Nitrogen Metabolism of Pea Seedling

J.W. Barnett

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BARNETT (J.W.)

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PLAN OF THESIS

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I. Introduction.

The Nitrogen metabolism of plants has been the subject of much experimental investigation, since the time of Schulze, whose extensive researches in this field laid the basis for further work, but, partly owing to the many changes which have taken place in the experimental methods used for determining nitrogen distribution, a great deal of this experimental work has been done under such different conditions that comparison with similar results of other workers is hardly possible. Moreover, many of the methods used have not been standardized sufficiently rigidly to admit of their being repeated by other investigators. A determination of the nitrogen distribution in the Lupin seedling at various stages of growth had been made in Westfield College laboratory (see McKie, 1930) and it was thought that a similar quantitative study of the nitrogen metabolism of the pea seedling, carried out under parallel conditions, as far as possible, might yield interesting results, which at the same time might be useful in conjunction with the results of other workers in the same field.

The pea was chosen for this work, because in

the first place, it was closely allied, genetically, to the Lupin, both belonging to the Leguminosae, and because, further, the Leguminosae are known to be rather exceptional with regard to their nitrogen metabolism, accumulating, in general, a larger proportion of asparagine during protein hydrolysis. No recent data for the Nitrogen metabolism of the green pea are available; it was hoped, therefore, that such a study would serve to indicate whether the pea resembled other members of the Leguminosae in this respect.

A similar investigation of the nitrogen metabolism of the etiolated pea seedling was made in 1916, by Sure and Tottingham, who studied the nitrogen distribution in the cotyledon and in the shoot, separately. In the present research two lots of etiolated pea seedlings were also grown and analysed, under conditions exactly parallel to those prevailing for the green seedlings, in order to determine at what stage the nitrogen metabolism of the plant was affected by the presence of light. These results have been compared as far as it was possible, with Sure and Tottingham's data for etiolated pea seedlings.

Since it was known that the present piece of
research could not extend over more than one year,
only the simple problem of determining nitrogen
distribution at various stages of growth, was
attempted, but several other problems for further
study, which suggested themselves during the
investigation, have been mentioned at the end of
this dissertation.

II. Treatment of Seed.
1) Germination.

Garden peas (Pisum sativum), Little gem
variety, were grown in large earthenware pots
(about 6-12 in a pot) in a warm greenhouse from
January - April 1935.   The seeds were planted
about 1½cm. deep in a good potting mixture, containing
much leaf mould and a little sand, the same mixture
being used throughout.   In earlier experiments,
the peas had been soaked for 24 hours before planting,
but it was found that this treatment tended to cause
bacterial decomposition of the seeds, and was
therefore discontinued in later sowings.   The
seedlings were freely watered daily.

Shoots usually appeared above the ground after

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TABLE 1.

Comparison of Results of N Distribution in Duplicate Extracts.

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; % of)tal]	N estimated as:		Insol. N.	Coag protein N.	Proteose N.	Total protein N.	Amm- onia N.	Amide N.	Amino N.	Total N estim- ated.
.ginal)ed	Lot I.	A	11.48	75.22	9.68	96.38	0.13	1.48	2.18	100.17
	" "	B	10.37	77.22	8.32	95.91	1.13	1.48	2.40	99.92
days)wth)	Lot II.	A	72.97	15.95	6.65	95.57	0.47	1.92	4.29	102.25
	" "	B	80.56	13.33	0.91	94.8	0.55	1.43	4.38	101.16

about a week.   Tendrils occurred first on the
22nd. day, while nodules did not appear on the
roots until the 32nd. day.

The temperature of the greenhouse varied from
10°-20°C. (50°-67°F.).

2) Harvesting.

About 50 seedling's were harvested from one
batch.   Except in two cases, (Lots I and VII)
all the seedlings were used for a single extraction;
but in these two sets, duplicate determinations of
nitrogen distribution were made on parallel
extracts in order to test the accuracy of the
procedure.   As seen in Table I, the results agreed
reasonably well and as the time available for the
present research was limited, no subsequent
duplicate extractions were made.

The earth was gently loosened from the roots
and the seedlings immediately washed carefully in
cold, running water and cleaned with the aid of a
small camel hair brush.   They were gently dried
between blotting paper, before being measured for
total length of root and shoot.   Their general
appearance, (number of leaves, appearance of

tendrils, nodules etc.,) was noted, and any obvious abnormalities were discarded.

In order to prevent subsequent enzymic hydrolysis, the seedlings, wrapped in a muslin cloth, were immersed in boiling water for 1 minute. This treatment should deactivate any enzymes present. In case any soluble nitrogenous compounds should have been extracted from the seed during this treatment, the boiling water residue was evaporated to small bulk on a water bath, treated with ten times its volume of alcohol, and the white, flocculent precipitate filtered off on a Jena glass crucible, washed .with spirit, dried and weighed. The filtrate was evaporated to small bulk and analyzed as a whole for total N, with a negative result. Only in lot Vi was the precipitate, which weighed less than 0.1gm. sufficient in amount to allow of further analyses. Quantitative analysis of this residue for amide and amino acid nitrogen again yielded a negative result. Unfortunately, after these analyses had been performed, there was not sufficient of the precipitate left to carry out a determination of total N.

The seedlings were dried as completely as possible between blotting paper and transferred to 97% alcohol in a stoppered bottle until required for analysis.

3) Etiolated Seedlings.

Seedlings were also grown in the dark under conditions (of temperature, soil and watering) similar to those employed for growing the normal seedlings. Darkness was secured by covering the roof and sides of the greenhouse with black, polished paper, thick black curtains being hung round the bench which was used for this series of seedlings. The seeds were watered at night, the curtains being pulled back to allow access of fresh air to the soil. The whole process of harvesting, washing and of subsequent operations was carried out in the light and the seedlings were finally bottled in spirit, as before. The seedlings in most cases appeared almost completely etiolated, though their stems were occasionally pale yellow in colour.

4) Residue in Spirit.

The seedlings were removed from spirit and any residue in the alcohol jar was analyzed for amide N (since asparagine, being insoluble in alcohol, might

appear here. Cf. /McKie (1931)). In Lot IV this
residue, when dry, weighed 0.025gm. and was found
to contain no amide N. In no other set was there
sufficient insoluble residue for analysis. Since
this residue under the microscope, appeared amorphous,
it was considered that it was probably some trace of
protein material, insoluble in alcohol, and since
its amount was small compared with the total protein
present, it was neglected. In lot VIII, there was
no solid residue, but much chlorophyll had been
extracts from the leaves. The alcohol solution
was evaporated to small bulk and analyzed as a
whole for total N, which was found to be about 2.5%
of the total N, (although this N had not, of course,
been included in the estimate of total N of the meal).
In no other case were the seedlings left for a long
enough time in alcohol for much chlorophyll to be
extracted.

5) Drying of Seed.

Various recommendations have been made with
regard to the optimum conditions for drying, when
least change in the nitrogen distribution takes place.

TABLE II.

Osborne and Wakeman: 1920. J. Biol. Chem. (42) p.

Comparison of N distribution in fresh and dried leaves

of spinach.

	% extracted as % of total nitrogen.	
	Fresh leaves	Dry leaves.
Total protein	67%	71%
Coagulable protein	3.8	5.9
Colloidal protein	58.7	53.8
Proteose	4.8	11.3
Non-protein substances	25.9	26.4
Soluble in ether and alcohol	2.3	2.3
	95.5	99.7

Brief reference will be made to the contributions
of recent workers towards the solution of this
problem:

During an investigation on the protein nitrogen
of spinach leaves, Osborne and Wakeman (1920)
compared the distribution of nitrogen in fresh
leaves and in leaves dried in a current of air at
60°. Some of their results are reproduced in
Table II. They concluded that "Spinach leaves
dried at a low temperature .....yield results so
similar to those obtained with the fresh green leaf
that evidently the constituents of the cells are
altered to only a slight degree by drying".
(However, as Link & Schulze pointed out later (1924)
(see below) this result may be due rather to
compensated error, than to absence of change.)

Chibnall (1922), on the other hand, concluded
that drying had a pronounced effect on the
nitrogenous distribution in the cells, and that the
results of many previous workers on the amounts of
non-protein N in plants, were seriously invalidated
by the uncertain methods of drying to which their
material was subjected. He gives data in which

TABLE III.

as of al °	Total protein N	Total water sol.N	NH3 N.	Aspar. amide N.	Nitric N.	Humin N.	Basic N.	Mono amino N	Other N.
sh leaves	74.8	21.6	0.4	0.5	2.7	0.99	2.0	7.7	2.7
ves dried air curr- at 40° 50hrs.	61.1	33.7	5.3	1.1	1.6	0.8	2.8	13.2	4.2

TABLE IV.

N as % dry weight (Sugar beet seedlings)

	Green tissue	Room temp.	45°	65°	90°
Protein-proteose free N.	0.7	1.0	0.8	0.75	0.5
Proteose	0.19	0.22	0.1	0.1	0.15
Amino N	0.25	0.35	0.32	0.32	0.28

the N distribution in leaves of the runner bean
is estimated both for fresh and for dried leaves.
One series is reproduced in Table III.   Chibnall
therefore prefers to express his results in terms
of total fresh weight of the leaves, and does not
dry his material before use.   The procedure does
eliminate the uncertain error due to change while
drying, but it means that the fresh material must
be worked up for extraction immediately after
harvesting.   Such a method was not practicable in
the present instance.

Link and Schulz (1924) also compared the
quantitative distribution of N compounds in fresh
and dried material.   Some of their results, working
at different temperatures, are given in Table IV.
They concluded that, while drying at a high
temperature (80^{0} and 95^{0}) caused coagulation of
soluble N constituents in all tissues, slow drying
at a low temperature (32^{0} and 45^{0}) allowed
proteolytic decomposition to take place.   At 65^{0}
however, the only significant change was a decrease
in the water soluble N, due to coagulation, and   .
they therefore recommend this temperature as the
optimum temperature for drying.   They also point

out that when, as in some cases, the apparent
change in distribution is small, it is nevertheless
quite possible that proteolytic decomposition has
been balanced by coagulation of soluble compounds,
the net result being only a slight alteration in
distribution.

In the present research, the temperature
recommended by Link and Schulz was adopted, viz.
65°C. (The treatment with boiling water would
probably have killed any enzymes present, but it
was thought advisable to take all the precautions
possible for preventing proteolysis). The
seedlings were dried for 3 hours at 65°, then ground
to a fine powder in a mill, and the meal spread out
in a thin layer on filter paper and dried in an
incubator at 65° for 48 hours. During the drying
of the etiolated seedlings, a crystalline substance
appeared on the outside of the stems and seeds;
this substance, probably asparagine, was not
analysed separately, but was ground up with the
rest of the seedlings and the whole analyzed. No
such crystalline compound appeared during the
drying of the green seedlings.

6) **Extraction of Seed Meal.**

In order to determine the nitrogen distribution in the meal, it is essential that all the soluble constituents shall be separated from the insoluble material. Two methods have been described for extracting the soluble substances.

Osborne and Wakeman's method (1920) consists essentially of grinding the plant material in a "Mixtamil" mill, then mixing the pulp so obtained with water, and returning twice to the mill. The cellular matter is removed by centrifuging, then the protein matter flocculated by addition of alcohol.

Chibnall and Schryver's method (1921) differs from the above method in that the leaves are not ground to powder but are minced, treated with ether water, and then pressed out through muslin yielding a green colloidal liquid, from which the protein is removed by warming and filtering the coagulum. Chibnall later (1922) improved upon this method by grinding up the minced leaves in a mechanical pestle and mortar, then filtering through lawn. The ether-water method was later discarded as no advantage was derived from it. By this

method, Chibnall found that only 11% of the total
N remained behind in the insoluble residue.

In the present research, it was not possible
to use Osborne's method as only a small centrifuge
was available, and this would have entailed dealing
with the plant material in successive small
quantities, thus increasing the experimental error,
so a modification of Chibnall's method was used.
The method adopted was as follows:

The dried meal, about 6-7gms. was weighed out
into a mortar, ground up carefully, by hand, with
50cc. distilled water for about 15 minutes, then
filtered through fine muslin (12" sq. having an
internal mesh of about 0.017cm.) and spread out
over a large fluted funnel. The muslin had been
previously washed, dried at 105° and weighed,
together with the Petrie dish in which the insoluble
residue was subsequently to be weighed. A fresh
piece of muslin but from the same strip, was used
in each set of experiments. The liquid was
expressed from the muslin by gently twisting the
ends of the muslin, the lower part being held firm
in the funnel by means of a rubber tipped glass rod.
The residue on the muslin was transferred to the

mortar with the aid of the glass rod and ground
up again with 30cc. water, for about 5 minutes, the
process of grinding and filtering being repeated,
using 6 lots of water in all.   After the final
extraction, the pestle and mortar were rinsed out
with a few ccs. of distilled water, the washings
filtered through the muslin, and the residue pressed
as dry as possible, then transferred to a weighed
Petrie dish, and dried at 105°.

In the experiments on the original pea meal,a
temperature of 110° was used for drying, but owing
to the difficulty of absorption of moisture before
weighing, it was eventually decided to dry the
material at 105°.   (Use of a filter press for the
last stage of pressing out the residue had been tried,
but it was found that a great deal of cellular
matter was expressed in this way, so its use was
discontinued.)

In the earlier experiments on the original
pea meal, parallel extractions were tried, in one
case using 6 lots of water, and in the other, only
4 lots.   In the latter case, 11.5% of the total N
of the meal remained in the insoluble fraction,

while in the former, only 10.4% was left.   As it

was desirable to extract as much of the nitrogenous

material as possible from the insoluble residue,

(since the chemical character of the latter was not

definitely known) the process involving 6 water

extractions was adopted.   Although in these

preliminary experiments only 10.5% of the total N

was left in the insoluble fraction, it was found that

after the seedlings had been soaked for several days

in alcohol, a larger proportion of the total N

remained in the insoluble part, after the same

extraction treatment.   Unfortunately, no

determination of the N distribution was made on the

original seeds after having been soaked in alcohol.

Since the pea contains the proteins legumin

and vicilin which are insoluble in water but soluble

in solutions of sodium chloride, it was thought that

extraction with 10% salt solution might remove more

of the nitrogenous material from the insoluble

matter, than did the water extract method.   30cc.

10% NaCl were therefore added to the dried meal, the

mixture ground up and allowed to stand for 2 hours

in order to dissolve the legumin and vicilin.   After

TABLE V.

	Sodium chloride extraction of seed meal.		Water extraction of seed meal.	
	N as % dry wt.	N as % total N.	N as % dry wt.	N as % total N.
soluble protein N	0.55	14.6	0.45	11.4
tal protein N (by difference)	3.75	93.6	3.85	96.5
tal water soluble N	0.25	6.4	0.14	3.5
ino N.	0.188	3.7	0.08	2.1
ide N.	0.07	1.5	0.06	1.5
monia N.	–	–	0.005	0.1

this, it was filtered through muslin and the
extraction repeated (without further standing) as
before.   It was found, however, that this treatment
allowed 14.5% of the total nitrogen to remain behind
in the insoluble fraction so that even less of the
soluble material had been removed by this treatment.
Further, the amino N shewed a decided increase,
(from 2.2% - 3.7% total nitrogen) suggesting that
the prolonged standing necessary for salt extractions
caused some autolysis to take place.   Some of the
data relating to this extraction are given in Table V.
In addition, the subsequent coagulation of water
soluble protein by heat, was rendered more difficult
in the presence of sodium chloride and filtration of
the coagulated protein proved more laborious than
usual.   This method was therefore abandoned, in
favour of the water extraction process.

7) Insoluble Residue.

After being dried in an electric oven at 105°
for 48 hours, the residue, together with the muslin
and the Petrie dish, was transferred to a dessicator,
and weighed after 40 minutes.   It was found
impossible to obtain a constant weight unless it was
cooled always for the same length of time.   When

the weight of the residue had thus been found,
the latter was removed as completely as possible
from the muslin, ground up finely in a mortar,
spread out in a thin layer or filter paper and dried
again at 105°. for at least 24 hours, to drive off
any moisture that might have been acquired during
grinding. The powder was transferred to a weighing
bottle, cooled in a dessicator for $\frac{1}{2}$ hour, then
weighed. About 0.5gm. of the powder was introduced
into the Kjeldahl flask, the weighing bottle
reweighed approximately, then reheated for $\frac{1}{2}$ hour at
105°. cooled for $\frac{1}{2}$ hour and reweighed. The powder
was analyzed for total N, about 3 determinations
usually being made.

With regard to the nature of the insoluble
residue, it has been assumed that no non-protein N
remains in the insoluble fraction, but that all the
N in the latter is of protein origin; this seems
probable, in view of the rather thorough method of
extraction, but its justifiability is by no means
proved. It is a useful assumption, however, since
by including the "insoluble N" fraction with the
other two protein fractions, in the value for "total

protein N" a more representative value is thereby
obtained.

8) <u>Coagulation and Filtration of Protein.</u> The
next step was to remove from solution any water
soluble or other proteins which had been extracted
from the insoluble residue. Before this was
attempted, Osborne's work on the proteins present
in the pea seed was consulted in order to find out
the properties of such proteins as were likely to
occur.

According to Osborne (1895, 1896, 1907, 1908)
the proteins in the pea are:

(i)   Legumelin, an albumen, coagulable by heat,

(ii)   Vicelin, a globulin, insoluble in water,

(iii)   Legumin, a globulin, soluble in water
        containing a trace of acid, not completely
        coagulated by heat, nor by prolonged boiling.
        It is partially soluble in aqueous extracts,
        owing to the natural acidity of the pea
        plant, although it is insoluble in pure
        water.   According to Osborne (1896) the
        only reagents which bring about complete
        precipitation are Na_2SO_4 (saturation at 34°)
        and ammonium sulphate.

TABLE VI.

Methods of Filtering Coagulated Protein.

	Used by	Results with present extract.
Ordinary filter paper	Bure & Tottingham (1916)	Clogged almost immediately.
Abundum crucible RA 760		Too coarse; protein came through.
RA 84		V. satisfactory. Protein easily washed with boiling water. Clear filtrate. But could not remove protein without traces of crucible as well.
Jena glass crucible.	Robinson (1929) McKie (1930) 291.	Too coarse. Filtrate cloudy.
	293	Clogged at once.
	292	Rapid at first. Clear filtrate. Clogged after a time.
Gooch crucible		Very slow in filtering. Cannot separate protein from asbestos.
Soxhlet extractor thimble.	Chibnall (1922)	Rapid filtration. Easily washed. Filtrate not clear. Difficult to separate small quantity of precipitate from thimble.
Whatman filter paper.		Filtered very slowly, but with slight pressure, gradually got all liquid through. Clear filtrate.

Owing to the presence of legumin, it was therefore to be expected that, in order to precipitate all the water soluble protein from the extract, some method other than, (or in addition to,) coagulation by heat, would be needed. Before this could be quantitatively investigated however, it was necessary to find some satisfactory method for filtering the precipitated protein. Accordingly, the extract from the original seed meal was heated gradually to the boiling point and the filtration of the coagulum attempted various methods, as tabulated in Table VI.

The filtration of the coagulated protein was the most substantial difficulty encountered in this piece of research. The method used by Sure and Tottingham during an investigation of N compounds in pea seedlings of filtration through an ordinary filter paper, had to be abandoned, as the filter paper became clogged soon after the beginning of the filtration. The method adopted by Chibnall, of filtration through a Soxhlet extractor thimble, which provides a very thorough method of washing the protein, was not possible here, as the protein came through the pores of the thimble. Glass crucibles,

used by Robinson (1929) and McKie (1930) also were found unsuitable. Abundum crucibles proved the most satisfactory, but it was found that in removing the filtered protein from the crucible some powder from the walls tended to be scraped off with the protein, so this method also had to be given up.

Use of a centrifuge was tried. The centrifuge available, was, however, a small one, with tubes about 1.5cm. in diameter, and 10ccm.long which was worked by hand, making about 1,400r.p.m.

This process was, of necessity, very laborious and was unsatisfactory since such a long time had to elapse between coagulation and having the extract in a stoppered bottle ready for use. Further, at the rate of rotation possible by hand, it was not possible to secure very thorough settling of the protein. Moreover, after the protein had been dried (in the centrifuge tubes) it had to be either removed from them for weighing, which was impossible on a quantitative scale, or else all the centrifuge tubes had to be weighed with their contents, a cumbersome process, which was not likely to give accurate results. The use of a centrifuge was therefore discontinued.

The method finally adopted was filtration

TABLE VII.

	Method of precipitation.	Titration.	Gm.N2left in 500cc.	Residual N as % total N (Tot.W.sol.N=24.6)	N removed as % total N.
1.	2.5% CCl_3COOH (10cc A)	27.3cc.	0.069gm.	13.4	11.2
2.	Saturated w. $MgSO_4$ at 16^0	27.9cc.	0.069gm.	13.4	11.2
3.	Saturated w. $ZuSO_4$	27.25cc.	0.147gm.	28.6	-
4.	10% + 5 drops N/10 HCl	27.6cc.	0.104gm.	20.3	4.3
5.	10% NaCl + 5 drops n/10 H Ac.	27.5cc.	0.115gm.	22.4	2.2
6.	Saturated w. N a$_2$SO$_4$at 33^0	27.9cc.	0.069gm.	13.4	11.2
7.	Saturated w. Na$_2$SO$_4$ at 35^0 after adding NaCl to 10%	23.0cc.	0.04gm.	7.8	6.8
8.	Saturated w. Na$_2$SO$_4$ at 35^0 after adding NaCl to 10% & neutralising pH 7.	23.1cc.	0.03gm.	5.8	18.8

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TABLE VII (continued)

9.	Saturated w. Na_2SO_4 after NaCl to 10% & neutral to litmus (pH 7)	27.7cc.	0.08gm.	15.6	9.0
10.	Saturated w. Na_2SO_4 at 33° after bringing to pH4.5 & adding NaCl to 10%	28.05cc.	0.03gm.	6.4	18.2
11.	Saturated w. Na_2SO_4 at 33° after bringing to pH 4.5 (no NaCl)	27.95cc.	0.047gm.	9.1	15.5
12.	Repeated 10 standing 5 hrs.	28.0cc.	0.36gm.	7.01	17.5
13.	Repeated IX standing 5 hrs.	28.25cc.	0.026gm.	5.1	19.5

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through a toughened filter paper (Whatman No.50 11cm)
on a Buchner funnel, under slight pressure. (The
filter paper was washed, dried at 105° and weighed in
a Petrie dish before use). The filtration was in
all cases slow, sometimes extending over 12 hours,
(the time for filtration varied considerably from one
extract to another) but the filtrate was usually
clear. This method was, on the whole, more
satisfactory and reliable than any other method
tried. On one or two occasions, the filter paper
clogged so badly that the rest of the solution was
filtered through another, similar, weighed filter
paper, while the protein on the original filter
paper was washed thoroughly with hot water, and the
washings filtered through the second filter paper.

9) Precipitation of Protein from Aqueous filtrate.

It was next necessary to study the various
methods of precipitating protein from solution in
order to see which one removed the maximum amount
of protein from this extract.

Results are shown in Table Vl.

Precipitation of protein by 2.5% trichloracetic
acid was tried, since this reagent had been used
with success very recently by McCulla (1934) for

removing protein from extracts of pea and wheat
seedlings. This again was not entirely
satisfactory as only 11% nitrogen was removed by
this treatment, out of a possible 20%. Saturation
with Mg SO_4 and with $NaSO_4$ at 33^0 were found to
remove about the same amount of protein as the
treatment with trichloracetic acid, while saturation
with $ZnSO_4$ removed rather less. Similarly, addition
of solid sodium chloride to a concentration of 10%.
followed by a few drops of HCl, or H̲A̲c was also less
satisfactory than the former reagents. However,
according to Osborne, saturation with $NaSO_4$ at 34^0
in the presence of 10% NaCl , throws out all the
legumin except a trace; this method was therefore
tried, and found to remove more protein than any
method yet tested. Further, if the pH were adjusted
either to 4.5 or to 7, a still lower N content in
the filtrate was obtained. Allowing the mixture
to stand for 5 hours after saturation with Na_2SO_4 in
the presence of NaCl did not seem to affect the
amount of protein precipitated. The above method
involving the use of sodium sulphate, was therefore
adopted, for precipitating protein.

TABLE VIII.

Comparison of Results of Van Slyke Amino Nitrogen
Determination before and after removing protein.

	Vol. of N 2	Temp.	Pres.	mgm N= 1cc. gas	Wt. of N in 250cc. extract	This wt. after correcting for blank.	Amino N as dry wt.	% of Total nitrogen
thod I.								
ank	0.11	19°	755mm.	.5683	0.0521gm.			
cc.	0.93	16.5°	756mm.	.5737	0.4462gm.	0.0374gm.	0.418%	10.4%
cc.	0.88cc	17.5°	756mm.	.5730	0.4195gm.	0.0368gm.	0.392%	9.8%
thod II.								
cc.	0.56cc	16°	755mm.	.5747	.0499gm.	.0398gm.	0.424%	10.58%
cc.	0.58cc	16°	755mm.	.5747	.0499gm.	.0414gm.	0.441%	11.0%
cc.	0.58cc	16°	755mm.	.5747	.0499gm.	.0414gm.	0.441%	11.0%

The presence of so much sodium sulphate in the filtrate, (10cc. saturated at 33° contains about 5gm. anhydrous Na_2SO_4) caused serious bumping during the subsequent Kjeldahl distillations, and its presence was inconvenient also for the Van Slyke determination of amino acid. However, some data were available, which indicated that the presence of protein in the amino acid solution, did not raise the value of the amino N as found by the Van Slyke method.

During earlier experiments on the original seed meal extract from which protein had not yet been removed by coagulation some determination of amino acid were made by the Van Slyke method (method 1, without removal of protein, Table VIII) it was found however, that this protein was coagulated during the treatment in the reaction vessel, and was difficult to remove afterwards. 10cc. of the extract was therefore taken, treated with 90cc. alcohol, shaken for 5 minutes then allowed to stand for 36 hours. The precipitated protein was coagulated by warming in a water bath at 100°, and was then filtered through an crucible. The filtrate was evaporated under reduced pressure (about $50^{o}C$) to a bulk of

10cc. then washed out into a burette and made
up to a known volume.   3cc. of this was used
for Van Slyke determinations of amino N (method
II, Table Vlll).   From the last column, it is
seen that the amino N estimated has not decreased
after removal of a large part of the protein (the
fact that the amino N estimated after removal of
protein was rather higher than before filtration,
is probably due to the inhibiting effect of the
coagulation in the reaction vessel, or the thoroughness
of the shaking up of the solution).   It was
therefore concluded that estimation of amino N by
the Van Slyke method in the presence of a small
quantity of protein and proteose N would be
permissible since the presence of protein did not
seem to raise the amino N value.

The determination of water soluble N was
therefore carried out on the filtrate after
filtering off coagulated protein, the further
precipitation with sodium sulphate being carried
out on a small portion of the filtrate only.

The pH of the extract was adjusted to about
4.5 by addition of a few drops of acetic acid.

(neutralisation to pH 7 was not used as it was
thought that loss of ammonia might occur during the
boiling of the neutral solution).   The extract was
then heated gently to boiling, allowed to boil for
one hour, filtered hot, as already described, the
precipitate washed many times with hot water, then
transferred with a filter paper to a weighed dish,
and dried at 105° to constant weight.   The protein
was then separated as completely as possible from
the filter paper, (care being taken to see that no
shreds of filter paper adhered to the protein)
ground to a fine powder in a mortar, returned to the
oven, dried at 105° for 48 hours, then analysed for
total N as described for the insoluble residue.

(10) Water Soluble Nitrogen.

The filtrate, with the washings, was transferred
to a graduated flask, 2cc. phenol solution added as
a preservative, and the solution made up to the mark
with distilled water.   A layer of toluene was added
to prevent contact with the air.   In every case
this procedure was followed by analysis, without
delay.   The analysis of N distribution was usually
completed in about 6 days after extraction.

This aqueous extract (A) was then analysed for

total nitrogen; 25cc. portions, to which a few cc.
of concentrated sulphuric acid had been added, were
evaporated to a volume of about 5cc. then incinerated
as usual.

In one case, namely the extract from the original
seed meal, (X2) some portion came out of the solution,
after standing for 24 hours. The amount of this
protein was determined by taking 40cc. of the well
shaken extract filtering through a Jena glass crucible,
and making up the residue to 50cc. and determining
total nitrogen in this filtrate, which was then used
for subsequent determinations of the various water
soluble nitrogen fractions, the protein removed in
this way being estimated by difference. But in no
subsequent case did sufficient protein appear on
standing as to make a second filtration necessary.

5cc. portions of extract (A) were treated with
NaCl to a concentration of 10%, then saturated with
anhydrous sodium sulphate at 33°. in an incubator,
the mixture filtered after standing for some time,
the precipitate washed three times with small
quantities of saturated sodium sulphate solution, and
the total N in the filtrate determined. Thus the N
removed by sodium sulphate precipitate was found by

difference. Since saturation with this reagent also precipitates proteose, (Masteneys and Borsook, 1917) it was impossible to separate proteose N from the protein N remaining in solution after coagulation. In tabulating the results, therefore, proteose N had been included with protein N. The fraction headed "total $\underset{\wedge}{N}$" PROTEIN in the tables of nitrogen distribution, is therefore composed of insoluble nitrogen, coagulable protein, and nitrogen of substances precipitated by saturation with sodium sulphate at 33^{0}.

The filtrate was further analysed for ammonia N, amino N, amino acid N and nitrate, by the methods to be discussed in the next section. In most cases the sum of the nitrogen estimated in all these separate fractions, did not account for the whole of the nitrogen present, as calculated from the weight of meal taken, and the percentage of nitrogen in this meal. In tabulating the full data, therefore, any deficiency between the sum of the soluble nitrogenous constituents, and the total soluble nitrogen estimated in extract A, has been added in, as "other soluble nitrogen". It may be

represent small quantities of substances such as urea, purine bases, etc., which were not estimated. On the other hand it must include the sum of the errors on all of the determinations of soluble nitrogenous constituents.

Any variation of the sum of insoluble nitrogen, coagulable nitrogen and total water soluble nitrogen, from the total nitrogen value, indicates the magnitude of the experimental error on that set.

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111. EXPERIMENTAL METHOD OF N ESTIMATION.

1. Total Nitrogen

Total nitrogen was estimated by the Kjeldahl-Gunning method in which any nitrogen present is converted into ammonium sulphate by incineration with concentrated sulphuric acid, in the presence of copper sulphate as catalyst. Quantities of the dried meal, weighing about 0.2 gm, (or 25cc. of the extract.) were incinerated with 10cc. concentrated sulphuric acid, to which about 2gms. of the incineration mixture, recommended by Robinson (1929) (10gms. K_2SO_4: 1gm. $CuSO_4$: 0.75gm. H_2SO_4) had been added. A clear green fluid was usually obtained after 20 minutes, and boiling was continued for another 5 minutes. The liquid was cooled, diluted with 20cc. water and 20cc. alcohol. After cooling again, the flask and contents were set up in the apparatus shown in diagram 1, and 50cc. of 40% caustic soda run in slowly from the tap funnel, with aeration, followed by 5cc. of 25% sodium sulphide to decompose mercury ammonium compounds. The temperature of the liquid was raised to boiling, while aeration was continued then 5cc. 2N copper sulphate was added and the ammonia which distilled over was collected in

DIAGRAM I.　　　　　KJELDAHL　NITROGEN　ESTIMATION

12.

DIAGRAM I.

C →

→ to water pump

D →

A →

B →

E →
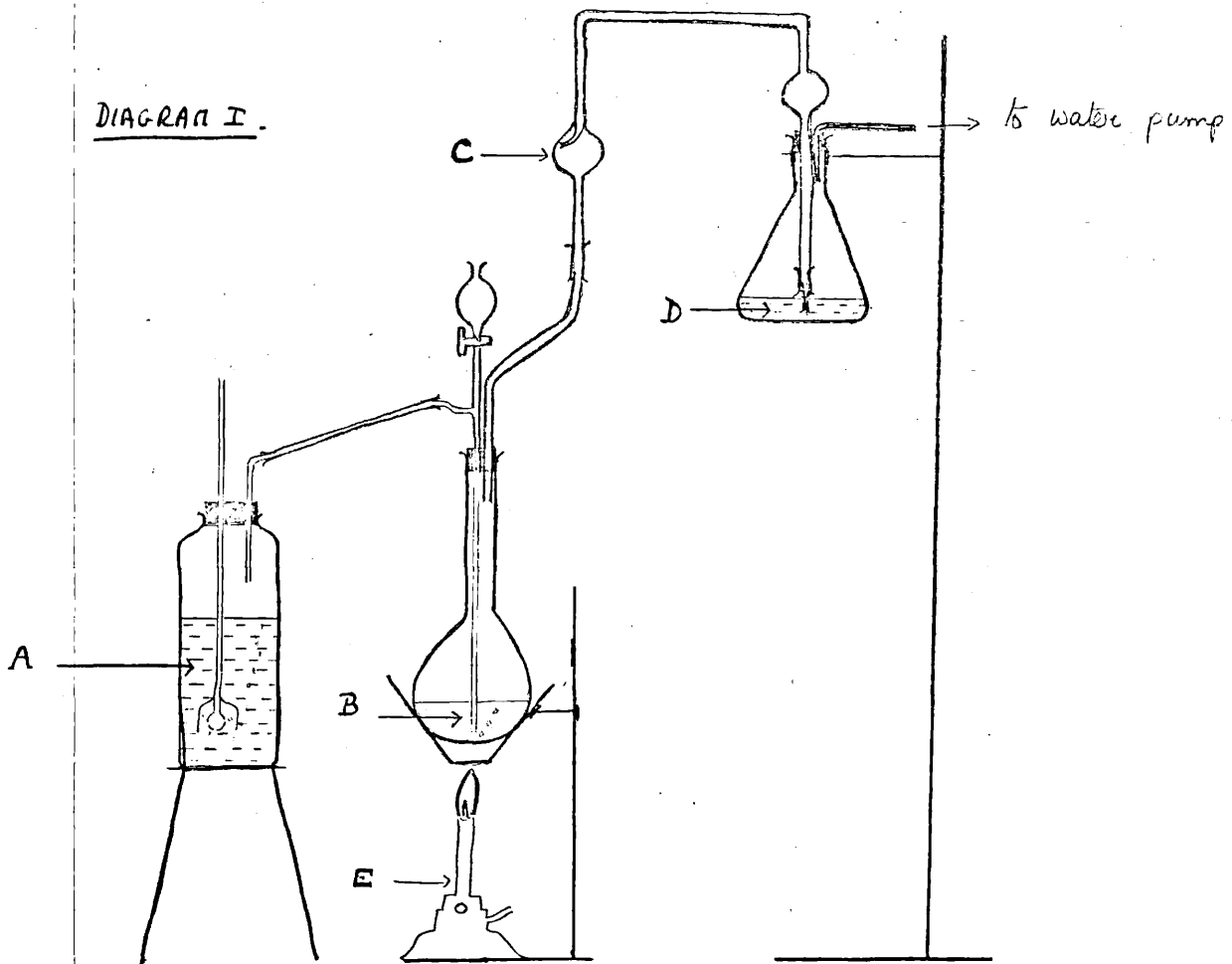
A = Dilute H_2SO_4 to remove NH_3 from aeration current.
B = Kjeldahl flask, w. drop funnel for delivering NaOH.
C = Trap to prevent any NaOH from reaching acid in)
D = Erlenmeyer flask cont. $\frac{N}{10}$ HCl (25 cc) + 5 drops methyl red.
E = micro-burner.

TABLE 1X

andardization of Kjeldahl's Method   Total N in   (Theoretical % N =
                                        Glycine.   18.66).

NaOh = 0.7716 $\frac{N}{10}$ Acid = 1.237 N.   Blank expt. 50cc. acid =
                                                        62.1cc. NaOH

Wt.of glycine taken	Titration	ccNaOH used up	Gm.N	%N in glycine	% yield
0.2010gm.	23.8cc.	33.3cc.	0.0359	17.9	96
0.2355gm.	23.5cc.	38.6cc.	0.417	17.7	94.8
0.2156gm.	27.2cc.	34.9cc.	0.0377	17.5	93.2
				mean	94.7%

Yield from Kjeldahl method   : 94.7%

$\dfrac{N}{10}$ acid in an Erlenmeyer flask, the end of the

delivery tube being detachable so that it could be

removed and washed·out. The distillation was

continued for 20 minutes, then the residue was

titrated against $\dfrac{N}{10}$ NaOH, to methyl red as indicator.

A control experiment, omitting the meal, was

carried out for each new lot of caustic soda used.

Since this technique does not estimate nitrate N,

the modified method described by Moore (1920) in

which nitrate N is converted into a nitro derivative

by heating with salicyl sulphonic acid, and this

reduced with sodium thiosulphate, before incinerating

as usual, was adopted for analysis of the meal and

aqueous extracts from lots Vll,Vll,Vlll in which

nitrate had been found to be present.

The results for the standardization of this

method are reproduced in Table lX. It is seen that

the average yield is 94.7%.

The methods of estimating the total N present

in the insoluble and coagulable protein factors, and

in the aqueous extract (A), have already been dealt

with. (cf 11/7-10).

2. Free Ammonia.

Free ammonia was estimated eventually by Folins

DIAGRAM 11.

DIAGRAM II

Dilute H_2SO_4

A

D E

B

EXTRACT

To PUMP

$\frac{N}{100}$ H_2SO_4
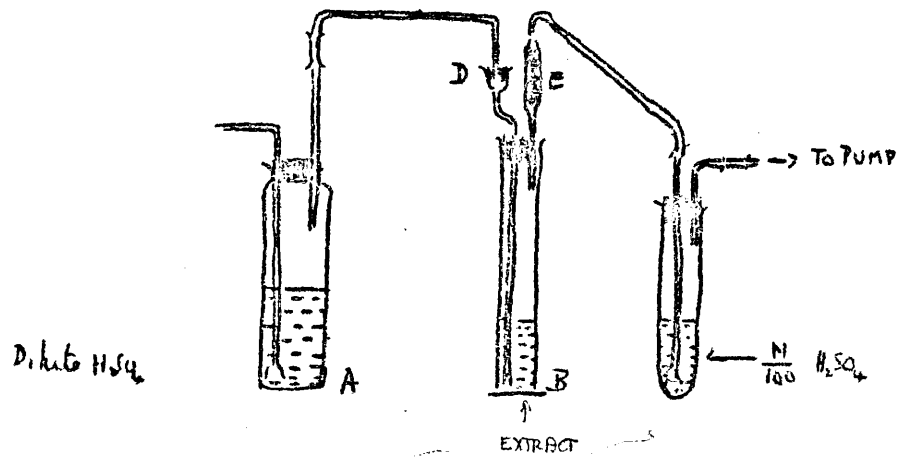
TABLE X.

% N as Ammonia yielded by various methods.

Solution of ammonium chloride made up. Contained 0.096gm.
AmCl in 100cc. Solution standardized by titrating against
standard AgNO. Found to be $1.76 \frac{N}{100}$. 5cc. solution
taken for each estimation. 25cc. $\frac{N}{acid}$ = 23.9cc.NaOH.

	Conditions.	Titration.	cc.used up.	% yield.
1.	Aerated for 1 hr. (Folin)	17.4cc.	6.5cc.	73%
2.	" " 1½hrs.(Folin)	16.5cc.	7.4cc.	83.6%
3a.	" " 1 hr. with slow aeration at first	19.7cc.	4.2cc.	47.4%
3b.	Aerated for 1 hour followed by 1 hour rapid aeration.	20.2cc.	3.7cc.	89.7%
4.	Aerated 2 hours (rapid)	15.9cc.	8cc.	90.8%
5.	" 4 hours "	15.9cc.	8cc.	90.8%
6.	In Stanford apparatus at 45° and 27mm. pressure, as described.	15.85cc.	8.05cc.	91%

aeration method, though several other methods were previously tried. The apparatus used for the Folin aeration method is shown in diagram 11. The alkali used was sodium carbonate, which was added from a large tap funnel D, instead of adding the anhydrous salt, in order to eliminate any error due to loss of ammonia during exposure to air. Various modifications were tried in order to improve the yield of ammonia. The results are shown in Table X. Since the ammonia present in the plant extracts was, at all stages of the investigation, very small, it was considered that a yield of 91% involved an error which, being generally less than 0.4% of total N, could be neglected as within the experimental error of the total determination.

Data showing ammonia and amide content at various stages are given in Table XX.

An attempt was made to improve the yield by using Stanfords apparatus, involving distillation in a vacuum at 40°, (as used by Stanford, 1921, and see also Chibnall (1922). The water pressure available in Hampstead however, was too low to give an efficient vacuum, the lowest pressure that was attainable being 27mm. (even with a Geryk pump, this

this was the lowest pressure recorded.)   Following

Stanfords technique the flask containing the

ammonical solution was submerged in a water bath

kept at a temperature of 45°; after evacuation to

27mm. the apparatus was closed, saturated sodium

carbonate solution was run in gradually and the

distillation allowed to proceed for 10 minutes in the

closed apparatus, with no possibility of any loss of

ammonia.   This was followed by aeration for $\frac{1}{2}$ hour,

still under reduced pressure.   The maximum yield

obtained, however, was still only 91%.   This method

was therefore abandcned in favour of the more

practicable Folin method.

3. Amide Nitrogen.

Amide N was estimated by Sachss's method (1873)

with the modification suggested by Vickery and Pucher

(1931), viz hydrolysis of the amide by boiling for

6 hours with 2N sulphuric acid, instead of 4% hydro-

chloric, in order to minimize the error due to

reduction of nitrates present, to ammonia, by unknown

substances in the plant.   The ammonia formed on

hydrolysis was estimated by Folin's method, as

described under free ammonia.

Standardization of the method using, pure

recrystallized asparagine, showed a yield of 94%,

TABLE XI

Percentage Amide N of Asparagin Estimated by Sachsse's Method.

Normality	cc.hydro-lysed soln. taken.	cc.acid taken.	cc.NaOH = 25cc. acid.	Titra-tion.	cc.NaOH used up.	Normal-ity (estimated)	%Yield
1. 1.12	10cc.	75cc.	37.0cc.	102.1cc.	8.9cc.	1.01	*90.6
2. 1.12	10cc.	25cc.	37.0cc.	27.7cc.	9.3cc.	1.06	94.6)
3. 1.36	25cc.	50cc.	37.0cc.	45.8cc.	28.2cc.	1.28	94.1)

* During this experiment, the stopper came out of vessel B, and remained unnoticed for some time. There was therefore some loss of ammonia.

TABLE XII.

ESTIMATION OF AMINO NITROGEN BY SORENSON'S METHOD.

Amino Acid	Wt.in 100cc.	Titration (25cc.glyc erine Sol.)-ated	Normality of solution 1.calcul	2.found	% Yield	Normality of NaOH used.
Glycine 1.	0.7310gm	30.75	0.973	0.949	97.5	.7716 N/10
2.	0.4360gm	(1) 18.35cc.				
		(11)18.3cc.	0.581	0.565	97.2	"
3.	0.7510gm	(1) 30.3cc.	1.001	0.935	93.5	"
		(11)30.3cc.				
4.	0,7762gm.	(1) 31.6cc.			93.9	"
		(11)31.4cc.	1.035	0.972		
Alanine 5.	0.5561gm.	(1) 15.4cc.	0.625	0.4775	76.20	.7802 N/10
		(11)15.2cc.				
6.	0.510gm	(1) 16.8cc.	0.5786	0.5243	90.6	"
7.	0.6560gm	(1) 21.6cc.	0.739	0.673	90.4	"
Tyrosine 8.	0.6296gm	(1) 44.7cc.	∴ estimated wt. of tyrosine =			

0.6313gm = 100%"

as shown in Table XI.   Two separate solutions of

asparagine were made up and portions of each were

estimated separately in the Folin apparatus, after

hydrolysis for 6 hrs.

4. Amino Acid Nitrogen

Various methods were tried for the estimation

of amino acid nitrogen.   Recently Richardson (1928)

has carried out a thorough, quantitative investigation

of the methods available for the determination of

of amino Nitrogen in plant extracts, from which he

concludes that the formalin method of Sorenson is

the most satisfactory.   This method was tried on

standard amino acid solutions, but as will be seen

from Table XII the results were variable.   A

solution of the amino acid was made up, approximately

N/10 and 25cc. of the solution mixed with 30cc. of

neutralized formaldehyde.   This mixture was then

titrated against N/10 caustic soda, with phenol

phthalein as indicator.   When dealing with the

turbid plant extract, a control was set up by mixing

the same volume of water with powdered talc to the

same degree of turbidity as that of the extract.

A few drops of methyl orange were also added to

reproduce the colour of the extract, and then adding

TABLE XIII

COMPARISON OF RESULTS OF AMINO N ESTIMATION BY 2 DIFFERENT METHODS ON SAME PLANT EXTRACT.

oremans method.	Titration	Control	Required by amino acid	Amino N as % dry wt.	Amino N as % total N.
1.	2.35cc.	0.45cc.	1.9cc.	0.55%	13.7%
2.	2.3cc.	0.45cc.	1.85cc.	0.535%	13.4%

n ykes thod.	Vol.of gas.	Temp.	Pres.	Gm N in 250cc. extract (corrected)		
1.	0.58cc.	16°	753mm	0.0414	0.441%	11,0%
2.	0.58cc.	16°	753mm	0.0414	0.441%	11.0%
3.	0.56cc.	16°	753mm	0.0398	0.424%	10.6%
ank	0.11cc.	19°	755mm			

the same number of drops of phenol phthalein. The
end point was, however, indefinite,although the
method itself was rapid to use, so a modified method
was next tried.

Foremans method (1920), (see also Jodidi, 1918,
and 1926, and Harris 1924 for discussion of this
method) in which titration of the carboxyl groups
of the amino acid is carried out in the presence of
85% alcohol, was found to give a better end point,
and more consistent results were obtained. However,
the results obtained by this method in one extract
were compared with the results given by the Van
Slyke method on the same extract, and were found to
be some 22% higher than the latter, as is seen from
Table XIII. E.M.Richardson has shown that the
alcohol method applied to plant extracts always gives
results for amino N higher than those obtained by
the Van Slyke method (by about 18% in his experiments)
and he demonstrated that magnesium was present in
just sufficient quantity in the extract to account
for the difference, some of the alkali added being
used up in precipitating the insoluble magnesium
hydroxide. However to make an allowance for
the magnesium factor in this determination would
have involved tedious quantitative magnesium

TABLE XIV

Estimation of Amino Groups in Alanine (Van Slyke)

Alanine N Wt. = 89   N = 15.7

Wt. (i)= 10.2474gm.
    (ii)= 10.1488gm.

      0.0986gm.   This was dissolved in 100cc. & 5cc. taken.

	Vol.	Temp.	Pres. mm.	M gm.N = 1cc.gas	Wt.amino N in 100cc.	Wt.Alanine in 100cc.	% yield
Blank	0.11cc.	19°	755	0.5682	0.00125gm.		
Soln.1.1.35cc.		13.5°	760	0.5867	0.0153gm	0.9281	94%
2.1.33cc.		15°	760	0.5830	0.0155gm	0.9076	92%

estimations so it was decided to discard this method
and to try the Van Slyke method.

The manipulation of the Van Slyke apparatus,
once it has been set up is rapid, and the results
accurate,(see Van Slyke 1911,1912 and 1913, also
Mitchell and Ebstein 1918 and Wilson,1923). Capryl
alcohol was used to minimize foaming although even
with this precaution a certain amount of foaming
occurred. The standardization of the method, using
alanine is shown in Table XIV, but as Van Slyke has
demonstrated, the accuracy of the method varies with
the specific nature of the amino acid used.

Since the plant extracts contained amide nitrogen
as well as amino acid nitrogen, it was necessary to
test the former in the Van Slyke apparatue to see
whether, under the conditions of reaction, the amide
nitrogen reacted with nitrous acid.(see Van Slyke 1911)
it was also thought advisable to test quantitative
mixtures of amide and amino acid in the Van Slyke
apparatus, to ensure that the presence of amide in
the reaction mixture did not affect the amount of
nitrogen evolved by the amino acid. Results are
shown in Table XV.

From Table XV. it is seen that, in the first

TABLE XV

To show the Effect of the Presence of Amide in the Amino
Acid solution for Van Slyke Analysis.

.Amide only. Acetamide was used. 0.180gm.dissoved in 100cc. (N.Wt. 59).
∴.% N= 23.7% Amide N in 100cc. = 0.0427gm.
5cc.solution taken for every estimation. 300 r.p.m.

	Time	Temp.	Pres. mm.	Vol.of gas.	Mgm.N =1cc.	Gm.N in 100cc.	Gm.N after correcting for balnk.	% amide N estimated
cc.water								
lank	1.5min.	17°	746	0.31cc.	0.5665	0.0058		
	2.10min.	16°	746	0.38cc.	0.5690	0.0072		
	3.15min.	16°	746	0.43cc.	0.5690	0.0081		
clut.1	5min.	18°	746	0.32cc.	0.5640	0.0060	0.002	0.4%
2.	10min.	17°	746	0.40cc.	0.5665	0.0075	0.0003	0.7%
3.	15min.	17.5°	746	0.47cc.	0.5652	0.0088	0.0007	1.6%
.Amino Acid								
hly. 1.	5min.	15°	746	1.41cc.	0.5720	0.0269	0.0211	
2.	10min.	15.5°	746	1.45cc	0.5705	0.0276	0.0204	

|							Found	Calculated	Difference.
.Amide & Amino(Soln.contd. equ.vols.of amino acid									
id	Soln. B & double strength amide soln.								
1.	5min.	17°	753	0.91	0.5720	0.0174	0.0115	0.0106	+.0009
2.	10min.	20°	753	1.02	0.5637	0.0191	0.0119	0.0108	= 0011
3.	15min.	18°	753	1.08	0.5692	0.0205	0.0124	0.0111	+0.0013
4.	5min.	15°	746	0.77	0.5720	0.0147	0.0089	0.0106	-0.0017
5.	10min.	15°	746	0.85	0.5720	0.0162	0.0090	0.0108	-0.0018
6.	15min.	14.5°	746	0.90	0.5732	0.0174	0.0093	0.0111	-0.0018

place amides do not react appreciably with nitrous

acid under the conditions of the determination, and

the amount of nitrogen evolved from any amide present

in the extract, (since it was never present to a

greater extent than 13% of the total nitrogen),

would therefore be negligable.

In the second place, from Table XV.C it will be

seen that a mixture of amino acid and amide does not

yield a volume of nitrogen aggreeing quantitatively

with that calculated.   However two sets of determination

were made with the mixture one at a temperature of

about 15°, and the other at about 18° and in the

former case the results were lower, in the second

higher, than the calculated amounts, so that it

seemed likely that the difference is due to the fact

that although temperature is brought into account

in relation to the volume of the gas evolved there is

still another factor influenced by temperature viz.

rate of reaction.   Since the blank determination was

made at a higher temperature than the second determin-

ation with the mixture, it might be expected that

the calculated value (involving the blank determination

at 17°,) might be higher than the observed volume

from the mixture at 15°. Conversely, it might also

be expected that observed volume from the mixture at

DIAGRAM III.   NITRATE ESTIMATION.

DIAGRAM III

water at 45°

A

E

B

C

D

$\frac{N}{100}$ acid
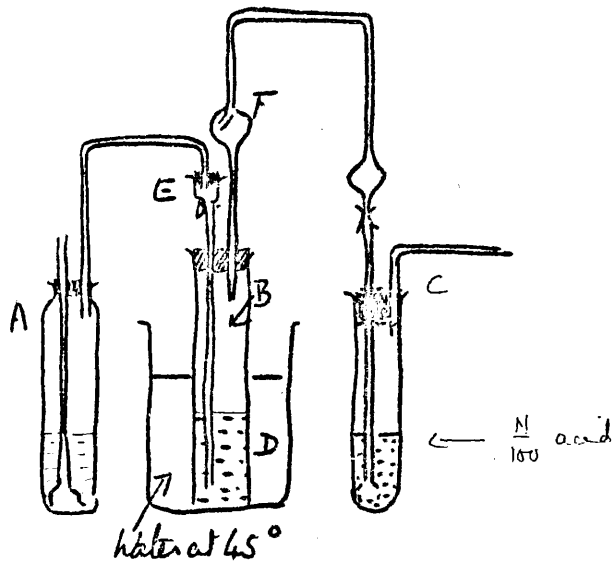
18° would be soemwhat higher than a calculated

volume involving a determination of nitrogen from

amino acid at 15°.

The conclusion drawn from this piece of work

was that in carrying out amino nitrogen determinations

it is advisable to make the blank determination at

the same temperature as that at which the main set

of experiments is to be performed.

No correction was made for the free ammonia

present, when calculating the amino content, since

the former was at all times, almost negligable.

5. Nitrate Nitrogen.

Nitrate nitrogen was tested qualitatively by

the diphenylamine test and by the brucine test, but

no trace of it was found except in the extracts of

Lot VI,VII and VIII. In these solutions, it was

estimated by reduction with Devarda's alloy,

(also used by Chibnall 1922; Robinson,1929 and

McKie 1931). The methods involved in the technique

of the former two workers were,however, not practic-

able, owing to unsufficient water pressure. A

variation of Chibnalls method was therefore used.

The apparatus shewn in diagram III was set up

in duplicate. In the reaction vessels D were placed

25cc. of the extract and a few drops of capryl

TABLE XVI

Standardization Method for Estimating Nitrate.

Standard solution contained 0.214gm.in 250cc.

∴ Normality = 1.008 N/10 ∴ 27.3cc. NaOh should be used up.

corresponding to amount of acid used up by NH_3 evolved.

Conditions	Titration	cc.used up.	% yield
1. 25cc. solution aerated 1 hour in cold with 2gm. anhydrous NaCO	45.6cc.	3.0cc.	11%
2. 25cc. solution aerated in cold with 10cc. 40% NaOh.	28.7cc.	19.9cc.	73%
b.Residue (a) aerated 1 hour more in water bath at 45°.	22.8cc.	<u>1.5cc</u>	
		20.4	78.4%
3. 10cc. taken aerated 2 hrs. at 40°.	14.8cc.	8.95cc.	89%

alcohol; into E, 10cc. 40% NaOh and into C, 25cc.
N/100 acid, 2 drops capryl alcohol and a few drops
of methyl red. Into one vessel B only, was placed
about 10gm. Davarda alloy. The connections were
closed, E being connected to a sulphuric acid washer
and B to a head F, for preventing any trace of strong
alkali from reaching the acid in C. The latter was
connected to a water pump. Then the alkali was run
into both vessels and left to stand overnight. The
following day, the reaction vessels B were surrounded
by a water bath kept at $40-45^{\circ}$, aeration started and
continued for 2 hours. The difference between the
quantities of ammonia coming over was taken as that
due to nitrate nitrogen.

It was found difficult to obtain a good yield
from dilute solutions of nitrate corresponding to its
concentration in the plant extract. The conditions
adopted for obtaining the maximum yield by this method
are shewn in Table XVI. Since the amount of nitrate
present was at all times small a yield of 89% was
considered good enough for the present research.

The determination of proteose N (and other N
precipitated by sodium sulphate at 33°) has already
been described (see Wasteneys & Borsook 1916).

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IV. STATEMENT OF EXPERIMENTAL RESULTS.

I. Seeds grown in light

The variation of the N distribution in the pea seedling at various stages of growth is summarized graphically in Figure I., and numerically in Table XVII.

Figure I shews the N distribution estimated as a percentage of the total N at each stage. The most significant fact which emerges from a study of this graph is the parallelism between the variation of the amino acid and the protein N (shewn here as the sum of insoluble, coagulable and proteose N) Amino acid N accumulates during protein breakdown, increasing from 2% of the total N in the original seed to 25% at the 18th.day, while protein N decreases from 96.4% in the original seed, to 59% at the 18th. day. Hence the minimum protein N value coincides with the maximum amino N content. After the 18th. day, resynthesis of protein becomes predominant, protein N increasing again to 84% of the total N on the 32nd. day, while amino N decreases after the 18th. day, falling to 6% of the total N on the 32nd. day.

The variation of insoluble, coagulable protein and proteose N is tabulated respectively in Tables

TABLE XVII

Distribution of Nitrogen

Lot.	Days of growth.	Insoluble N	Protein N (coagulable)	Proteose N.	Total Protein. N
X_1	0	11.44	71.88	11.72	95.04
X_{11}	0	10.35	77.22	8.36	95.93
A_1	3	73.33	15.95	6.65	95.93
A_{11}	3	80.56	13.33	0.74	94.63
B	5	42.11	34.9	11.64	88.65
C	9	37.79	33.53	4.97	76.29
D	13	25.97	36.2	11.71	73.88
E	18	12.24	29.7	16.8	58.74
F	22	31.01	31.1	13.8	75.91
H	32	60.9	12.5	11.43	84.83

as % of Total N.

Amino N	Ammonia N	Amide N	Nitrate N	Total N in fractions estimated	"Other soluble N"
2.17	0.14	1.49	–	98.84	+ 1.16
2.40	0.27	1.01	–	99.61	+ 0.39
3.50	0.47	1.13	–	101.03	(– 1.03)
2.21	0.55	0.93	–	98.32	+ 1.68
6.97	0.59	0.94	–	97.15	+ 2.85
11.90	0.48	0.89	–	89.56	+10.44
22.13	0.69	3.41	–	100.11	(– 0.11
35.28	1.14	2.43	4.43	92.07	+ 7.93
10.58	1.27	2.22	3.28	93.26	+ 6.74
6.09	0.55	4.28	–	95.75	+ 4.25

TABLE XVIII.

VARIATION IN INSOLUBLE N.

Lot.	Days of growth.	% N in insoluble residue	No. of determin-ations made.	Residual N as % of Total dry wt.	Total N
X_1	0	1.46	3	0.457	11.44
X_{11}	0	1.41	1	0.415	10.35
A_1	3	4.29	2	3.029	73.33
A_{11}	3	4.59	2	3.328	80.56
B	5	3.86	3	1.983	42.11
C	9	4.74	2	2.032	37.79
D	13	4.55	3	1.469	25.97
E	18	3.26	2	0.744	12.24
F	22	4.43	3	1.78	31.01
H	32	5.3	3	2.940	60.9

TABLE XXI

Variations in Amino N

Lot.	Days of growth.	No.of determin -ations made.	Amino N as % total dry wt.	% total N
A_1	0	1	0.087	2.17
A_{11}	0	2	0.012	2.40
A_1	3	2	0.145	3.50
A_{11}	3	2	0.091	2.21
B	5	2	0.328	6.97
	9	9	0.639	11.90
D	15	3	1.254	22.13
	18	2	1.535	25.28
	22	2	0.606	10.53
	32	4	0.292	6.09

TABLE XXII

Variation in Ammonia and Amide N

Lot.	days of growth.	No.of deter-minations made	Ammonia N as % dry wt.	Ammonia N as % total N	No.of deter-minations made.	Amide N as % total wt.	Amide N as % total N
X₁	0	1	0.005	0.14	2	0.059	1.49
A₁₁	0	1	0.011	0.27	2	0.041	1.01
A₁	5	2	0.020	0.47	1	0.047	1.13
A₁₁	3	2	0.023	0.55	1	0.038	0.93
B	5	2	0.028	0.59	2	0.044	0.94
	9	3	0.026	0.48	3	0.048	0.89
	13	2	0.039	0.69	3	0.194	3.41
	18	3	0.067	1.14	3	0.150	2.48
	22	2	0.073	1.27	2	0.127	2.22
	32	2	0.027	0.55	2	0.207	4.28

TABLE XXIII

Insoluble and Protein N as Percentage of Total Protein

(including Proteose)

Lot.	Days of growth.	Insoluble N	Coagulable Protein N	Proteose N	Coagulable Protein & Proteose
IA	0	12	75.6	12.3	87.9
IB	0	10.8	80.5	8.7	89.2
IIA	3	84.0	13.9	2.1	16.0
IIB	3	85.1	14.1	0.8	14.8
II	5	47.4	39.3	13.8	53.1
IV	9	49.6	43.9	6.5	50.4
V	13	35.2	49.0	16.8	65.8
VI	19	20.9	50.5	28.6	79.1
II	23	40.8	41.0	18.2	59.2
IX	32	71.8	14.7	15.5	28.2

TABLE XXIV

Soluble N fractions as % of Total water soluble N

(Sum of soluble components not including Proteose)

Lot.	Days of growth	Amino N.	Amide	Ammonia	Nitrate.
IA	0	57.4	38.9	3.7	–
IB	0	60.3	37.2	3.5	–
IIA	0	72.7	30.4	7.9	–
IIB	3	63.3	23.1	8.6	–
III	5	82.1	10.5	6.9	–
IV	9	88.7	7.8	3.5	–
V	13	84.6	12.8	2.6	–
VI	19	67.5	10.5	3.3	8.7
VII	22	56.9	11.8	6.7	24.6
IX	32	55.5	39.5	5.0	–

TABLE XXV

Lot.	Days of growth.	No.of seed-lings harvested.	Average length of root in cm.	Average length of shoot in cm.	Total wt. of meal in gms	Total N. as % of dry wt.
X	0	–	0	0	12.23	4.007
A	3	116	2.3	0	A. 6.047 B 8.763	4.13
B	5	56	6.0	1.8	5.007	4.71
C	9	48	15.4	3.1	7.198	5.375
D	13	52	15.6	5.5	4.651	5.67
E	18	38	19.6	7.1	3.564	6.075
F	22	32	22.5	8.3	6.022	5.7
G	26	34	26	10.5	4.310	–
H	32	32	33.5	11.9	7.707	4.85

XVII,XIX and XX., and the variation of amino N in

Table XXI.

Amide nitrogen (Table XXII.) shows no

significant change during germination.   It is very

low at first, increasing to about 5% over the 13-18

day period and at the last stage (32nd. day) increases

to 4.3% of the total nitrogen.

Similarly, ammonia nitrogen (Table XXII.)

remains consistently low, never rising above 1.4%,

 perhaps shewing a slight maximum over the 18-22 day

period, though this is almost within the experimental

error.

Nitrate nitrogen (Table XVII.) only appears on

the 18th., 22nd., and 27th. day periods, when it

represents some 3-5% of the total nitrogen.

The total nitrogen of the seedling, expressed

as percentage of dry wt, shows some variation

as shewn in Figure II. and also Table XXVII,

increasing gradually from 4% in the ungerminated seed

to a maximum of 6.07% on the 13th.day. then falling off

again to 4.8% on the 32nd. day.   This increase may be

due partly to an intake of nitrates from the soil,

since nitrates do appear in the plant at the stages

which are associated with the maximum total nitrogen

content.   Another factor which probably functions continuously throughout the investigation is the change in carbohydrate content.   During the early stages of germination the carbohydrate supply is being used up, and is not being augmented since there are at this stage no leaves.   This decrease of carbohydrate in the seedling will be reflected as an increase of the total nitrogen expressed as a percentage of dry weight;   this will be followed by an apparent decrease in the total nitrogen when the carbohydrate content begins to increase again, after photo-synthesis has started.

The inter-realtion between the three protein fractions is shewn graphically in Figure III. as % of total N.   Insoluble N (see Table XVIII.) shews a continuous decrease from 80% in the seed to a minimum of 12% on the 18th. day, hence coinciding with the minimum value for total protein.   It then increases again to 61% of the total nitrogen on the 32nd.day period.

The curves for the other two protein fractions coagulable protein N (see Table XIX.) and proteose N (see Table XX.) do not shew this regularity. However, if these two fractions are added together

and the sum plotted graphically, this protein apparently
remains fairly constant over the 0-22 day periods,
except for a low value at the 9 day period, which is
probably due to the fact that the total N estimated
on that set was rather low, being only 89% of the
total nitrogen and part of this deficiency may well
have been due to an error in the protein N determin-
ation.

If, however, protein nitrogen is plotted as in
Figure V, in which the three fractions are represented
as percentages of the total protein estimated, then
it is clear from the figure, that both coagulable
protein N and proteose N (and of course, their sum)
shew an increase from the original seed to the 18th.
day when they are at their maximum value, followed
by a decrease after the 18th.day, to the 32nd. day.
This increase of coagulable protein is a surprising
result, since it indicates that "coagulable protein"
increases during the stage when protein breakdown
as a whole, is proceeding and then decreases when
protein resynthesis begins.   There is one factor
which might influence the percentage of protein
appearing in the insoluble fraction and which would
affect it in the right direction to account for the

above observation; namely, the amount of protein
extracted from the insoluble portion will be greater,
the smaller the amount of total protein present,
since the extraction will necessarily be more thorough
if the same treatment is preserved throughout.   Thus
it is possible that as total protein decreases to
a minimum at the 18th. day, so the amount of protein
extracted will increase and when total protein
increases again after the 18th. day, the percentage
of total protein extracted from the insoluble portion
might decrease.   However, it does not seem likely
that this can account altogether for the variation of
"coagulable protein", since in the 32nd. day set,
a particularly thorough and lengthy extraction was
made, but the amount of protein N appearing in the
insoluble portion is still considerably greater
than in any sets since the 3rd. day period.

There is another possibility which might explain
the fluctuation of the "coagulable protein" curve.
It is conceivable that protein might be broken down,
in the first instance, by the splitting off of a
non-nitrogenous portion of the protein leaving the
residue richer in nitrogen.   This is borne out by
the fact that the percentage of nitrogen in the

"coagulable protein" fraction does increase at first, although it does not show a maximum at the 18th. day In this connection, it is interesting to note that McCulla (1934) in his studies of germinating seeds, obtained evidence that in a variety of seeds, the amide nitrogen of the protein was more readily removed than any other group in the protein, but he found that this did not apply to the pea.

It seems, however, more probable, that the increase in coagulable protein nitrogen as a percentage of total protein N and also as a percentage of dry weight as shewn in Figure IV. is in reality only an apparent increase, due to the decrease in some other nitrogenous fraction, viz. insoluble protein nitrogen. From Figures IV. and V. it is seen that insoluble protein N decreases to a minimum on the 18th. day, then increases again. If this insoluble protein were to be broken down before or more quickly than "coagulable protein" then the latter, expressed as percentage of total protein N or as percentage of total dry weight, (since part of the protein broken down would probably be lost as CO_2) would appear to increase. If it were expressed as a percentage of the total nitrogen, however, it would

TABLE XXVI.

Nitrogen as % total Nitrogen.	Etiolated.	Green.	Etiolated.	Green.
Ammonia N	0.6	0.48	0.96	1.1
Amide . N	3.7	1.05	13.4	3.7
Amino N	5.6	12.01	20.6	25.3
Nitrate N	–	–	–	4.4
	9.9%	13.5%	34.2	34.5

| | 9th. day | | 13th. day | |

not appear to increase under these conditions,
thus agreeing with the data given.

The accumulation of proteose in increasing
amounts up to a maximum at the 18th. day, as already
referred to on the previous page (see Figures IV and V)
would appear to indicate that proteose formation by
hydrolysis of protein takes place at a higher rate
than the further breakdown of proteose to form
simpler hydrolysis products e.g. amino acids etc.,
and so proteose accumulates.

2. Etiolated Seedlings.

Results with etiolated seedlings shew some
interesting results (see Table XXVI.)

On the 18th. day the total water soluble nitrogen
estimated, expressed as percentage of total nitrogen,
is approximately the same, both in etiolated and in
green seedlings, from which it can be deduced that
until the 18th. day, proteolysis in the light
continues to the same extent as in the dark.   The
difference between the results on the 8th. day, is
partly accounted for by the fact that normal 9 day
seedlings were soaked for 24 hours before sowing,
while the later batch was not.

However, some doubt as to the validity of the

above explanation is introduced by the fact that
although the total nitrogen of the seedling as a
percentage of the dry weight, is the same as the 9th.
day, both for etiolated and for normal seedlings,
indicating probably similar stages of development
at the 18th. day, the total N of the green plant is
6% while that of the etiolated plant is only 5%.
This difference is more than can be accounted for by
the nitrate present in the former. It is, however,
possible that more nitrate was taken in from the
soil in the green plant than was found as free
nitrate, the nitrate being synthesised into other
compounds. It is also possible of course, that the
growth in the two sets of plants in the light and
in the dark, was not strictly parallel.

If this agreement between the two sets of figures
for green and for etiolated plants on the 18th. day,
is not deceptive, it can be deduced that the increase
in amide nitrogen to 13% of the total nitrogen, in
the etiolated plant, must take place partly, at
least, at the expense of amino acid nitrogen which,
in the etiolated plant at the 18th. day is only
20.06% while in the green plant, is 25.3%.

Ammonia in both sets, is about the same
in ammount, being slightly greater in both sets
at the later stage.

V.   COMPARISON OF THESE RESULTS WITH THOSE OF OTHER INVESTIGATORS.

As has already been observed, the most
significant fact which emerges from the study of
the nitrogen metabolism of the pea seedling, is the
accumulation of amino acid nitrogen which takes place
in the green seedling during protein breakdown.
A study of the relevant literature has therefore
been made to ascertain whether this behaviour of
the pea is common to other plants also, or whether
it is exceptional, and to find out, if possible,
what factors govern the behaviour of different plants
in this respect.

The accumulation of amino acid, in the presence
of light, has been observed by several investigators
who have studied the metabolism of germinating seeds.

Thompson (1915) studied the distribution of
amino nitrogen in seedlings of the Alaska pea, over
a period covering 38 days.   Some of his results
are shown in Table XXVll.   He does not estimate
protein, nor amide nitrogen.   His results however,
indicate an accumulation of amino acid during the
first two weeks of growth.   The fact that his
maximum observed value for amino nitrogen, on the
7th. day (viz: 28% of total N) is considerably

TABLE XXVII.

Thompson (1915) Amino N in seedlings of Alaska Pea

	Day	Total N (% dry Wt.)	Amino N (% total N)	Amino N (% total N) (1935 Barnett)
	1.	3.27	6.58	2.2
	2.	3.22	8.23	
	3.	3.28	10.52	4.4
	4.	3.35	11.52	
Whole	5.	3.44	18.46	7.0
seedling	6.	3.48	21.46	
	7.	3.53	28.27	
leaves & part of	14.	9.43	19.41	22
stems & roots	2 1.	9.29	16.09	25
only.	38.	8.35	14.32	6

higher than any similar value recorded in the present investigation is probably due to the total nitrogen of the seedling being somewhat lower in the former investigation than in the latter.

Jodidi (1925) noticed an accumulation of amino nitrogen in etiolated corn seedlings up to the 8th. day of germination accompanied by a decrease in protein nitrogen.   His observations only covered 8 days however.

Suzuki (1925) in an investigation of the germination of the Lima Bean (Plasiolus lumatus) extending over a period of 12 days, found that while protein decreased in the cotyledon throughout the period studied, amino acids had increased in the cotyledon at the 6th. day period and decreased slightly at the 12th. day stage, while in the stem, the increase at the 6th. day and decrease at the 12th. day of amino nitrogen was very much more marked, both for the etiolated and green seedling.   In the stem, protein nitrogen decreased at the 6th. day, and increased at the 12th. day period.   His results indicate that translocation of water soluble nitrogen takes place from the cotyledon of the stem, and he suggests that this is due to the

TABLE XXVIII.

N as % total N	Seed	5 days	13 days		19 days		26 days	
			Shoot	Cot.	Shoot	Cot.	Shoot	Cot.
soluble N	75.31	44.8	40.4	41.3	35.4	38.7	40.1	43.3
luble Protein N	20.3	25.5	9.6	13.7	9.1	7.3	5.1	10.6
onia N	0.2	3.8	3.7	4.4	3.7	9.4	1.0	3.0
ide N	0.6	3.1	13.8	0.2	15.1	17.3	16.8	12.3
ino N	0.7	8.0	10.1	14.6	9.5	–	5.1	–
st N	3.4	14.5	22.3	25.7	27.1	27.3	31.1	30.5
tal N (% dry wt.)	4.81	5.2	5.6	4.66	9.86	3.7	10.27	3.77

49.

transportation of amino acid nitrogen.   He does

not give data for amide nitrogen, except for the

6 day period, when it only represents 4% of the

total nitrogen, in the cotyledon, and 11. in the

stem.

Sure and Tottingham in 1916, in a study of the

amino nitrogen of the etiolated pea seedling found

that in the cotyledon amino nitrogen increased

until the 13th. day, then suddenly diminished, while

in the shoot amino nitrogen showed a less steep

maximum at the 13th. day, and afterwards decreased

gradually.   In neither shoot nor cotyledon did the

amino nitrogen become as high as in the present set

of results, which is only to be expected, since they

worked with etiolated seedlings, the results with

the present set of seedlings having shown that the

amino acid content is lower in the etiolated

seedling than in the corresponding green plant.

Some of their data are given for comparison in

Table XXVIII.   It is seen from their data however,

that the amino acid content of the shoot shows a

maximum at the 13th. day, differing in this respect

from the present set of data for the pea.

Bonnet (1929) grew lupin seeds on distilled

water, in darkness, and noted that protein nitrogen

decrease was accompanied by an accumulation of amino and amide nitrogen, the former increasing more than the latter.   He states that after a certain time, resynthesis of protein took place, at the expense of the amino nitrogen, (although this cannot be deduced from the small amount of data published.)

Priarischnickov (1896) noticed a similar accumulation of amino and amide nitrogen, during the breakdown of protein in germinating seeds of Vicia Sativa.

Zaleski and Shatkin (1913) studied the nitrogen distribution in the bulbs of Allium Cega.   After 25 days the protein N had increased, while the amino acid originally present in considerable quantity in the bulbs, decreased during growth. They concluded that protein was found at the expense of the reserve amino acid of the bulb, and that there was no apparent connection between the amount of amide present, and protein formation.

Pettibone and Kennedy (1916) on the other hand, found no accumulation of amino acids during the germination of maize seedlings, the amino nitrogen remaining fairly constant.

Webster (1928) also found no relation between
amino or amide nitrogen, and protein nitrogen, in
his investigation of the nitrogen metabolism of the
Soy Bean, germinated up to 3 weeks.   He found,
however, that the total soluble nitrogen had a
maximum at the 2-3 weel stage, which coincides with
the present data, which show a maximum at the 18 day
stage, for total water soluble nitrogen.

The results of Suzuki, Bonnet, Jodidi,
Prianischnickov, and Zaleski and Shatkin mentioned
briefly above, are confirmed by the present results
with the green pea seedling, in so far as protein
resynthesis appears to take place at the expense of
amino acid nitrogen.

But many investigators, however have noted an
accumulation of asparagine or glutamine in
germinating seedlings, more especially in etiolated
seedlings, but occasionally in normal plants.   The
pea seedling, however shows no such accumulation of
amide in the normal state, nor to any considerable
extent in the etiolated plant, according to the
results of this research.

The data obtained by Gure and /Tottingham in
1916, when studying nitrogen distribution in the

TABLE XXIX

	Amide N	Amino N	Day	Amide N	Amino N	
tyledon	0.2%	14.6%	13	13.8%	10.6%	Shoot
	17.5%	0%	19	15.1%	9.5%	

partially etiolated pea seedling, may be compared
with the data obtained in the present investigation
(TableXXIX). (Some of their data have also been
set out in a previous page, see TableXXVIII). They
found that amide accumulated continuously in the
shoot, representing about 17% of the total nitrogen
by the 26th. day. In the cotyledon, amide
accumulated up to 17% on the 19th. day, then
decreased to 12% on the 26th. day. Further, they
noticed that amide accumulation was parallel to
amino nitrogen decrease, and interpreted this as
indicating that amides were formed at the expense
of the amino acids in the etiolated pea plant.

This same conclusion has already been reached
from a study of the comparative data for the green
and etiolated pea seedling in the present research.
With regard to the actual amounts of amide and
amino acid nitrogen present, it is impossible to
compare the two sets of data, since in the present
research, the whole seedling was analysed complete,
while in Jure and Tottinghams investigation the
shoot and the cotyledon were analysed separately,
and no data are presented for the relative amounts
of each part. However the amount of amide present

does seem to be greater than in this present
research, while the amino acid nitrogen content
of the etiolated plant on the 19th. day was found
to be absent completely from the cotyledon, whereas
in this case it was found to represent 20% of the
total N at this stage. In both cases, amino acid
was estimated by Van Slyke's method. This considerable
difference is partly explained by the fact that in
the shoot only total nitrogen was found by Sure and
Tottingham to be as high as 9.86% of the dry weight
while at the same stage, the total nitrogen in the
seedling as a whole, in the present research was
only 5%.

The amino nitrogen expressed as a percentage
of the dry weight was, according to Sure and
Tottingham's data 0.96% of the dry weight of the
shoot alone, and in the present research 1.00% of
the dry weight of the total seedling, a remarkably
close agreement. However there is really no
justification for comparing results for the shoot
alone, with data relating to the whole plant.

No other quantitative data seem to be available
for the nitrogen metabolism of the pea seedlings, but
comparable results with other types of seedlings may
be mentioned here:

TABLE XXX

...ie (1930). N Metabolism of Lupin Seedling (N as % of total N)

Total N (% dry wt.)	Ammonia N	Amide N	Protein N	Proteose N	Amino N	Aspara gine N	Insoluble N
6.42	0	0	5.92	0	0	0	41.1
9.89	0.26	–	–	–	–	–	43.9
8.24	1.02	3.15	20.2	0.85	0.92	21.2	–
8.7	0.93	2.76	23.4	1.72	0.93	22.3	41.5
19.16	0.22	1.41	9.81	14.2	0.29	53.7	20.7
16.25	0.41	1.41	27.8	10.3	0.25	29.4	24.9
14.56	0.33	1.65	12.0	3.7	0.30	20.5	43.5
14.49	0.19	1.34	15.0	–	0.17	14.6	38.9
12.64	0.37	1.11	18.5	13.1	0.13	10.6	26.3

McKie (1930) in an investigation on the
nitrogen metabolism of the lupin seedling
(lupinus luteus), grown under normal conditions,
similar to those pertaining to this present research,
noted an accumulation of asparagine during the early
stages of germination until the 18th. day, when the
asparagine nitrogen reached the remarkable value of
53% of the total nitrogen.   This was followed by
a diminution of asparagine nitrogen, parallel to an
increase of protein nitrogen.   Even at the 82nd.
day, however, asparagine still represented 10% of
the total nitrogen.   Hence it is clear that the
nitrogen metabolism of the lupin follows a very
different course from that in the pea.   Moreover,
the amino nitrogen in the normal lupin seedling
never became appreciable, remaining consistently
below 1% of the total nitrogen, while in the pea
amino nitrogen represents the chief portion of the
nitrogen from protein degredation, while amide was
always very low in the green pea seedling.   Some
of these results are reproduced in Table XIX
A possible cause for these differences will be
discussed later.

The nitrogen metabolism of the lupin seedling

was first investigated thoroughly by Schulze (1878 et
seq.).   Briefly his results are as follows: he
found an accumulation of asparagine both in the
light and in the dark.   Under the latter conditions,
on the 21st. day 73% of the protein degredation
products were represented by asparagine (in the
present investigation, on the 14th. day, 70% of the
protein degredation products was in the form of
amino acids.).   Schulze also found that the amino
acid content was very low in both normal and
etiolated seedlings, a result which is confirmed by
the recent data of McKie for the lupin.

Schulze also found an accumulation of asparagine
during protein hydrolysis in Vicia Sativa, Soya
seedlings, (when asparagine nitrogen accumulated up
to 7% of the total nitrogen) and in Trefolium,
800gms. of which, (fresh weight) gave rise to 1.7gm.
asparagine) in plants not belonging to the
Leguminosae, be found a similar accumulation of
asparagine in seedlings of Tropoelin majus, Pinus
sylvestris and Papaver somniferum.   In some seedlings
however, he found that glutamine took the place of
asparagine, e.g. Ricinus communis, Sinapis alba,
Picaea excelsa, and various species of cruciferae

and ferns. In etiolated oat plants, he found
that after 7 days germination, amide accounted for
59% of the total decrease in protein nitrogen. In
grass however he found no such accumulation.

Jodidi (1925) investigated the nitrogen
metabolism of the etiolated corn seedling, over a
period of 8 days, after sowing and found that amide
(not specified as asparagine) accumulated while
protein decreased.

An examination of the above group of results
will show that in every case, when the nitrogen
metabolism of etiolated seedlings was studied,
an accumulation of amide asparagine was noted;
this accumulation does therefore, seem to be general
phenomenon. However, in normal seedlings, as the
results of Suzuki, Priavischnickov, Zaleki and
Shatkin, Pettibone and Kennedy, Webster, McKie and
Schulze have shown, sometimes amide sometimes amino
acid, may accumulate, while in a few cases neither
predominate particularly. It is not therefore,
possible to generalise in this case; the nature of
the products which accumulate probably depends upon
various internal factors in the seed itself, e.g.
the nitrogenous and non nitrogenous reserve products

of the seed etc.   These factors will be discussed

in more detail later.

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VI.   <u>Significance of Results.</u>

1) <u>Problem of Protein Synthesis in relation to its</u>
<u>primary starting material.</u>

In section IV it was pointed out that the most
important experimental observation arising out of
the present investigation is the accumulation of
amino acids which occurs during protein breakdown in
the germinating seed.   This result has been compared
and contrasted with results obtained by other
investigators on similar material.   The significance
of these results will now be discussed briefly.

When proteins are hydrolysed <u>in vitro</u>, either by
enzymes, acid or alkali, the chief product is always
amino acids.   This fact has to be taken into account
in any theory which is put forward with regard to
the structure of proteins, but although some grouping
such as $R-CO-NH-CH$ R which could give rise to amino
acids on hydrolysis, must necessarily be present
it is not justifiable to assume that the protein
molecule must therefore have been built up <u>in vivo</u>
only from amino acids.   Chemical evidence has been
obtained shewing that protein-like substances can
be prepared purely from amino acid derivatives
(cf. E. Fischer, Abderhalden, etc.).   One possible

biochemical method of approach to the problem of
the nature of the primary starting material for
protein synthesis in plants is the study of the
variation which takes place in the non-protein
compounds present in the plant when the protein is
undergoing change.   The simplest case to study is
that of the germinating seed, since in the initial
stages of growth protein change is due entirely
to one process, namely, the hydrolysis of protein,
so that the products of hydrolysis accumulate and
can be identified.   After photosynthesis has begun,
the non-protein nitrogenous compounds decrease, as
protein increases.   The assumption has been made
here that any nitrogenous substance which appears
to decrease in quantity after protein synthesis
has begun is being used up directly or indirectly
for protein formation.

It is possible that one specific amino acid,
or an amino acid derivative (e.g. acid amide) may
be used preferentially for protein synthesis rather
than a whole range of amino acids.

Hence the accumulation of asparagine (which is
an amino acid amide) in plants under certain
conditions noted by many investigators, during
proteolysis, could be considered to have some

possible bearing on the problem of the elementary

nitrogenous substance necessary for protein formation.

The function of asparagine in the nitrogen metabolism

of plants and the factors which decide whether amide

or amino acid shall accumulate in the germinating

seedling, are two of the most important questions

in this field of biochemistry at the present time.

Various hypothesis which have been put forward with

regard to these problems will be briefly outlined

here, and the bearing of these present results on

any of these theories will be considered.

2) Asparagine as direct precursor of protein.

Schulze, about thirty years ago, put forward

the theory that asparagine is a more favourable

material than amino acids for protein synthesis.

This theory depended, for its evidence, chiefly

on the interpretation which Schulze put upon his

results.   In his work upon germinating lupin

seedlings already mentioned, he found a considerable

quantity of asparagine present, even when the plants

were grown in the light, and the amide persisted

even after the resynthesis of protein had begun.

Schulze suggested that the metabolite which was

present in the largest amount (viz. asparagine here)

was the one which served as the most favourable for
protein synthesis.   Moreover, he noticed that, in
the lupin, the amino acid content in the normal
seedling was actually lower than in the corresponding
etiolated plants, and since he contended that in
the normal plant, in which the conditions were more
favourable for growth than in the etiolated plant,
the essential metabolic processes would take place
preferentially, the fact that amino acids were lower
in the normal seedling than in the etiolated one
again pointed to the conclusion that the formation
of amino acids was not an essential step towards
protein formation.

On the other hand, these results would be
interpreted by Prianischnikov, (whose own theory
will be referred to later) as indicating that the
amide which remains in considerable quantities while
protein synthesis is proceeding, is not required
for synthesis, but that the favourable synthetic
material is that which remains at a low concentration
purposes.   It is clear that the evidence which
Schulze puts forward, namely the accumulation of
amide during germination, cannot be taken as proof
of his hypothesis, as it can serve equally well as

evidence in favour of Prianischnikov's theory. (It
may be noted here that most modern workers prefer
to adopt Prianischnikov's interpretation of the
observations, rather than Schulze's and this is also
the view adopted here.)

There is, however, some other evidence for this
theory of the direct importance of asparagine in
protein metabolism, and it will be discussed breifly
here.

Prianischnikov grew etiolated seedlings of
barley on media of ammonium salts and found that
the plants absorbed nitrogen in the form of ammonia,
and synthesized asparagine. It might therefore be
concluded that asparagine is of first importance as
a step in the sequence of protein formation.
(although Prianischnikov himself did not take this
view). But it must be noted that these seedlings
did not actually synthesize protein, so that the
conditions may well have been abnormal.

Schulze and others had found that in the pods
of ripening seeds, asparagine was the predominant
nitrogenous compound present, and he therefore
concluded that, since one of the most important
processes in the ripening seed is the formation of

reserve protein, the asparagine present must be the material par excellence for protein synthesis.

Further evidence can be sought in the work of Hansteen (1896) who examined nitrogenous compounds in the plants of Lemna minor, by a micro chemical technique. He showed that if the plants were placed in glucose solution, starch accumulated in the plants, but that if asparagine was added to the glucose solution, no starch reaction was obtained, and moreover, the plants gave a protein reaction. If asparagine was added to a solution of cane sugar, no protein reaction appeared. Addition of single amino acids to the glucose solution did not seem to have the same effect as asparagine as a direct precursor of protein. Moreover, Osborne showed that asparagine and glutamine probably exist as such in the protein molecule.

However survey of the above work will suffice to show that there is very little direct evidence for Schulze's point of view, and that the function of asparagine as a direct precursor of protein has yet to be proved. The present results with the pea do not lend any support to this theory, as there is no indication that, in the normal seedling,

asparagine has any significance as a metabolite, since
at no time is it present in appreciable quantities
and shows no maximum or minimum at any period.

3) Asparagine as translocatory medium for protein
nitrogen.

Another theory which regards asparagine as
playing a very important part in protein metabolism,
put forward originally by Pfeffer and supported
recently by Chibnall, maintains that asparagine is
the chief (if not the sole) translocatory nitrogen
compound, by means of which nitrogen in a form
available for protein synthesis, is transferred from
the place of nitrogen storage, or absorption, to
the seat of synthesis.   Asparagine is then converted,
in some way, into protein.   This does not assume that
asparagine is transformed directly into protein (i.e.
without the intermediate formation of amino acids)
although this possibility is not excluded.

The most important evidence for this theory is
to be found in Chibnall's work on the nitrogen
metabolism of the runner bean (1924).   He showed,
by controlled experiments with green leaves of the
runner bean, that during the night there was a decrease
in the protein nitrogen in the leaves, and also of

the water soluble nitrogen and concluded that the
water soluble nitrogenous products were translocated
from the leaves at night.   He subsequently found,
by controlled experiments with leaves, isolated from
the plant, with their petioles in water, that a
similar decrease in protein was accompanied by an
accumulation of asparagine.   He considered that his
experiments warrented the conclusion that this
accumulation was not due to starvation and lack of
carbohydrate, but that it was the natural course of
protein metabolism;   in the complete plant the
asparagine would not accumulate, but would be
translocated away from the leaf,to other parts of the
plant where its nitrogen could be used for protein
synthesis.

Sure and Tottingham also conclude from their
results (1916) on the N distribution in the etiolated
pea seedling that they had obtained evidence for the
translocation of amides from the cotyledon to the
shoot after five days growth.

Haskell and Mason's well known experiments
(1930) on translocation in the cotton plant, can be
interpreted to indicate that asparagine is the
chief translocatory medium for nitrogen, but the
results are not conclusive, and, as they themselves

point out, could be taken equally well to indicate that amino acids are the medium for nitrogen transportation.

Contrary to this point of view, there is Thompson's work (1913), with the Alaska pea, when direct evidence was obtained of the existence of amino acids in the plant sap.

Pettibone and Kennedy (1916) also obtained direct evidence of amino acids being concerned with nitrogen transportation, since the plant sap of germinating maize seedlings was found to contain amino acids.

Further, Suzuki (1907) working with the Lima Bean, found an indication that, at the 12th. day of germination, translocation of amino acids from the cotyledon to the stems took place, and this was confirmed by an analysis of the stems in which an increase of amino acid was found to have occurred.

Consideration of the evidence outlined above leads one to the view that, while asparagine quite probably can serve as a medium for the translocation of nitrogen in the plant, it is unlikely that it is the only medium, since it seems probable that amino acids can also function in that respect. The

present results cannot have any bearing on this theory as to the role of asparagine, since no attempt has been made to study the nitrogen distribution in different parts of the plant.

4) Asparagine as storage product for excess ammonia.

The theory of Prianischnikov has already been mentioned. He considers that asparagine is not essential for protein formation, but merely serves as a storage product for rendering innocuous the excess of ammonia, formed during the degradation of amino acids, which would otherwise be harmful to the plant; in this respect, he suggests that the function of asparagine in plant metabolism resembles that of urea in the animal. Prianischnikov criticizes much of the evidence brought forward with regard to asparagine as a precursor of protein, and prefers to consider amino acids as the more favourable material for protein synthesis. His method of interpretation of experimental results differs from that of Schulze as has already been mentioned, viz. Prianischnikov considers that an accumulation of asparagine which continues to exist in considerable quantity after resynthesis of protein has begun, indicates rather that it is not required for protein

TABLE XXXI.

Showing evidence for protein formation at expense of amino

acids. (Schulze)

. (1906)	Lupin	15 days		24 days	
	Protein	1.71		1.78	
	Asparagine	4.02		5.09	
	Basic compounds	1.22		1.03	
	Other "	2.39		1.40	

. (1899)	Lupin	15 days in dark	15 days in dark, then days in light.		
	Protein	18.35	13.96		
	Asparagine	43.02	54.47		
	Other N	38.63	26.57		

. (1898)	Lupin Luteus	6 days	15 days	24 days
	Protein	5.9	1.71	1.78
	Asparagine	1.16	4.02	5.09
	Basic N	0.97	1.22	1.03
	Other N	1.72	2.39	1.40

formation, and that the amino acid may be present only in low quantity because it is being continuously used up in synthesis of protein.

Evidence for the formation of protein at the expense of amino acids is to be found in the observations of several different investigators. Schulze in his early work on nitrogen metabolism, found that amino acids and asparagine increased during the early stages of germination of lupin seedlings (see data in Table XXXI), and later, amino acids decreased when protein synthesis began. Although Schulze himself put a different interpretation upon these facts, the latter can, however, be considered to afford evidence for the formation of protein at the expense of amino acids.

The results of Suzuki (1907) on proteolytic changes in the Lima Bean during germination, show a similar accumulation of amino nitrogen on the sixth day after germination, followed by a decrease of amino nitrogen on the 12th. day.

Zaleski and Shatkein's results (1913) (quoted in the previous section) indicate that the increase of protein noted in bulbs of Allium Cepa, shortly after the beginning of growth, takes place at the expense of the amino nitrogen.

Thompson (1913) in his study of the total amino nitrogen in Seedlings of the Alaska Pea (data already quoted in Table XVII) obtained results which showed that the amino N increased (as % of total nitrogen) up to the 7th. day, then decreased after the 14th. day. However, since he did not estimate protein nitrogen, it is not possible to say whether this fluctuation corresponds with the breakdown and resynthesis of protein.

The results of Jure and Tottingham (1916) have already been quoted in Table XVIII. Since they worked with etiolated seedlings, it is not to be expected that their results will bear upon the above problem, but a small amount of protein does seem to have been resynthesized on the 26th. day (this may be accounted for by the fact that the seedlings were only partially etiolated.) Their values for amino nitrogen show a maximum, both in the shoot and in the Cotyledon, on the 15th. day. The decrease of amino nitrogen which follows must indicate that the amino N is transformed, partly or wholly into amide nitrogen, since the latter remains high until the 20th. day. Thus these results, while they do not afford any evidence for the resynthesis

of protein at the expense of amino acid nitrogen do
bear out Prianischnikov's suggestion that asparagine
is formed only as a storage product for preserving
excess nitrogen in an innocuous form.

Bonnet (1929) in a paper on the evolution of
nitrogen during germination, working with lupin
seedlings, in the dark, stated that, when the plant
had attained a certain development, proteins are
formed at the expense of amino nitrogen. However,
as has already been pointed out, his published
results do not bear out this statement, nor is it
clear how any protein resynthesis took place, since
growth was in the dark.

The present results with the pea give support
to the view that protein is formed at the expense
of amino acids, since protein regeneration is
accompanied by a fall in amino acids, which had
accumulated during protein breakdown, reaching a
maximum at the point of minimum protein content,
before photosynthesis set in. Amide nitrogen,
however, showed no relation to protein nitrogen
variation. Moreover, when the seedlings were
etiolated, it was noted that, while the amino
acid content increased continuously (showing no
maximum as with the green seedlings) the amino

nitrogen was less at any one period, than at

the corresponding period with the green seedlings,

while this decrease of nitrogen was reflected in

an increase of amide nitrogen, since the amide

nitrogen value was higher in the etiolated seedlings

than at the corresponding period in the green plant.

This may be taken as evidence that amides are formed

from amino acid in the etiolated plant.   There is

not any evidence from these results, however, to

indicate that amides are formed from amino acids,

as intermediate products in the synthesis of proteins.

It remains to discuss the possible cause of

the difference in behaviour between the pea and the

lupin seedling, with regard to their nitrogen

metabolism, as has already been observed, is the

fact that, in the pea, grown in the light, the

nitrogenous product which accumulates while protein

decomposition is taking place, is chiefly amino

acid, while in the lupin, grown under similar

conditions, asparagine accumulates to a very high

value, amino acid being present only in very small

amount.

There is much evidence, arising out of the

observations of earlier workers, that the nature of

TABLE XXXII

(Schulze (1908).

Seedling	% of original protein lost.	% of original seed weight as reserve carbohydrate.
Lupinus Luteus	80.4	18
angustifolius	75.7	39
Curbita	27.9	52 (fat)
a mays	27.4	Rich in starch

the products which accumulate is affected by the
concentration of carbohydrate present in the growing
plant.

Some early work of Schulze (1898) on the
relation between the extent of protein degredation
and the original non-nitrogenous reserve compounds
in various plants, for which some data are given in
Table XXII, indicates that the greater the store of
non-nitrogenous reserve material the less extensive
is the protein breakdown, or conversely, that
asparagine accumulation is greater in seedlings which
have poorer non-nitrogenous reserves (this was also
shown by Schulze).

Further, some work by Suzuki (1902) and Palladin
(1912) indicates that asparagin formation occurs at
the expense of amino acids, and can only take place
in the presence of oxygen, since they found that in
certain seedlings in which asparagine accumulated
in the presence of air, did not form asparagine if
grown in the absence of oxygen. Moreover, Butkewitsch
has shown (1909) that if seedlings were anaesthetized
by toluene, during growth, no asparagine was formed,
but ammonia accumulated instead, and further, the
latter only formed if oxygen was present, indicating

that ammonia is formed from amino acids by oxidation
and subsequently the ammonia is transformed into
asparagine.

Further evidence for the effect of carbohydrate
concentration on asparagine accumulation is found
in experiments on starved leaves and shoots, (e.g.
Monteverde, 1889, Prianischnikov 1895-1910) in which
it is shown that starved shoots, e.g. of pisum
formed asparagine when left in water only but if
left in glucose solution, no asparagine was found.

From these results, it would appear probable
that amino acids, formed by proteolysis, can
breakdown in the presence of a low concentration of
carbohydrate, to ammonia, which is then transformed
into asparagine, possibly by combination with non-
nitrogenous residues formed either by oxidation of
carbohydrate or else according to recent work by
Ruhland and Wetzel, by deamination of amino acids.
In the presence of much carbohydrate, asparagine does
not accumulate. Whether the cause of this is that
in the presence of high carbohydrate concentration,
amino acids do not undergo further breakdown, but
are somehow directly transformed into protein, with
the aid of carbohydrate, or whether an equilibrium

TABLE XXIII

Showing constituents of seeds as percentage of dry weight

(Schulze 1891)

Lupin	Lupin	Pea	Pea
Protein	36.8	22.5	Protein
Nuclein	.7	1.1	Nuclein
Alkaloids	1.1	-	-
Lecithin	1.6	1.2	Lecithin
Cholesterin	.1	.1	Cholesterin
Glycerides	4.6	1.9	Glycerides
Ether. Soluble substances	.2	40.5	Starch
Galactan	7.6	6.2	Cane sugar & Galactan
para galactan	11.7	-	
Crude fibre	18.2	6.0	Crude fibre
Organic acids	1.6	.7	Organic acids
Ash	3.6	3.5	Ash
Undetermined	12.1	17.3	(para galactan and undetermined)

is set up in the reversible set of reactions shewn
below:

Amino Acid

Protein   Ammonia
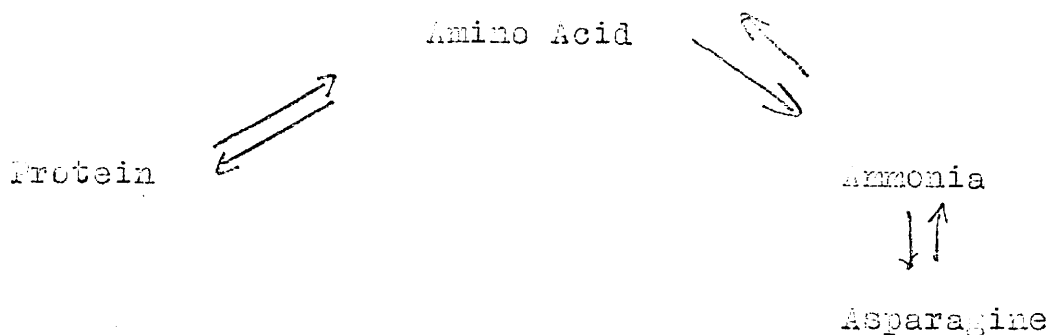
                                                Asparagine

so that asparagine, although it may be a metabolite,
yet does not accumulate, cannot be decided from the
available data, since either interpretation would
fit the facts.

In the case of the pea, the reserve
carbohydrate supply is known to be comparatively
high, while in the lupin it is definitely lower
and consists mainly of hemicelluloses which are not
easily available for oxidation.   The data of Schulze
(1891) given in Table XXIII illustrate this
difference.

It is suggested, therefore, that the probable
cause, (possibly not the only one) of the difference
between the nitrogen metabolism of the pea and the
lupin is that in the pea, reserve carbohydrate

concentration is high and is therefore available
for protein synthesis, so that asparagine accumulation
does not take place, but in the lupin, the store
of available carbohydrate is comparatively low, so
that protein hydrolysis proceeds further before
photosynthesis of carbohydrate and hence of protein
resynthesis, begins, resulting in the accumulation
of asparagine.

This suggestion is born out by the fact that in
etiolated seedlings of the pea, in which the
carbohydrate reserves become considerably depleted
as growth continues, asparagine does accumulate to
some extent, but not in such large quantities as in
the normal lupin seedling, in which the initial
carbohydrate content is very low.

The reason why asparagine is formed at all
cannot be settled by the evidence so far available.
The facts at hand seem to lend support to Prianis-
chnikov's theory that asparagine serves as a
storage product for rendering innocuous excessive
ammonia, which would otherwise be injurious to the
tissues. The present results do not disagree with
this theory, and while they give no support to
Schulze's theory that asparagine is essential for

protein formation, they do seem to give positive
evidence in favour of Prianischnikov's theory that
amino acids are the essential intermediate step
towards protein synthesis.

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                                                     der Kernungsvorgange.

Schulze   see references from previous section.

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VII.   Summary.

1.   Experiments have been made to determine the nitrogen distribution in the pea seedling grown in the light, at various stages of growth, from the ungerminated seed to the 32nd. day seedlings, with a view to throwing some light on its nitrogen metabolism, in relation to the results of other workers in the same field.

2.   A detailed account of the method used, has been given, with data for the quantitative standardization of the methods.

3.   The chief feature of the nitrogen metabolism of the pea seedling is that protein breakdown is accompanied by an accumulation of amino acid, up to 25% of the total nitrogen, until the 18th. day when protein resynthesis begins, and amino acid content falls.

4.   Amides remain at all times negligible, and do not show any relation to the change of protein content of the seedling.

5.   Ammonia also is almost negligible at all times.

6.   Nitrates are absent from the seedling except at the 22nd. day stage.

7.   In control seedlings, grown in the darkness,

amino acids still represent the largest part of the water soluble nitrogen, but are less than in the green plants.

8. In the etiolated plants, amide accumulates up to 13% on the 18th. day, but the amount present was less than that reported by many workers for other seedlings.

9. In the green seedlings, total nitrogen as a percentage of dry weight of material increases to a maximum on the 18th. day, then, falls again continuously.

10. These results with the pea have been compared with data obtained by other workers on the nitrogen metabolism of germinating seedlings, and in particular, with those obtained by McKie (1930) for the lupin seedling.

11. The role of asparagine in the nitrogen metabolism of plants has been discussed, and the bearing of these results on this problem has been considered.

12. It is concluded that these results support the theory of Prianischnikov, that amino acids are the direct precursor of protein and that asparagine is not used directly in the formation of protein, but serves for storing excessive ammonia which

FIGURE I

FIG. I

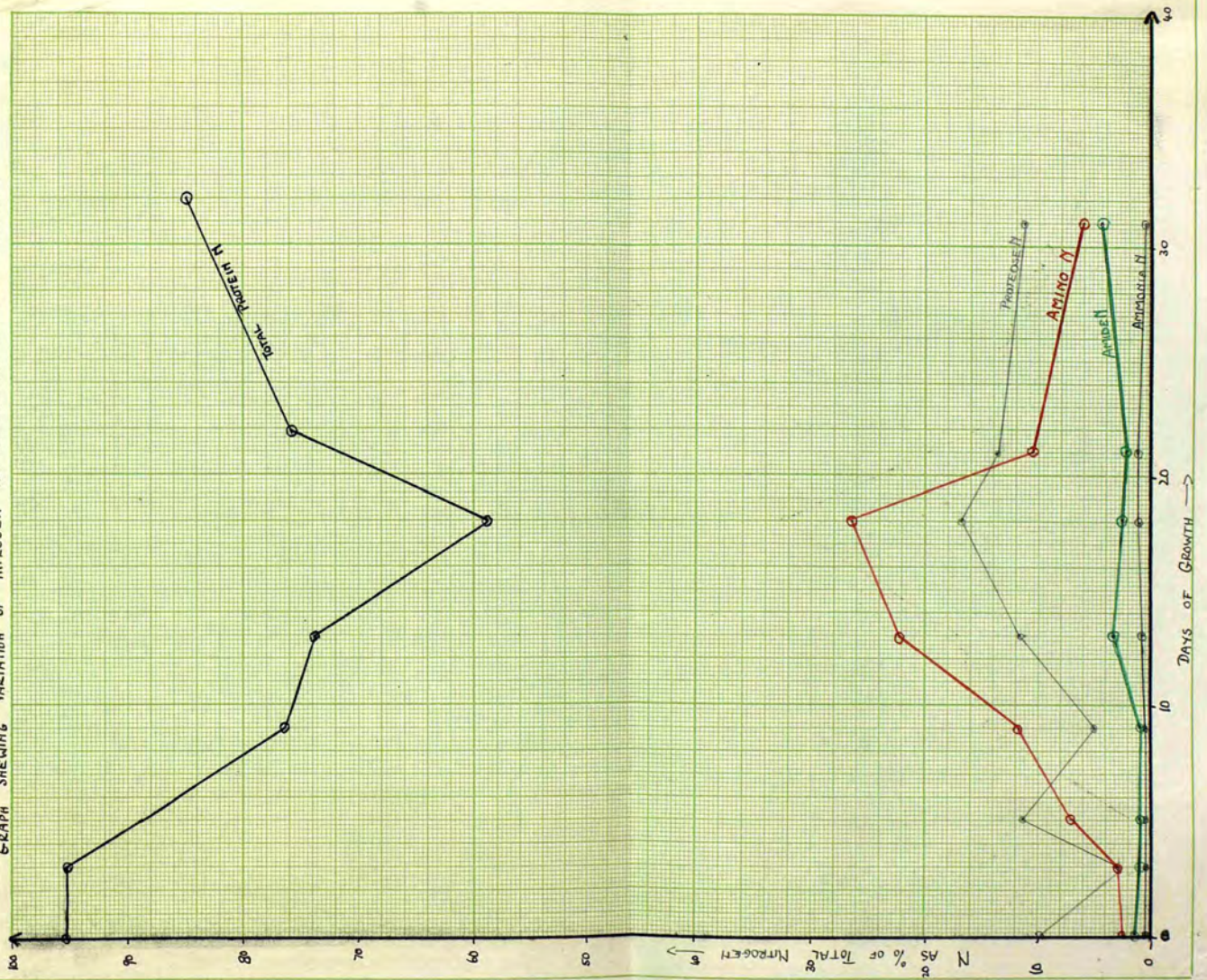GRAPH SHEWING VARIATION OF NITROGEN AS PERCENTAGE OF TOTAL NITROGEN

FIG: II .

FIG II

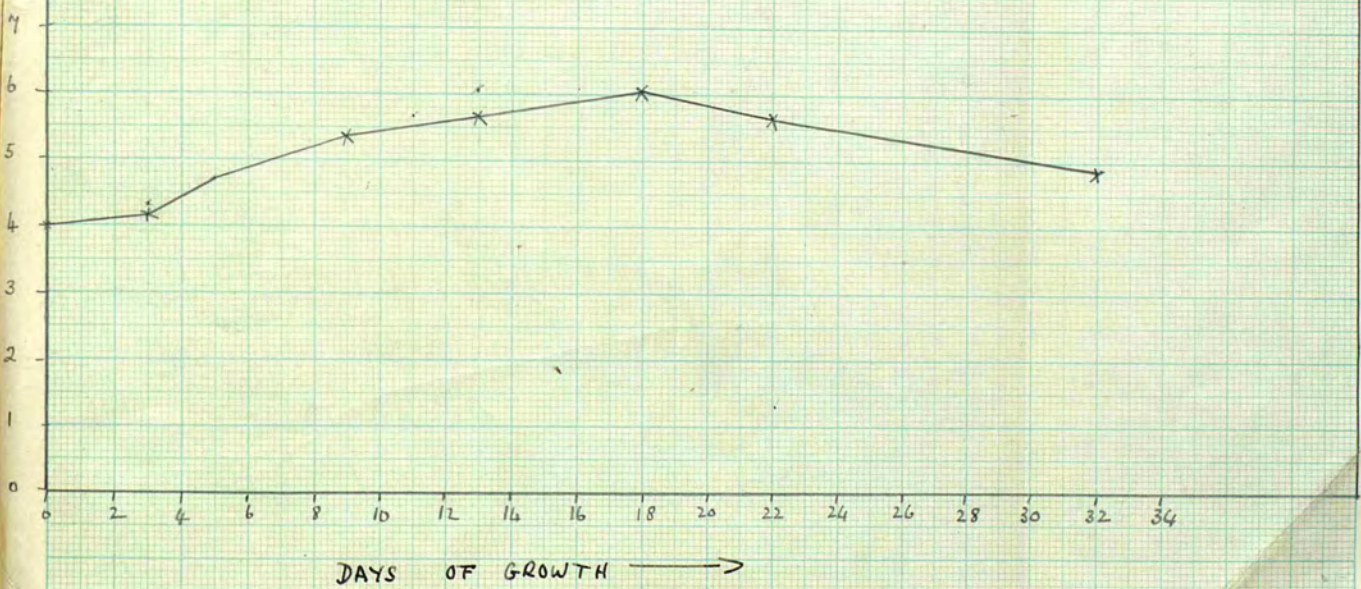GRAPH TO SHEW THE VARIATION IN TOTAL NITROGEN OF PEA MEAL, EXPRESSED AS % OF DRY WEIGHT

DAYS OF GROWTH ⟶

FIG III

GRAPH TO SHEW THE RELATION BETWEEN VARIOUS PROTEIN FRACTIONS

FIG: IV

GRAPH TO SHEW THE RELATION BETWEEN VARIOUS FORMS OF PROTEIN NITROGEN.

TOTAL PROTEIN

INSOLUBLE N

COAG. N + PROTEOSE N

COAGULABLE N

PROTEOSE N
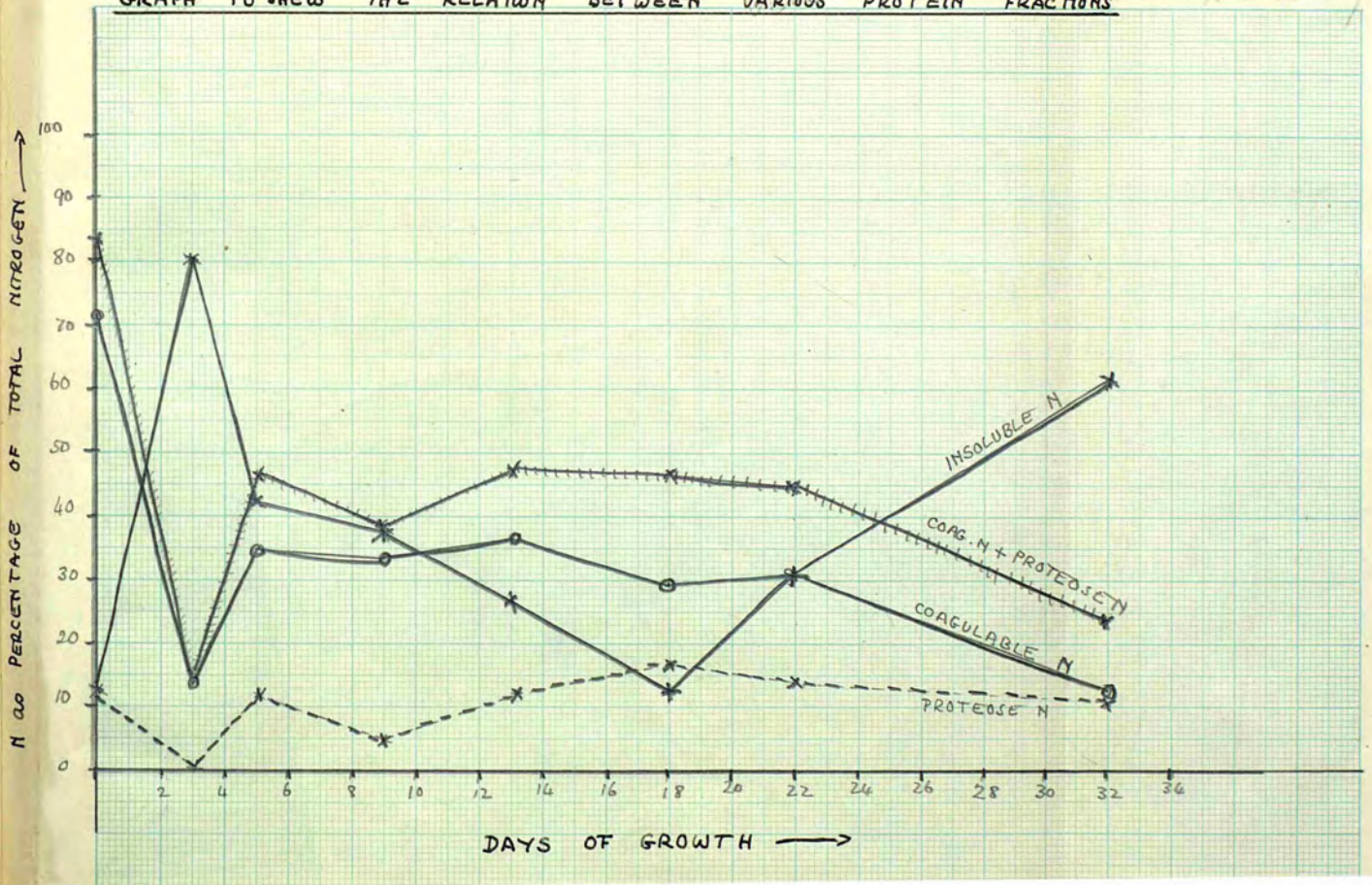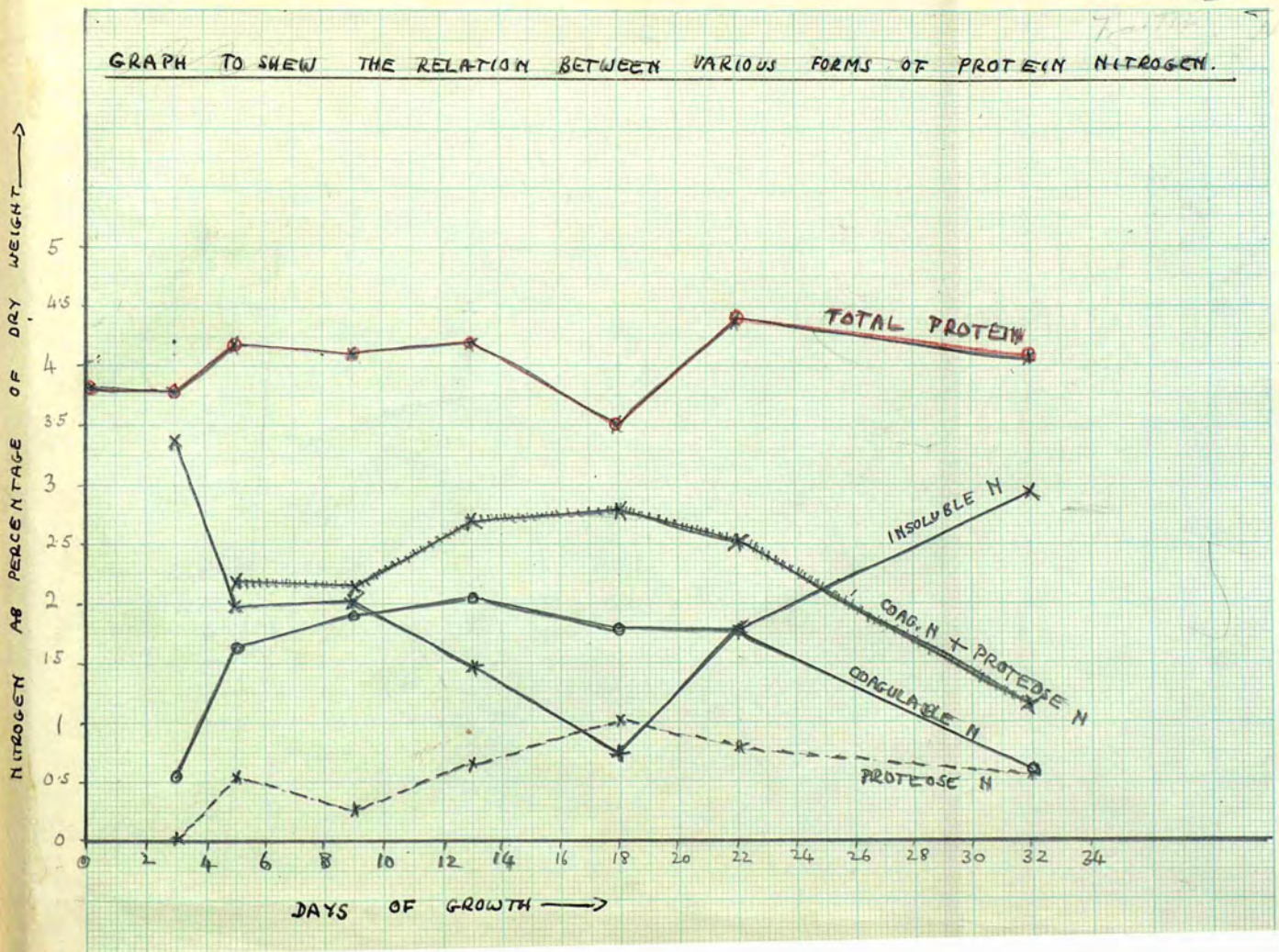
NITROGEN AS PERCENTAGE OF DRY WEIGHT →

DAYS OF GROWTH →

FIG IV

Fig. V — GRAPH TO SHEW RELATION BETWEEN VARIOUS FORMS OF PROTEIN NITROGEN