitrogen. . . (tungstic acid). . . .	0.160 0.165	—	—
Crop 1934			
Alkaloids	0.110	0.012	0.122

Rather striking is the increase of alkaloids in plumule + radicle. The amounts present in plumule + radicle of 100 ungerminated seeds were respectively 0.0011 and 0.0009 gram. After three days germination in darkness, respectively 0.0118 and 0.012 gram were found. The amount had multiplied ten times in that period. The cotyledons on the other hand had lost alkaloids in the same period.

The increase in soluble nitrogen in the cotyledons after 3 days germination was small. Determined by the trichloracetic acid procedure it amounts to 19.1%, by the tungstic acid method to 19.7% of the total nitrogen present, against respectively 16 and 12% in the ungerminated seed.

5. THE ALKALOID PROTEIN METABOLISM IN THE GERMINATION IN LIGHT AND IN DARKNESS ON SOIL FREE FROM NITROGEN.

The seedlings were cultivated in coarse sand which had been previously treated with hydrochloric acid and thereafter washed till the last traces of acid had disappeared. Germinating pans, sand and water were previously sterilised.

Germination in the light and in darkness took place in the same surroundings, i.e. under equal conditions of temperature and moisture.

The number of seeds germinating amounted on an average to 85%. After 11 to 13 days the average length of the parts of the etiolated seedlings above ground was 14 cm. In some plants the cotyledons were then still folded close together, the testa on the top being torn. Most of them, however, had shed the testa completely and the first pair of colourless leaflets was just appearing. The length of the part of the green seedlings above ground after 12 days was about 12 cm. The first pair of leaves were then entirely spread.

The seedlings were removed from the germinating pans with care to prevent the roots from being damaged and were freed from adhering sand by washing. The grains of sand stubbornly adhering to the roots were not removed. Drops of water were removed with filter paper.

100 Seedlings were always simultaneously dried in a drying oven by means of a hot air current of 70° C. The testas shed were collected and dried for the determination of the non-alkaloid nitrogen fractions.

The dried seedlings were pulverized in a mortar and sieved, or ground in a grinder. The powder was carefully mixed and weighed.

TABLE IV.

Duration of germination	GERMINATION IN DARKNESS (CROP 1933)				GERMINATION IN THE LIGHT (CROP 1933)			
	Alkaloids	Total N	Protein N	Soluble N	Alkaloids	Total N	Protein N	Soluble N
Determination 1. 16—27 June 1934 . . 11 days.	0.108 0.108	—	—	—				
Determination 2. 16—28 June 1934 . . 12 days.					0.133 0.135	0.842 0.840		
Determination 3. 26 July—6 Aug. . . 11 days.	0.115 0.116	0.861 0.857	—	—				
Determination 4. 26 July—7 Aug. . . 12 days.					0.130	0.863	trichloracetic acid 0.217 0.219 tungstic acid 0.225	 0.634 0.632 0.634
Determination 5. 30 Aug.—10 Sept. . 11 days.	0.106 0.106	0.845 0.845	trichloracetic acid 0.291 0.294 tungstic acid 0.399 0.398	 0.554 0.553 0.447 0.447				
Determination 6. 30 Aug.—13 Sept. . 14 days.	0.113 0.112	0.878 0.878	trichloracetic acid 0.239 0.238 tungstic acid 0.262 0.258	 0.634 0.632 0.611 0.618				
Determination 7. 5 Sept.—16 Sept. . . 11 days.	Cotyledons 0.074 Plumule + radicle 0.035							

TABLE V.

Duration of germination	GERMINATION IN DARKNESS Alkaloids per 100 seedlings			GERMINATION IN THE LIGHT Alkaloids per 100 seedlings		
Crop 1934.	Cotyledons	Plumule + radicle	Total	Cotyledons	Plumule + radicle	Total
Determination 8.						
11—16 Sept. 1935.	—	—	0.106			
5 days			0.108			
Determination 9.						
11—21 Sept. 1935.	—	—	0.121			
10 days			0.120			
Determination 10.						
11—25 Sept. 1935.				0.068	0.071	0.139
14 days				0.068	0.071	0.139
Determination 11.						
11 Sept.—1 Oct.	0.0623	0.0648	0.127			
20 days	0.0627	0.0645	0.127			
Determination 12.						
11 Sept.—6 Oct.				0.041	0.120	0.161
25 days				0.042	0.121	0.163
Determination 13.						
9—14 Oct. 1935.	—	—	0.110			
5 days			0.111			
Determination 14.						
9—15 Oct. 1935.				—	—	0.135
6 days						0.135
Determination 15.						
9—19 Oct. 1935.	—	—	0.119			
10 days			0.119			

On a few occasions the fresh seedlings were ground with sand and directly analysed for alkaloids.

Alkaloids, total nitrogen, protein nitrogen and soluble nitrogen were determined. The alkaloids were calculated as lupinine.

The results are summarised in table IV for seeds of the 1933 crop and in table V for those of the 1934 crop.

6. ALKALOID METABOLISM IN SEEDLINGS AFTER ONE OF THE COTYLEDONS HAD BEEN REMOVED.

Of plants cultivated in darkness one of the cotyledons was removed after 9 days' germination. Of this cotyledon the alkaloid content was determined. The cotyledons of 100 plants collected in this manner contained 37.7 mg of alkaloids.

The seedlings stood the amputation very well, in their subsequent development they were not noticeably backward compared with seedlings of the same age of which no cotyledon had been removed. The wounded spot did not exude moisture.

When the plants were 21 days old, the alkaloid content of the remaining cotyledon was determined, as also that of plumule + radicle. In the 100 cotyledons 27.4 mg of alkaloids were found, in plumule + radicle of 100 plants 26.5 mg.

TABLE VI.

	Alkaloids expressed in terms of lupinine	
	abnormal germination in darkness	normal germination in darkness
in 100 cotyledons after 9 days.	37.7 mg	38.1 mg
in plumula + radicle after 9 days . . .	—	42.5 mg
in 100 cotyledons after 21 days	27.4 mg	29.8 mg
in plumule + radicle after 21 days. . .	26.5 mg	67.3 mg

The results were compared with those of a simultaneously proceeding normal germination in darkness (see table VI). The alkaloid content in the remaining cotyledon after 21 days fairly well agrees with that of a cotyledon of a normal seedling of the same age. The removal of one of the cotyledons therefore has no noticeable effect on the metabolism of the alkaloids in the other cotyledon. It has on the other hand a very great effect on the metabolism in plumule + radicle. Here there must undoubtedly have occurred an alkaloid breakdown.

7. THE RESULTS.

The results of the investigation as to the alkaloid metabolism are shown in fig. 3. To be able to compare the observations which were carried out with seeds of the 1933 and 1934 crops, the alkaloid content of 100 seedlings is expressed in percentages of the quantity per 100 seeds. The lines connect the arithmetical means of the alkaloid values concerning observations of an equal germination period, respectively in light and in darkness.

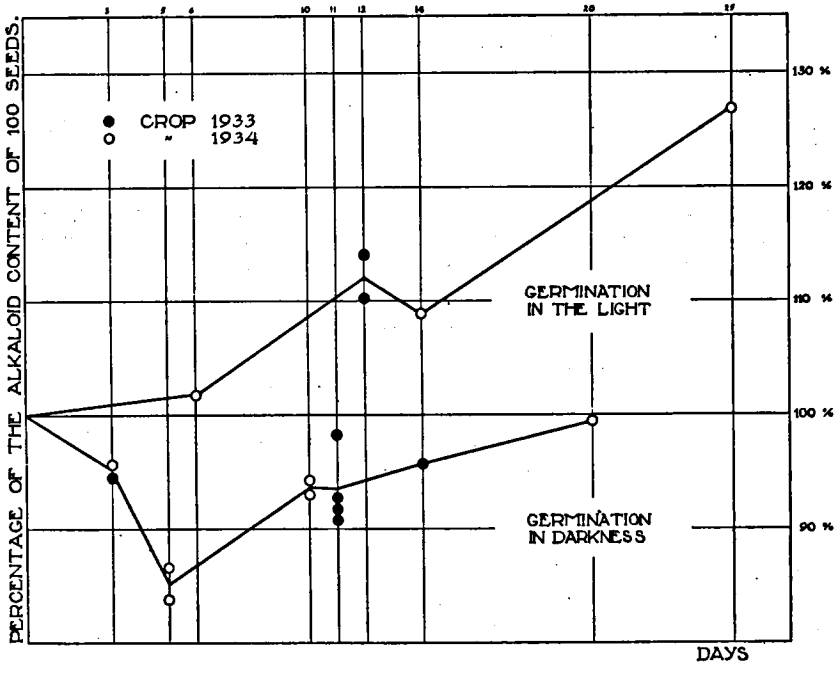


Fig. 3.

Observations 1, 3, 5 and 7 (table IV) were all carried out with seeds of the 1933 crop. The germination took place in darkness for 11 days. 100 Seedlings then contain resp. 108, 116, 106 and 109 mg of alkaloids. The mean is

$$M_2 = 109.8 \pm 3.8 \text{ mg}$$

while the standard error amounts to

$$\mu_2 = \sqrt{\frac{\Sigma \delta^2}{n\,(n-1)}} = 2.1 \text{ mg.}$$

In the ungerminated seed there is found for these quantities

$$M_1 = 117.6 \pm 3.5 \text{ mg}$$
$$\mu_1 = 1.7 \text{ mg}$$

from which may be calculated:

$$\frac{M_1 - M_2}{\sqrt{\mu_1^2 + \mu_2^2}} = 2.9.$$

The figure derived from the above formula allows to accept that the alkaloid content of the seedlings germinating in darkness for 11 days has really decreased (see J. H. BURN) (8).

If the results of the observations 8 and 9 are analysed in the same manner (table V), it appears that also after germination for five days in the dark the alkaloid content has decreased, probably even more than after 11 days. After 14 (table IV, determination 6) and 20 days (table V, determination 11) the difference with the alkaloid content of 100 seeds has become too small to attach a real significance to it.

The result may be summarised in the following manner: During the first days of germination in darkness the alkaloid content of the seedlings decreases whereupon a rise follows, so that after two to three weeks the content has again become about equal to that of the ungerminated seed.

The results are different with germination in the light. Here from the beginning of the germination the alkaloid content increases, after 10 days the increase is about 10%, after 25 days about 30%.

If parts of the seedlings are analysed (table IV and V, det. 7, 10, 11 and 12), then it appears that the alkaloid content in the cotyledons decreases, that in plumule + radicle increases. This is equally true for germination in darkness as in light. But while in the dark during the first days of germination the decrease in the cotyledons is greater than the increase in plumule + radicle, in light the increase in plumule+radicle exceeds the decrease of these compounds in the cotyledons.

Whether these concentration changes are chiefly the result of a translocation of the alkaloids, or must be ascribed to breakdown

on the one hand and to synthesis on the other hand, cannot be decided on the basis of these observations.

WEEVERS (124) studied the problem of translocation for the xanthine derivatives caffeine and theobromine. Both in Coffea liberica as in Paullinia cupana, during a period in which a considerable decrease of xanthine bases occured in the leaves as they grew older, no increase of these compounds was noticeable in the bark. The fact also that no transport over a longer distance could have taken place, WEEVERS proved by ringwound experiments in Thea assamica. While the caffeine content of the leaves had strongly decreased, only an insignificant increase was noticed in the bark above the ringwound.

WEEVERS concluded from this that there cannot be any question of a transport of the xanthine derivatives as such.

These observations make a transport of alkaloids less probable, although what is valid for xanthine bases may not without further investigation be applied to other groups of alkaloids.

If the concentration changes observed in the cotyledons and in plumule + radicle in germination of Lupinus luteus are the result of alkaloid breakdown on the one hand and the formation of these compounds on the other hand, then there is from the very beginning of germination an alkaloid synthesis in plumule + radicle. This is entirely in accordance with the behaviour of the xanthine bases. WEEVERS (ibid.) was able to establish the formation of alkaloids always when cell division and growth took place, in primary as well as in secundary growing points, in normal as well as in wound cambium. In his germination tests with Thea assamica and Coffea liberica, xanthine bases were formed in plumule + radicle of the young seedlings, whereas the compounds in the cotyledons broke down.

Of importance is also what WEEVERS (123) observed in the germination of Ricinus. The ricinine content of the ungerminated seed is very slight and amounts to only 4 mg per 100 seeds. After 3 weeks' germination in the dark the amount increases to 72 mg. Alkaloid synthesis must have occurred here from the very beginning.

The supposition that decrease in concentration of alkaloids in the cotyledons of the yellow lupin when germinating must be considered to be due to breakdown of these compounds, gains in probability if it can be demonstrated in a convincing manner that the organism is capable of decomposing its alkaloids. This possibility is still more apparent from the observations summarised in table VI, than from the small fluctuations in the total alkaloid content per 100 seedlings

during the first days of germination. If after 9 days one of the cotyledons of the seedling is removed, then the alkaloid decrease in plumule + radicle during the next 12 days is a considerable one, whereas the alkaloid content of the remaining cotyledon decreases as in a normal seedling during that period. For these phenomena there is no other explanation than a breakdown of alkaloids in plumule + radicle.

If these results are compared with those of SABALITSCHKA and JUNGERMANN (163-164), who also studied the germination of Lupinus luteus in darkness and in light, one is struck by the fact that these investigators noticed in their germination tests in darkness a far greater decrease in alkaloid content. In the investigation described here the decrease in the total alkaloid content is at its highest after 5 days and at that moment amounts to about 15% of the quantity present in the ungerminated seed, while after 14 days the decrease is still only about 4%. SABALITSCHKA and JUNGERMANN noticed after 14 days a decrease of 26.5%. If one calculates from their observations the alkaloid decrease in the cotyledons, then this amounts after 14 days' germination in darkness to 58%. I was able to establish after 11 days (table IV, det. 7) in the cotyledons an alkaloid decrease of about 36% and after 20 days (table V, det. 11) a decrease of about 50%.

More striking than in germination in darkness are the differences in germination in light. In their germination tests in light SABALITSCHKA and JUNGERMANN did not use nitrogen-free soil, but let the seeds germinate in garden soil at a temperature of 28° C.

After 14 days they noticed an alkaloid decrease of about 20%, after 4 weeks, however, an increase of 43%.

In my investigations of germination in light, invariably an *increase* of the total alkaloid content was noticed. After 6 days this amounted to 6%, after 12 days to about 13%, after 14 days to about 10% and after 24 days to about 28%. After 14 days the alkaloid decrease in the cotyledons amounts to 46%. SABALITSCHKA and JUNGERMANN found after 14 days a decrease of 60%.

In order to establish the alkaloid content of the seedlings after 2 weeks' germinating in darkness and after 2 weeks' germinating in light, SABALITSCHKA and JUNGERMANN analysed respectively 70, 73 and 56 seedlings. The alkaloid content per 100 seedlings was calculated and this compared with the mean alkaloid content of 100 seeds. The standard error for the content per 100 seeds is not stated. Their opinion is: „Da später die Pflanzenmasse ja immer mehr zunahm waren auch immer weniger Pflanzen zur Untersuchung nötig". It

is evident that such a view, as also the fact of their not determining the standard error in the determination of small fluctuations in the alkaloid content is subject to doubt. The objections against the method followed by SABALITSCHKA and JUNGERMANN in the determination of the alkaloids have been discussed in the preceding chapter.

For the nitrogen distribution in the seed we found (table II):

	Total nitrogen	Protein nitrogen	Soluble nitrogen
Testa of 100 seeds	18 mg	—	—
Cotyledons of 100 seeds.	835 mg	710 mg	125 mg
Plumule + radicle of 100 seeds. .	16 mg	—	—

Of the total nitrogen about 15% is present in the seed in soluble form, in the cotyledons 16%.

After 3 days' germination in the dark (table III) the nitrogen distribution is:

	Total nitrogen	Protein nitrogen	Soluble nitrogen
Cotyledons of 100 seedlings . . .	816 mg	658 mg	151 mg
Plumule + radicle of 100 seedlings	45 mg	—	—

The percentage of soluble nitrogen in the cotyledons then amounts to 19%, while about 7% of the protein nitrogen originally present in the cotyledons has been converted into soluble nitrogen.

The nitrogen mobilisation in the cotyledons after 3 days' germination in darkness is still only small.

After 11 days' germination in darkness (det. 5) the seedlings show a quite different picture. According to the trichloracetic acid procedure 65% of the total nitrogen is then present in a soluble form, according to the tungstic acid procedure 53%. In this phase the nitrogen mobilisation is considerable.

A still higher value was found after 14 days' germination in darkness (det. 6). The fraction of the soluble nitrogen determined by the trichloracetic acid method is then 72%, by the tungstic acid method 70 % of the total nitrogen.

The seedlings contain an about equally large percentage of soluble nitrogen after 12 days' germination in light (det. 4), viz. 74%, both according to the trichloracetic acid procedure as to the tungstic acid method.

IV. ALKALOIDS AND METABOLISM.

A. THE CHEMICAL PROCESS OF THE FORMATION OF ALKALOIDS.

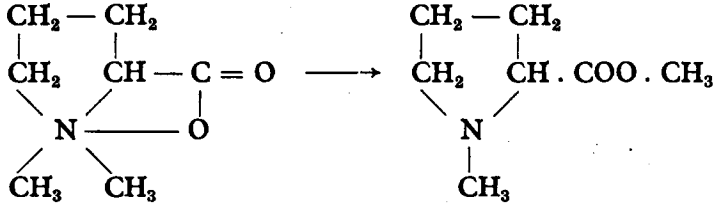
Knowledge of the chemical process of the formation of plant bases in the organism is as yet very limited. Biochemical processes in which certain compounds in the plant body are converted into alkaloids are not known with the exception of the formation of the proteinogenic amines. It was established that they originate from decarboxylation of amino acids through the influence of bacteria. The formation of these compounds, however, in the higher organism must also be accepted. HEINSEN (38) e.g. showed the conversion of tyrosine into tyramine in the pancreas, while HOLTZ (42) established the fermentative formation of tyramine from tyrosine by renal tissue.

The mechanism of this decarboxylation has not been studied any further. VON EULER and FRANKE (24) consider it likely that the first phase of the amine formation consists of a dehydration of the amino group which regresses after decarboxylation. There would be an intermediate formation of iminocarbonic acid, from which, as WIELAND and BERGEL (125) pointed out, carbon dioxide is eliminated with ease.

HOLTZ (41-43) pointed out the significance of ascorbic acid and sulfhydryl compounds for the formation of histamine by decarboxylation of histidine.

The structural relationship between alkaloids and amino acids has long been known. This relationship is very striking in a number of betaines which may be considered as methylation products of natural amino acids.

Such changes are easily realised chemically. TRIER (115) for instance as early as 1910 obtained, through distillation of stachydrine, the methyl ester of hygric acid, viz. N-methylproline.



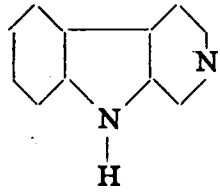
Inversely, through methylation of hygric acid as also of proline, stachydrine might be formed (SCHULZE and TRIER) (102-103).

In the year following, ROMBURGH and BARGER (94) by methylation of tryptophane, obtained hypaphorine, a betaine occurring in the seeds of Erythrina Hypaphorus.

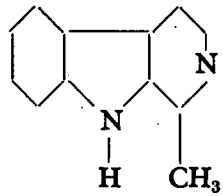
A number of more complicated plant bases, however, may also be formed from amino acids by simple chemical processes. In this manner for instance, starting from β-phenyl-aethylamine, the decarboxylation product of phenylalanine, the tetrahydro-isochinoline-nucleus occurring in numerous alkaloids is obtained by condensation with carboxylic acids, according to the reaction of BISCHLER and NAPIERALSKI (3). In this synthesis the acid may be replaced by the corresponding aldehyde according to PICTET (84).

This condensation has been used in the synthesis of the Anhalonium alkaloids by SPÄTH and his collaborators (108-109-111).

A similar condensation of tryptophane with aldehydes leads to compounds built on the ring system.



to which belong the Harmala alkaloids. In this way KERMACK, PERKIN and ROBINSON (52-53), by condensation of tryptophane with acetaldehyde and subsequent oxydation with chromic acid obtained harman an alkaloid ocurring in the Rubiacea Arariba rubra and in the Symplocacea Symplocos racemosa. SPÄTH and LEDERER (110) synthesized the same compound starting from tryptamine.



Also for the determination of the structure of plant bases, the knowledge of the close relationship between amino acids and alkaloids has very often been a guiding line. But although such knowledge may be of importance for the determination of the structure and for the

synthesis of plant bases, it remains an open question as to how far the organism follows the same ways in the formation of alkaloids.

TRIER (116) in a „Hypothesenschema" suggested the possible formation of a number of these compounds in the plant body. His view about the formation of nicotine e.g. from one single amino acid, viz. proline, by condensation with formic acid which may be formed by dismutation of formaldehyde according to the reaction of CANNIZZARO, is undoubtedly very fascinating. The same remark applies to his idea of the formation of evodiamine from tryptophane. Similar schemes may be drawn up for other alkaloids. WEEVERS (123) has given a survey for all those plant bases of which the structure has been established with sufficient certainty.

It must, however, not be lost sight of the fact that the experimental test of the conception that the organism builds up its alkaloids from amino acids, is as yet quite insufficient. By no means convincing in this respect are the results of some investigators who have tried to influence the formation of alkaloids by supplying such nitrogen containing compounds to the plant as might be expected to be capable of being changed by simple biochemical reactions into alkaloids characteristic of the organism concerned.

This applies in the first place to the experiments of CIAMICIAN and RAVENNA (9-10-11) who were able to establish an increase of the nicotine content in tobacco plants after inoculation with heterocyclic bases such as pyridine and piperidine. It was, however, subsequently proved that the same effect could be obtained with compounds not containing nitrogen as e.g. glucose. WEEVERS (123) remarks in connection with the investigations mentioned: „Es ist bei dieser Arbeitsmethode jedoch eine Schwierigkeit, dasz man oft nicht sagen kann, ob die Bildung oder Mehrbildung eines Alkaloids die Folge einer komplizierten Reizwirkung oder einer einfachen chemischen Umwandlung des einverleibten Stoffes ist".

KLEIN and LINSER (54) raised tobacco plants for 9-14 days on a 1% proline solution and compared the nicotine content with that of control plants the culture medium of which during the same period was water only. They found especially in the leaves of the first series of plants a higher nicotine content than in the control plants.

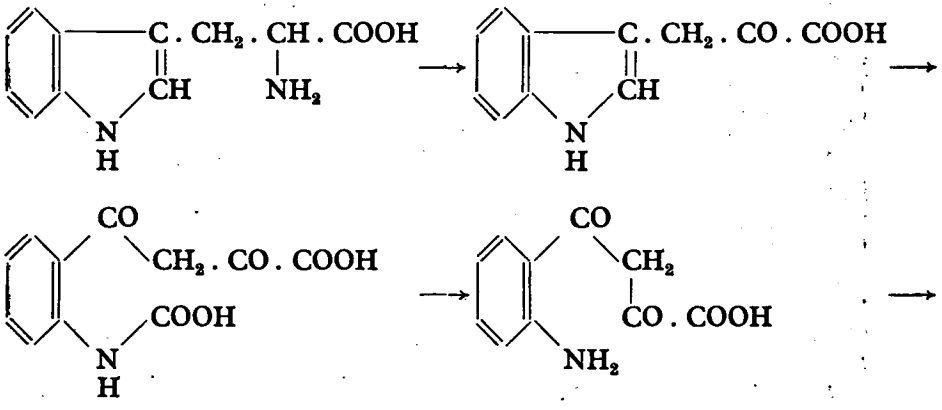
GORTER (30) was able to demonstrate a decrease in nicotine content in the leaves of tobacco plants which had been kept during 4 or 5 days on a twice diluted nutrient solution according to KNOP. If 0.5% of proline was added to the nutrient solution, also a decrease was noticed, to a lesser extent, however, than in the plants raised on a culture medium free from proline. This observation makes very problematical the conclusions of KLEIN and LINSER who infer from

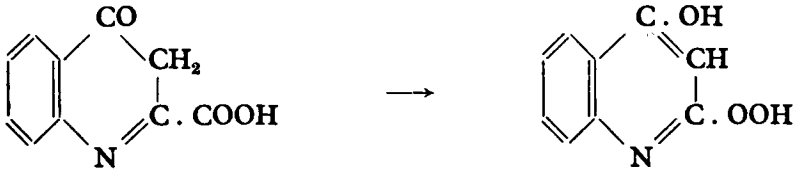
their results that the nicotine content increases on a proline solution.

Of more importance are the results of an earlier observation by the same investigators (55). After injecting a proline solution in the hollow stem of Stachys palustris, a plant which forms stachydrine and in those of Dahlia variabilis and Arachis hypogaea which both form trigonelline, the betaine content in the leaves increaeses amounting to 3-4 times that of the control plants. If together with proline also hexamethylenetetramine was given, the betaine formation was still further increased. An increase in trigonelline was also noticed after injecting glutaminic acid or ornithine.

The formation of stachydrine from proline and of trigonelline from this same amino acid, as also from glutaminic acid and ornithine is in complete accordance with the views of TRIER and WEEVERS mentioned above on the genesis of alkaloids from chemically related amino acids.

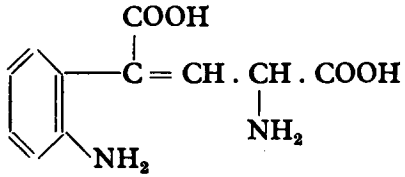
ELLINGER (17) as early as 1904 drew the attention to a biochemical relationship between tryptophane, a few years before discovered by HOPKINS and COLE and kynuric acid. The latter compound is a normal component of the urine of dogs. After supplying tryptophane to rabbits, its presence could also be demonstrated in the urine of these animals while in the same manner the amount of this acid in the urine of dogs was increased. According to ELLINGER and MATSUOKA (18), after intravenous injection of tryptophane and also of indolpyruvic acid in rabbits, part of these compounds is traced back in the urine as kynuric acid. On the basis of these observations the investigators mentioned give the following scheme for the formation of kynuric acid from tryptophane





MATSUOKA, TAKEMURA and YOSHIMATSU (70) found that this formation takes place in the liver. The surviving dog's liver was able to form kynuric acid from l-tryptophane as well as from indolpyruvic acid.

KOTAKE (62) suggests that kynuric acid is not formed via indolpyruvic acid or at least only to a very slight extent, but proceeds chiefly by way of kynurenin which was discovered by MATSUOKA and YOSHIMATSU.



After having caused in rabbits fed on hulled rice an experimental polyneuritis, KOTAKE, by subcutaneous injection of tryptophane, found in the urine considerable amounts of kynurenin which were larger in proportion to less kynuric acid being formed. Both by rabbits as well as by the surviving dog's liver kynurenin could be changed into kynuric acid.

These observations prove that it is possible for a chinoline nucleus to be formed by the organism from tryptophane and they give partly an insight into the manner in which this conversion is achieved. For the knowledge of the biogenesis of the alkaloids of the chinoline type they undoubtedly supply very important indications.

Against the conception that the alkaloids are formed in the organism via amino acids, there is the opinion of a number of investigators who envisage the synthesis of these compounds from components free from nitrogen or from simple amines.

EMDE (19) e.g. has developed views according to which the alkaloid molecule is built up from amines and biogenous breakdown products of the carbohydrates. His undoubtedly interesting speculations lack, however, any experimental basis.

The same may partly be stated of R. Robinson's theory about the

phytochemical alkaloid synthesis (for a survey, see P. KARRER, Lehrbuch d. org. Chemie, Leipzig 1936). It is true that this investigator assumes the formation from amino acids for some plant bases, his scheme, however, of the synthesis of the compounds of the papaverine type from methylglyoxal, formaldehyde, acetondicarbonic acid and ammonia subscribes to the opinion that biogenous breakdown products of the carbohydrates play a rôle in the formation of alkaloids.

B. THE PLACE OF THE ALKALOIDS IN THE NITROGEN METABOLISM IN GERMINATION.

The insight into the biochemical relationship between amino acids and alkaloids does, however, by no means solve the problem as to how and at which moment the alkaloids are formed in the so complicated system of biochemical mutations.

This relationship must certainly not be interpreted in such a way that wherever amino acids occur in the organism, also alkaloids will be formed. For, in that case, a considerable alkaloid synthesis in the cotyledons might be expected in the germination of those seeds of alkaloid forming plants whose cotyledons are rich in reserve protein. Such a synthesis was observed neither by SABALITSCHKA and JUNGERMANN, nor by myself in the germination of the yellow lupin. Neither did WEEVERS observe a formation of these compounds in the germination of plants containing xanthine bases, nor a ricinine formation in the endosperm in the germination of Ricinus.

In all probability, alkaloid formation in Lupinus luteus must be looked for, as in Ricinus, in the growing tips of the young seedlings. Given the intense metabolism in these places, it will be evident that the process of alkaloid formation is connected with that part of nitrogen metabolism on which point we are still very insufficiently informed. Our knowledge of the synthesis and breakdown of proteins in the vegetable organism concerns almost exclusively the reserve proteins, whereas in the growing tips it is just the formation of proteins of the protoplasm which are bound to play a prominent part.

Of importance is the question in which form alkaloids occur in the organism. The current opinion according to which the plant bases are supposed to be bound only to organic acids in the shape of alkaloid salts is, experimentally, very insufficiently founded. Numerous data point to the fact that the condition in the living organism is probably a much less simple one. Taking into consideration the character of the plant bases, apart from a salt formation, the forming

of complexes with a great number of compounds of the plant cell such as proteins, tannins etc. may be expected.

The methods followed in the extraction of plant bases do not tell us anything of the manner in which alkaloids are bound in the plant body. The use of strong bases or of ammonia in the extraction upsets the equilibrium in the tissues by which disturbance alkaloid salts as well as the complexes mentioned may be decomposed.

An indication of the existence of such complexes is supplied by fresh tea leaves. NANNINGA (73) established, that the quantitative extraction of caffeine with alcohol was possible only with black fermented tea. With green, fresh desiccated leaves, dependent on the tannin content, part of the xanthine bases remains in the material. This accounts for the existence of a caffeine-tannin complex in fresh tea leaves, which is decomposed on fermentation.

A further observation by NANNINGA shows the ready decomposition of such complexes. Four parts of fresh dries and pulverized leaves were moistened with one part of water, after the mixing the powder was allowed to stand 2 hours. After this time the caffeine could be completely extracted with chloroform.

Further investigation on this field will undoubtedly yield very important data for the physiology of the alkaloids.

When studying the nitrogen metabolism in germination, the first striking moment is the mobilisation of the reserve proteins in the cotyledons. In the ungerminated seeds of Lupinus luteus, about 85% of the nitrogen is present in the form of proteins which during germination are converted into soluble compounds under the action of proteolytic enzymes.

Plant proteases, such as for instance papain, are similarly to animal kathepsin, tissue proteases. Probably they are not single enzymes, but like the kathepsin to which they show great similarity, they contain in addition to a proteinase also a carboxypolypeptidase.

GRASSMANN (32) showed that plant proteases are activated by sulphydryl compounds occurring in the organism. The natural activator of yeast proved identical with glutaminyl-cysteinyl-glycocoll, e.g. the glutathion (33-51). At about the same time WALDSCHMIDT-LEITZ (119) isolated the zookinase of the kathepsin from the liver. This compound also could be identified with glutathion just like the natural activator of papain preparations (120).

According to BERSIN (2) who studied the mechanism of this activation, the number of free SH-groups in the papain molecule is a gauge for the activity of the enzyme. He succeeded in inactivating the enzyme reversibly with hydrogen peroxide. Such an inactivated

papain showed only after reactivation with cyanide the reaction with sodium nitroprusside, characteristic for the sulphydryl group.

In the course of proteolysis the reserve protein is broken down into amino acids via peptones and polypeptides. There are reasons for assuming that this splitting is in part immediately followed by other changes. The mixture of amino acids as occurring in young etiolated seedlings differs considerably in composition from the amino acid mixture which is obtained on hydrolysis from the reserve protein by acids. PASTEUR (80-81) observed that in the germination of leguminosae large quantities of asparagine were formed. SCHULZE and his collaborators (104-105) demonstrated that the quantity formed was greater than could be obtained from the reserve protein by hydrolysis and that the synthesis of the asparagine occurs at the cost of other amino acids formed in hydrolysis (100). It must be accepted that the amino acids are deaminated in this process and that from the ammonia and from a carbon skeleton of unknown origin the asparagine is formed.

In a milieu free from oxygen, asparagine is not formed (76). This phenomenon finds its explanation in the manner in which the deamination of the amino acids takes place in the organism.

After WARBURG and NEGELEIN (122) had pointed out the ready oxidability of amino acids on charcoal, WIELAND and BERGEL (125) have shown that even in those cases in which the value of the respiratory quotient made one suspect a complete combustion of the amino acid, as e.g. leucine, the combustion was not complete. The proportion of the ammonia and carbon dioxide formed was for monocarbonic acids always 1 : 1, for asparagine 1 : 2. Starting from an amino acid, in every case the aldehyde which is one carbon atom poorer was formed and in addition, in smaller quantities, the carboxylic acid corresponding to this aldehyde.

As activated charcoal proved unable to decarboxylate α-keto acids to carbon dioxide and aldehyde, WIELAND arrived at the following scheme for the oxidation of amino acids on the charcoal model.

$$\mathrm{R.CH(NH_2).COOH} \xrightarrow{-2H} \mathrm{R.C(NH).COOH} \xrightarrow{-CO_2} \mathrm{R.CH{=}NH} \xrightarrow{+H_2O} \mathrm{R.CHO} + \mathrm{NH_3}$$

It has been proved, however, that the deamination in the organism is effected in another manner than by activated charcoal. According to the earlier views of NEUBAUER (74-75) and KNOOP (56) the deamination of the amino acids in the organism takes an oxidative course

with formation of the corresponding keto acids. By administering amino acids foreign to the organism, the corresponding α-keto acids could be detected in the urine.

Through the investigations of KREBS the correctness of these views for the animal organism has been proved. KREBS (63-64) found that renal tissue when oxigen is present is able to deaminate amino acids according to the scheme.

$$\mathrm{R.\,\underset{\displaystyle NH_2}{\underset{|}{CH}}.\,COOH} \xrightarrow[+H_2O]{-2H} \mathrm{R.\,CO.\,COOH + NH_3}$$

In case of a normally proceeding deamination the keto acid formed is further decomposed and is withdrawn from observation. By inhibiting further decomposition by HCN or As_2O_3, α-keto acid has been detected as a primary product of the oxidation of amino acids.

From renal tissue, by grinding with sand and extracting with M/100 phosphate buffer, KREBS succeeded in isolating the enzyme which deaminates amino acids.

On continued investigation KREBS and JOHNSON (65) succeeded in tracing the breakdown of the amino acids still further. They found that the keto acids formed in the deamination could be converted by an oxidation-reduction process, which may proceed under anaerobic conditions, into hydroxy acid and carboxylic acid poorer by one carbon atom, in the following manner

$$\mathrm{R.\,CO.\,COOH + R'.\,CO.\,COOH + H_2O} \longrightarrow \mathrm{R.\,COOH + CO_2 + R'.\,CHOH.\,COOH}$$

This reaction which according to a more recent publication of the same investigators (66) has also been observed in bacteria, explains the occurrence of hydroxy acids in the breakdown of an amino acid mixture. This formation of hydroxy acids has been known for a long time and several investigators have considered this as proof of a hydrolytic deamination of the amino acids. KNOOP, however, on the ground of energetical considerations had earlier already raised doubts about the probability of hydrolytic deamination.

The question now remains as to how far the changes proceeding in animal tissue are similar to the amino acid breakdown in the plant body.

That in the vegetable organism also the deamination of amino acids may take an oxidative course, has been confirmed by recent

observations. VAN WAESBERGHE (118) in Aspergillus niger has shown the probability of an amino acid oxidodeaminase. He demonstrated that an "impoverished" Aspergillus mat in an alkaline milieu, in the absence of sugars, is able to affect aspartic acid. The breakdown does not go any further than deamination. The remaining carbon skeleton, in an alkaline milieu, is not decomposed at a measurable speed. For this deamination oxygen is required to the extent of 1 atom per molecule of aspartic acid. It could further be demonstrated that, also in an acid milieu, in the absence of sugars, the combustion was not complete. Only 3 atoms of oxygen were taken per molecule of aspartic acid, whereas for a complete combustion 6 atoms are required.

If for the yellow lupin too an oxidative deamination is accepted, then there is here an explanation of the absence of the formation of asparagine in surroundings free from oxygen, as observed by PALLADIN.

The reason of the non-formation of asparagine in narcotized seedlings may further be sought in a paralysis of the oxidodeaminase by the narcotic.

It has already been mentioned above that the origin of the carbon skeleton is unknown in the asparagine synthesis. Obviously one would think here of malic acid and of oxalacetic acid. According to KOSTYTSCHEW (61) these compounds are supposed to have been formed by the oxidative breakdown of sugars. There is here a certain contradiction, for the formation of asparagine would become considerable at the very moment when the carbohydrates become increasingly scarce.

RUHLAND and WETZEL studied nitrogen metabolism in Begonia and Rheum which differs in many respects from that of the yellow lupin. Whereas in Lupinus luteus and other so-called "amide" plants the ammonia formed by deamination of the amino acids is bound in the shape of asparagine, in the so-called "ammon" plants, of which Begonia and Rheum are typical representatives, the freed ammonia is bound to organic acids, especially to malic acid. RUHLAND and WETZEL showed that ammonia and l-malic acid were in this process formed in equimolar quantities and therefore they assume a chemical connection between the genesis of both substances. They presume that the ammonia as well as the l-malic acid are oxidative breakdown products of the amino acids.

It must here be pointed out that the plant organism is able to form aspartic acid still in another manner. QUASTEL and WOOLF (91)

demonstrated in 1926 the synthesis of this acid by coli bacteria under anaerobic conditions from fumaric acid and ammonia. The reaction was reversible. Under the influence of a thermolabile enzyme, called by WOOLF aspartase, the following reaction took place and an equilibrium was set up corresponding to

$$HOOC \cdot CH = CH \cdot COOH + NH_3 \rightleftharpoons HOOC \cdot \underset{\displaystyle NH_2}{\underset{|}{CH}} \cdot CH_2 \cdot COOH$$

COOK and WOOLF (12-13) found the enzyme also in a number of other bacteria of the facultative anaerobic type. VIRTANEN and TARNANEN (117) showed aspartase activity both in bacteria as well as in the higher plants (e.g. the cotyledons of the pea). These investigators succeeded in separating the enzyme from a dried preparation of B.fluorescens liquefaciens. The specificity shown by aspartase is absolute, according to VIRTANEN it attacks only aspartic acid.

It is probable that several enzymes of this type, which FRANK (27) calls "ammoniakasen", are found in vegetable life. RAISTRICK (92) noticed the transformation of histidine into imidazolacrylic acid by bacteria of the Colityphosus group and HIRAI (40) the formation of p-oxiphenylacrylic acid from tyrosine by B.proteus vulgaris.

For the vegetable organism, however, the function of the aspartase in the formation and decomposition of aspartic acid is probably a very secondary one.

The significance of asparagine formation in germination has become clear by the considerations of PFEFFER (83) and BORODIN (4), but especially by the classical research already mentioned of SCHULZE and his pupils. The translocation of nitrogen, specially in the Leguminosae, takes place chiefly in the form of this compound. Asparagine functions as the nitrogen source of protein generation in the tissue of the growing tip. In this process amido as well as amino nitrogen is consumed. It is well known that the amido group may be split, both in a chemical way as well as by an enzyme found in yeast and Aspergillus. GEDDES and HUNTER (29) discovered that yeast-asparaginase only affects asparagine and to a lesser degree glutamine. For the splitting off of the amino nitrogen from asparagine the oxidodeaminase mentioned above and discovered by KREBS must specially be considered.

The protein regeneration starts with a synthesis of the amino acids. As in the absence of carbohydrates no protein formation takes place, the carbohydrates probably form the source from which the carbon skeleton for the building of amino acids is drawn.

The amino acid synthesis has been extensively studied in animal organism. According to observations of KNOOP, EMDEN and SCHMITZ and other investigators it can be accepted that the amino acids are formed from keto acids or hydroxy acids and from ammonia obtained by the deamidation and the deamination of asparagine.

KNOOP (56) found in the urine of dogs fed with γ-phenyl-α-keto-butyric acid, the γ-phenyl-α-amino-butyric acid. EMDEN and SCHMITZ (20-21), by perfusing the isolated liver, were able to establish the formation of tyrosine, phenylalanine, leucine and alanine from the corresponding keto acids if these were added to the perfusing blood in the form of ammonium salts. At the same time they found that the liver free from glycogen is able to form alanine from ammonium lactate. The formation of this compound was also observed by FELLNER (25) in a liver rich in glycogen in the presence of ammonium salts, in which process an intermediary formation of pyruvic acid from glycogen may be accepted.

KONDO (60) using the EMDEN and SCHMITZ procedure, was able to establish the synthesis of such amino acids from the corresponding keto compounds which in normal conditions do not occur in the organism.

Further the experiments of KNOOP and OESTERLIN (57-58) are of importance, who in a purely chemical way achieved an amino acid synthesis by shaking keto acids and ammonia in solution with various reducing agents such as palladium and hydrogen, cysteine or ferrous sulphate, and obtained yields up to 66% of the quantity to be expected.

The formation of amino acids by a reductive amination of the corresponding keto acids becomes very probable judging by all these observations.

From the amino acids formed in this manner, the organism builds its proteins. The proteolyse, the hydrolytic splitting of the proteins in amino acids, as also the lipolyse and amylolyse most probably are reversible processes, although this is only known with certainty as far as lipolyse is concerned. In a homogeneous milieu such a process leads to an equilibrium of which the position is determined by temperature. The rapidity with which the equilibrium is set up, depends apart from temperature on the activity of the enzyme influencing the reaction. If it is accepted that the enzyme accelerates or retards the speeds of the forward and back reactions to the same extent, then the equilibrium is unaffected.

In a heterogeneous milieu a condition will generally arise which may differ considerably from the equilibrium in a homogeneous

phase. A number of factors will influence the course of the process among which may be mentioned differences in solubility and in degree of dispersion of the components in the different phases. Also adsorption and occlusion by which a separation between enzyme and substrate as well as between substrate and the reaction products becomes possible must be taken into account. Through the micro- and macroheterogeneity of the cell structure such factors will play an important rôle in the living organism. The reaction products which are formed may, moreover, be embodied in the cell structure and in this way be withdrawn from the reaction.

Through the development of the cytological technique a great variability of the protoplasm could be established so that the cell must be considered as a heterogeneous system subject to continuous modifications. It will be evident that in this way the organism has the disposal of a great number of possibilities for regulating synthesis and decomposition.

Some light has been shed on this subject by the investigations of LESSER, WILLSTÄTTER, v. PRZYLECKI, BUNGENBERG DE JONG and others. So far as the possibilities of synthesis are concerned, an observation of v. PRZYLECKI, GIEDROYÉ and SYM (90) is here referred to, who were able to demonstrate that the system maltase-glucose gives a greater yield in disaccharides in the presence of active charcoal, thanks to the greater adsorbing capacity of charcoal for di- than for mono-saccharides. The system lipase-charcoal-acetic acid-aethanol also showed a shifting of the total equilibrium in the direction of the ester synthesis.

v. PRZYLECKI (86) remarks in this connection: „Solche Verschiebungen, dank der groszen Adsorptionsfähigkeit verschiedener Substrate, sind auch im Zellinnern gut möglich und können als Synthese vergröszernde Momente angesehen werden. Selbstverständlich begrenzen sich solche durch Adsorption hervorgerufene Gleichgewichtsverschiebungen auf diejenige Zellteile, in denen das Enzym ausschlieszlich oder fast ganz in adsorbiertem Zustande anwesend ist und wo die Adsorbierbarkeit der Substrate der Synthese und des Zerfalls verschieden ist".

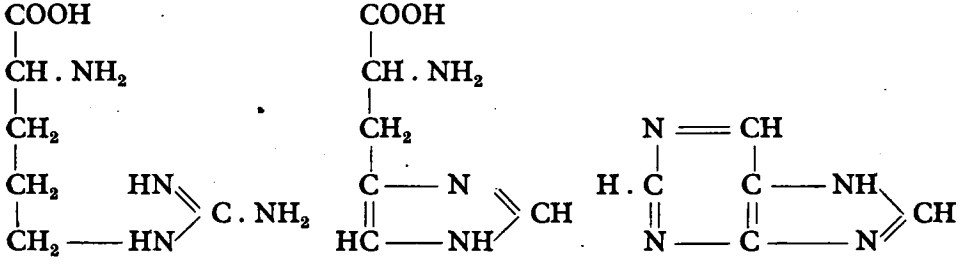
Recent investigations have shown that the proteins predominate in the protoplasm in the form of complexes, higher units which may have been built up from similar (homonomous complexes) as well as from different types of substances (heteronomous complexes). So there are proteino-proteins, nucleo-proteins, polysaccharo-proteins, lipo-proteins etc. which differ considerably in properties from the fundamental materials. Especially striking is the increased chemical activity of these "symplexes".

The manner in which complex formation arises may even differ for identically called members of this very extensive group of compounds. Symplexes are known both of the homo- and of the hetero-polar type. The stability of the compounds of the former type can be very great, as has been demonstrated by V. PRZYLECKI and his co-workers (87-88-89). The influence of electrolytes on the stability of symplexes of the hetero-polar type has been traced by BUNGENBERG DE JONG (6-7).

Tracing the moment when the alkaloids are formed in the metabolism, it appears that in germination of the yellow lupin the formation coincides with the synthesis of the amino acids. Both groups of compounds are built up in the organism not only simultaneously but also in the same place, viz. in the growing tips. This is in concord with the theory that there exists a genetic connection between these chemically also closely related substances. But how this connection is supposed to exist, directly or intermediarily, can at present only be guessed.

Let us consider for a moment the problem of the formation of the xanthine bases. Purine derivatives are of very general occurence in the organism, e.g. as components of the nucleic acids, the prosthetic groups of the nucleo-proteins, but which are also found in a free form in the cell plasm. Some derivatives of the adenylic acid play, as coenzymes, an important rôle in metabolism.

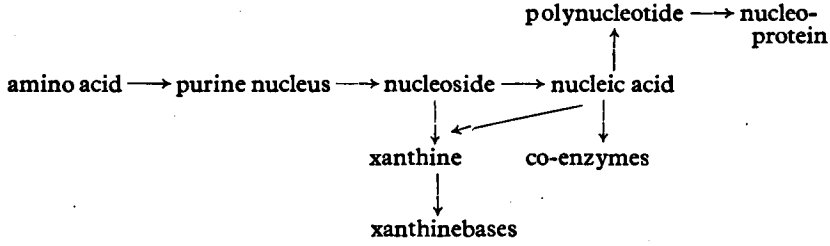
The purine nucleus must therefore be regularly formed in the organism, but it is not known how the nucleus comes into existence. Arginine and histidine have been considered as the starting point, but this is only a supposition and not an established view.



So far, two purine derivatives have been found in nucleic acids, adenine and guanine. The increased reactivity of these compounds is striking both in nucleotide- as well as in nucleoside-form, as is apparent e.g. in the deamination of these purine derivatives. Free adenine cannot be deaminated by the organism. According to

SCHMIDT (99) muscular tissue contains an adenylic acid deaminase which exclusively converts adenylic acid into inosinic acid and besides, an adenosine deaminase which can only effect the deamination of adenine bound in adenosine to hypoxanthosine. Two guanine fissuring enzymes are known, guanase which is able to deaminate guanine as well as guanosine, and guanylic acid deaminase which can only deaminate guanylic acid to the corresponding xanthine compounds.

As both purines are easily affected in nucleoside- as well as in nucleotide-form, the possibility is not excluded that we may see in these active nucleosides and nucleotides the starting point for the formation of the xanthine bases. The following scheme will make the matter clear

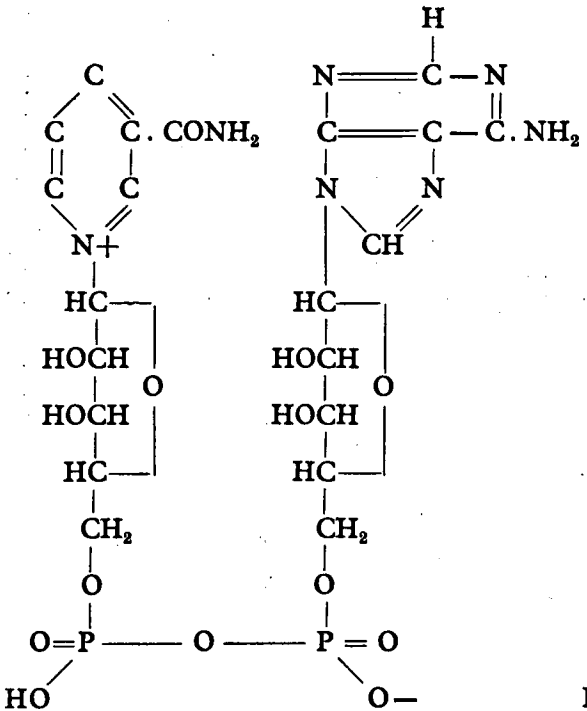


Some arguments may be adduced which justify the assumption that alkaloids other than the xanthine bases might find their origin in similar nucleotide-like compounds. Lately a number of physiologically very important substances have become known in which nitrogen-containing nuclei, other than the pyrimidine- and purine nucleus are nucleotide-like bound and which in this bond show an increased activity. Cases in point are the cozymase of yeast and WARBURG's „wasserstoffübertragendes Co-Ferment", both being pyridine nucleotides which, bound to proteins, play an important part in metabolism. Here we see for the first time pyridine compounds occurring in the organism of which the physiological significance is established.

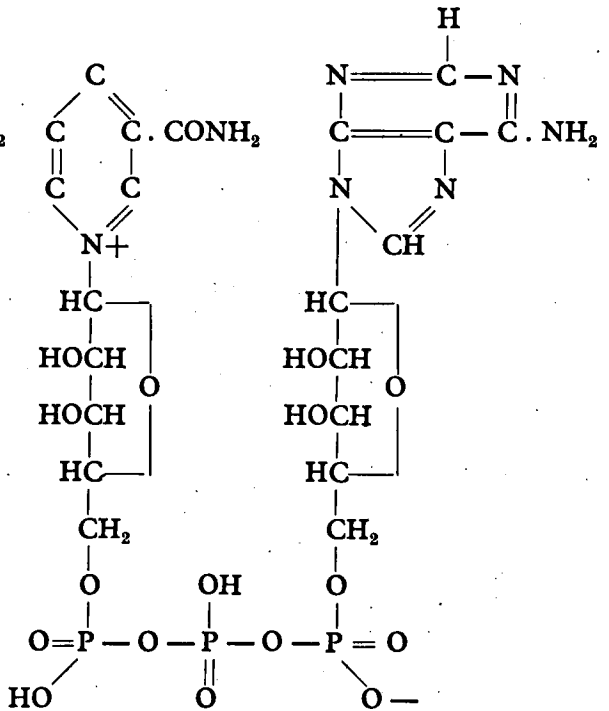
The most probable structure of these compounds according to v. EULER and SCHLENK (22-23) is given on the next page.

Another biologically important substance of this type is lactoflavine-phosphoric acid which bound to protein forms the yellow respiratory ferment.

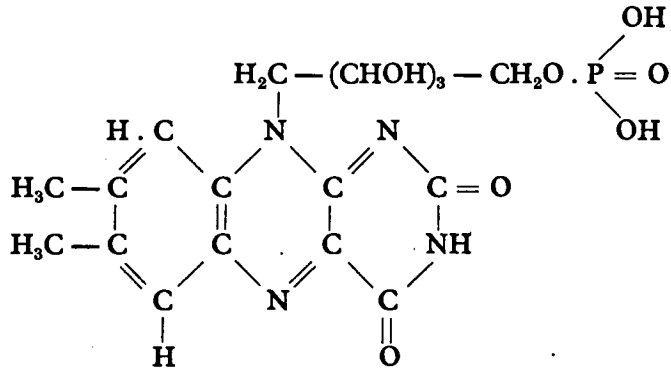
Lactoflavine (vitamin B_2) is a nucleoside-like compound of which KARRER and his co-workers have been able to establish the structure



Cozymase



WARBURG's „wasserstoffübertragendes Co-Ferment"

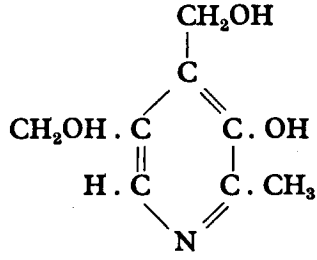


(49). It is 6-7-dimethyl-9(d-l'-ribityl)-iso-alloxazine which could be confirmed by synthesis (50).

WARBURG and CHRISTIAN (121) lately succeeded in isolating a lactoflavine-adenine-nucleotide in a pure form.

Finally a compound may be mentioned which e.g. is found in yeast and in rice bran and to which KUHN gave the name of adermine.

The composition could be established (67). It proved to be an oxypyridine of the following structure



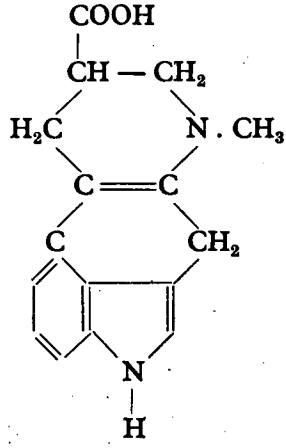
Young rats, deprived of this vitamin, do not grow and dermatitis develops at feets, corners of the mouth, eyes and ears. Adermine in yeast is bound to protein, from which it is easily liberated by acids. If one considers that the anti-pellagra-vitamin, the amide of nicotinic acid, is found in yeast as a subsidiary part of the cozymase, and that vitamin B_2 forms a part of lactoflavinephosphoric acid, the possibility must not be excluded of adermine also occurring in the organism in nucleotide form.

Such substances suggest a relationship between alkaloids and the nucleotide metabolism and at the same time throw light on the

significance of alkaloid structures for the building up of such biologically important compounds as vitamins and coferments. This leads to views which are directly opposed to the opinion of a number of investigators who consider alkaloid structures as a form of waste products which, once formed, have no further importance for the plant organism.

A remarkable group of compounds are the ergot alkaloids, the polypeptide character of which could be established by the investigations of SMITH and TIMMIS, JACOBS and CRAIG, BURCKHARDT and HOFMANN and others. Two isomeric series of these compounds are known. The one contains the pharmacologically active, laevo-rotatory alkaloids ergotoxine, ergocristine, ergotamine, ergosine and ergobasine (ergometrine), the other the inert, dextro-rotatory compounds ergotinine, ergocristinine, ergotaminine, ergosinine and ergobasinine (ergometrinine).

The substance common to all the alkaloids of the laevo-rotatory series is lysergic acid isolated by JACOBS and CRAIG (44) to which these investigators gave this structure (46)



The dextro-rotatory alkaloids on the other hand contain the isomerie isolysergic acid, obtained by SMITH and TIMMIS (107) from lysergic acid in which the double bond, according to CRAIG, SHEDLOVSKY, GOULD and JACOBS (15) is supposed to have been shifted one place to the left in the upper ring.

The other acids isolated from the polypeptide chains of the ergot alkaloids are pyruvic acid, isobutyryl-formic acid, l-leucine, l-pheny-

lalanine and d-proline. JACOBS and CRAIG (45) are of the opinion that isobutyryl-formic acid which is obtained from ergotoxine, respectively from ergotinine does not as such occur in these alkaloids but that during the hydrolysis it is formed from α-hydroxyvaline. Likewise pyruvic acid, by hydrolysis of ergotamine, respectively ergotaminine, is supposed to evolve from α-hydroxyalanine.

Ergobasine and ergobasinine are distinguished from other ergot alkaloids by a much simpler structure. The partial synthesis of these latter compounds was achieved by STOLL and HOFMANN (113). They were able to prove that ergobasine is identical with d-lysergic acid-d-isopropanolamide, ergobasinine with d-isolysergic acid-d-isopropanolamide. STOLL (112) draws the attention to the relationship of ergot alkaloids with the animal hormones, such as e.g. insuline. This relationship is not only a constitutional one but extends as far as the effect these compounds have on the animal organism: „Nous connaissons la nature polypeptidique de certains hormones animales, celle du corps thyroïde par exemple et avant tout celle de l'insuline qui consiste en une longue chaine d'acides aminés. Ce n'est donc pas par un simple effet du hasard que d'après les recherches d'ABDERHALDEN (1), l'ergotamine se comporte comme un antagoniste de la thyroxine, groupement prosthétique de l'hormone thyroïdienne et que dans certains cas de diabète l'ergotamine puisse seconder l'action de l'insuline."

It may be remarked that the alkaloids of the ergot type can hardly be considered merely as waste products of nitrogen metabolism. It is very improbable that simple polypeptides should not function any further in metabolism.

CONCLUSION.

In the light of recent biochemical research it seems to me very difficult to maintain the opinion that alkaloids are waste products of metabolism. It is unlikely that compounds which can greatly affect animal metabolism and moreover show a close relationship with substances of great importance for plant organism such as a number of co-ferments, should have no significance for plant life.

The study of changes in the concentration of alkaloids in metabolism leads also to views which cannot be reconciled with the opinion that the plant bases, once they have been formed, are no longer concerned in the metabolic process. This has already been conclusively demonstrated by WEEVERS for the xanthine bases and for ricinine.

My own observations made with Lupinus luteus point in the same

direction. I was able to establish that the alkaloids of the yellow lupin take an active part in metabolism. During germination the quantity of these compounds in the cotyledons decreases, that in the plumule + radicle increases. If germination takes place in darkness, then during the first three weeks the decrease in the cotyledons exceeds the increase in plumule + radicle. This cannot be explained otherwise than by a decomposition of the alkaloids in the organism.

If during germination one of the cotyledons of the seedling is removed, a far reaching alkaloid decomposition follows in plumule + radicle.

From this behaviour in germination it becomes clear that the alkaloids are not the rigid, immovable and unassailable waste products of metabolism for which a number of investigators take them, and that TSCHIRCH's qualification „an den Strand gespülte Verbindungen" cannot be applied, at least not to the alkaloids of the yellow lupin.

LITERATURE CITED.

1. ABDERHALDEN a. WERTHEIMER, Pflügers Archiv. ges. Phys. **216**, 697, 1927.
2. BERSIN, Zeitschr. f. physiol. Chem. **222**, 177, 1933.
3. BISCHLER a. NAPIERALSKI, Ber. d. d. chem. Ges. **26**, 1903, 1893.
4. BORODIN, Botan. Zeit. 1876.
5. BRAHM a. ANDRESEN, Zeitschr. f. angew. Chem. **39**, 1348, 1926.
6. BUNGENBERG DE JONG, Kolloid. Z. **79**, 223; 334, 1937.
7. —— Kolloid. Z. **80**, 221; 350, 1937.
8. BURN, Biological standardization, London 1937.
9. CIAMICIAN a. RAVENNA, Verh. Naturf. Ges., Wien 1913.
10. —— Annal. Chim. et Phys. **25**, 404, 1912.
11. —— Annal. de Chim. **5**, 1915.
12. COOK a. WOOLF, Bioch. Jl. **22**, 474, 1928.
13. —— Bioch. Jl. **23**, 472, 1929.
14. CORDES, Dissert., Dorpat 1888. See H. Beckurts, Archiv. d. Pharm. **228**, 330, 1890.
15. CRAIG, SHEDLOVSKY, GOULD a. JACOBS, J. biol. Chem. **125**, 289, 1938.
16. v. DIJK, Phytochemische onderzoekingen over alkaloiden in verband met het kiemen. Diss. Utrecht 1900.
17. ELLINGER, Zeitschr. f. physiol. Chem. **43**, 325, 1904.
18. ELLINGER a. MATSUOKA, Zeitschr. f. physiol. Chem. **109**, 259, 1920.
19. EMDE, Apotheker Zeitung 1932.
20. EMDEN a. SCHMITZ, Bioch. Zeitschr. **29**, 423, 1910.
21. —— Bioch. Zeitschr. **38**, 393, 1911.
22. v. EULER a. SCHLENK, Zeitschr. f. physiol. Chem. **246**, 64, 1937.
23. —— Naturwissensch. **25**, 318, 1937.
24. v. EULER a. FRANKE, Chemie d. Enzyme, H. v. Euler, Tl. II, Abs. III, p. 95, München 1934.
25. FELLNER, Bioch. Zeitschr. **38**, 414, 1912.
26. FOLIN a. WU, J. biol. Chem. **38**, 81, 1919.
27. FRANK, Chemie d. Enzyme, H. v. Euler, Tl. II, Abs. III, München 1934.
28. FROMME, Caesar & Loretz, See Dichgans, Apoth. Ztg. **29**, 308, 1914.
29. GEDDES a. HUNTER, J. biol. Chem. **77**, 197, 1928.
30. GORTER, Versl. koninkl. Akad. v. Wet. Vol. XXXIX, No. 1, 1936.
31. GOUWENTAK, Rec. d. Trav. bot. néerl., Vol. XXVI, 19, 1929.
32. GRASSMANN, DYCKERHOFF a. v. SCHOENEBECK, Zeitschr. f. physiol. Chem. **186**, 183, 1929.
33. GRASSMANN, v. SCHOENEBECK a. EIBELER, Zeitschr. f. physiol. Chem. **199**, 124, 1931.
34. GREENWALD, J. biol. Chem. **21**, 61, 1915.
35. —— J. biol. Chem. **34**, 97, 1918.
36. GRÜN a. HALDEN, Analyse d. Fette u. Wachse, Bd. II, Berlin 1929.
37. GSTIRNER, in W. Peyer, Analytische Praxis, Stuttgart 1934.
38. HEINSEN, s. LEHNARTZ, Einführung in die chemische Physiologie, p. 326, Berlin 1937.
39. HINSBERG, Medizinisch-chemische Bestimmungsmethoden Tl. I, p. 47, Berlin 1935.
40. HIRAI, Bioch. Zeitschr. **114**, 71, 1921.

41. HOLTZ, Zeitschr. f. physiol. Chem. **250**, 87, 1937.
42. —— Zeitschr. f. physiol. Chem. **251**, 226, 1938.
43. —— Naturw. **25**, 556; 589, 1937.
44. JACOBS a. CRAIG, J. biol. Chem. **104**, 547, 1934.
45. —— J. biol. Chem. **122**, 419, 1938.
46. —— J. Am. Chem. Soc. **60**, 1701, 1938.
47. KARRER, Helv. Chim. Acta **11**, 1062, 1928.
48. —— Helv. Chim. Acta **13**, 1073, 1930.
49. KARRER, SALOMON, SCHÔPP a. SCHLITTER, Helv. Chim. Acta **17**, 1165, 1934.
50. KARRER, SCHÖPP a. BENZ, Helv. Chim. Acta **18**, 426, 1935.
51. KENDALL, Mc. KENZIE a. MASSON, J. biol. Chem. **84**, 657, 1929.
52. KERMACK, PERKIN a. ROBINSON, J. Chem. Soc. **119**, 1602, 1921.
53. —— J. Chem. Soc. **121**, 1872, 1922.
54. KLEIN a. LINSER, Planta Bd. **20**, Heft III, 470, 1933.
55. —— Zeitschr. f. physiol. Chem. **209**, 1932.
56. KNOOP, Zeitschr. f. physiol. Chem. **67**, 489, 1910.
57. KNOOP a. OESTERLIN, Zeitschr. f. physiol. Chem. **148**, 294, 1925.
58. —— Zeitschr. f. physiol. Chem. **170**, 186, 1927.
59. KOLTHOFF, Säure-Basen-Indicatoren p. 118, Berlin 1932.
60. KONDO, Bioch. Zeitschr. **38**, 407, 1911.
61. KOSTYTSCHEW, Lehrb. d. Pflanzenphysiologie Bd. I, Berlin 1926.
62. KOTAKE, Zeitschr. f. physiol. Chem. **195**, 139, 1931.
63. KREBS, Zeitschr. f. physiol. Chem. **217**, 191, 1933.
64. —— Zeitschr. f. physiol. Chem. **218**, 157, 1933.
65. KREBS a. JOHNSON, Bioch. Journ. **31**, 645, 1937.
66. —— Bioch. Journ. **31**, 661, 1937.
67. KUHN, WENDT a. WESTPHAL, Ber. d. d. chem. Ges. **72**, 310, 1939.
68. MACH a. LEDERLE, Landw. Vers. St. **98**, 117, 1921.
69. —— Landw. Vers. St. **104**, 226, 1926.
70. MATSUOKA, TAKEMURA a. YOSHIMATSU, Zeitschr. f. physiol. Chem. **143**, 199, 1925.
71. MOTHES, Planta **1**, 472, 1926.
72. —— Planta **12**, 686, 1931.
73. NANNINGA, Med. 's Lands Plantentuin XLVI, 1901.
74. NEUBAUER a. FROMHERZ, Arch. f. klin. Med. **95**, 211, 1909.
75. —— Zeitschr. f. physiol. Chem. **70**, 326, 1911.
76. PALLADIN, Ber. d. botan. Ges. **7**, 126, 1889.
77. PARNAS a. KLISIECKI, Biochem. Zeitschr. **152**, 1, 1924.
78. —— Biochem. Zeitschr. **173**, 226, 1926.
79. PARNAS a. MOZOLOWSKI, Bioch. Zeitschr. **184**, 404, 1927.
80. PASTEUR, Ann. d. chim. et d. physique **31**, 70, 1851.
81. —— Ann. d. chim. et d. physique **38**, 457, 1858.
82. PETERS a. v. SLIJKE, Quantitative clinical chemistry, Vol. II, p. 63, Baltimore 1932.
83. PFEFFER, Jahrb. f. wiss. Botanik **8**, 530, 1872.
84. PICTET a. SPENGLER, Ber. d. d. chem. Ges. **44**, 2030, 1911.
85. PICTET a. GAMS, Ber. d. d. chem. Ges. **44**, 2036, 1911.
86. v. PRZYLECKI, Ergebn. d. Enzymforschung Bd. IV, 1935.
87. —— Biochem. Zeitschr. **271**, 173, 1934.
88. —— Kolloid. Z. **79**, 129, 1937.
89. —— Kolloid. Z. **83**, 51, 1938.
90. v. PRZYLECKI, GIEDROYÉ a. SYM, Biochem. Jl. **22**, 811, 1928.
91. QUASTEL a. WOOLF, Biochem. Jl. **20**, 545, 1926.

92. RAISTRICK, Bioch. Jl. **11**, 71, 1917.
93. ROBINSON, s. P. KARRER, Lehrbuch d. org. Chem., Leipzig 1936.
94. ROMBURGH a. BARGER, J. Chem. Soc. **99**, 2068, 1911.
95. SABALITSCHKA a. ZAHER, Zeitschr. f. angew. Ch. **37**, 299, 1924.
96. SABALITSCHKA a. JUNGERMANN, Bioch. Zeitschr. **163**, 443, 1925.
97. —— Bioch. Zeitschr. **164**, 279, 1925.
98. —— Pharm. Zentralhalle **66**, 146; 475, 1925.
99. SCHMIDT, Zeitschr. f. physiol. Chem. **179**, 243, 1929.
100. SCHULZE, Zeitschr. f. physiol. Chem. **24**, 18, 1898.
101. SCHULZE a. FLECHSIG, Landw. Vers. Stat. **137**, 1885.
102. SCHULZE a. TRIER, Ber. d.d. chem. Ges. **42**, 4654, 1909.
103. —— Zeitschr. f. physiol. Chem. **69**, 326, 1910.
104. SCHULZE a. UMLAUF, Landw. Vers. Stat. **18**, 1, 1875.
105. —— Landwirtschaftl. Jahrb. **5**, 821, 1876.
106. SCHULZE Trg., Planta **16**, 116, 1932.
107. SMITH a. TIMMIS, J. chem. Soc. 1440, 1936.
108. SPÄTH, Monatsh. f. Chem. **40**, 129, 1929.
109. SPÄTH a. GANGL, Monatsh. f. Chem. **44**, 103, 1923.
110. SPÄTH a. LEDERER, Ber. d. d. chem. Ges. **63**, 120, 1930.
111. SPÄTH a. RÖDER, Monatsh. f. Chem. **43**, 93, 1922.
112. STOLL, Journ. d. Pharm. et d. Chim. **28**, 226, 1938.
113. STOLL a. HOFMANN, Zeitschr. f. physiol. Chem. **251**, 155, 1938.
114. THOMS a. MICHAELIS, Jahrb. d. Ver. f. angew. Botanik **16**, 38, 1918.
115. TRIER, Zeitschr. f. physiol. Chem. **67**, 324, 1910.
116. —— Die Alkaloide, Berlin 1931.
117. VIRTANEN a. TARNANEN. Bioch. Zeitschr. **250**, 193, 1932.
118. v. WAESBERGHE, Rec. d. Trav. bot. néerl. Vol. XXXIV, 623, 1937.
119. WALDSCHMIDT-LEITZ, PURR a. BALLS, Naturwiss. **18**, 644, 1930.
120. WALDSCHMIDT-LEITZ a. PURR, Zeitschr. f. physiol. Chem. **198**, 260, 1931.
121. WARBURG a. CHRISTIAN, Biochem. Zeitschr. **298**, 150, 1938.
122. WARBURG a. NEGELEIN, Biochem. Zeitschr. **113**, 257, 1921.
123. WEEVERS, Rec. d. Trav. bot. néerl. Vol. XXX, 336, 1932/33.
124. —— Archives Néerl. d. Sciences exactes et naturelles IIIB, tome V, p. 111, 1930.
125. WIELAND a. BERGEL, A. **439**, 196, 1924.
126. WINTERFELD a. HOFFMANN, Arch. Pharm. **275**, 5; 65; 526; 630; 1937.