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STUDIES ON SOME CONSTITUENTS OF SOY-BEAN SEEDS AND THEIR TRANSFORMATIONS DURING GERMINATION

Shûiku SASAKI

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CHAPTER I

INTRODUCTION

From ancient times the soy-bean playd an important part in the Far East as food-stuffs, industrial materials, fertilizers, etc. In recenty years its export to Europe and America has increased greatly. The literature pertaining to the soy-bean is very extensive, but no attempt will be made to review it here as a whole.

The etiolated soy-bean seedlings germinated in the dark are used as a food in many parts of Japan and China; it is called "Moyashi" in Japan. MOLLIEX(1) studied its general constituents, SCHULZE,(2) (3) (4) wrote on some of its nitrogenous compounds and hemicellulose, YOSHIMURA(5) on its organic bases, and several other investigators on its asparagine contents.

The present investigation was performed to make further studies on the constituents of soy-bean seeds and the changes in those constituents during germination.

CHAPTER II

METHODS OF GERMINATION OF SOY-BEAN SEEDS

A) For small scale:—When seeds are germinated in the dark, they are usually infected by moulds or bacteria and cannot make normal growth without the use of an adequate method of sterilisation and of an apparatus for sowing. The following methods were used and good results obtained, being harmless for seeds and yet protecting them completely from infection.

A definite amount (10 ~ 20 g) of well selected seeds was placed in a beaker containing 70 per cent alcohol, and agitated several minutes: then the alcohol was poured off. The seeds were placed in a glass bottle containing 0.2 per cent mercuric chloride solution. The bottle was connected to a water sucker by means of a rubber tube provided with a sterilised cotton-wool air filter in the middle, evacuated, and shaken for 3 to 4 minutes. Then the mercuric chloride solution was poured off, and the seeds were washed thoroughly with distilled and sterilised water. The seeds were sowed in a loosely covered glass vessel which was previously sterilised, and contained silica sand 4 cm. thick, free from organic matter and moistened. After sowing, distilled and sterilised water was supplied from time to time so that the silica sand contained 12 to 14 per cent of moisture. The above procedure was performed in a HANSEN's inoculating box.

B) For large scale:—The following procedure was adopted and a comparatively good result obtained when a large quantity of seeds was germinated.

About 2 kg. of the seeds were steeped in water for 8 hours, and then sowed 3 cm. thick on a rice straw-mat in a wooden box, and covered with rice straws mat. For the first one or two days warm water (about 40°) was poured on twice a day. Excess of water was drained off through the several slits at the bottom of the box. After the seeds were germinated cold water was poured on. The young plants grew rapidly owing to the high temperature which was produced by their metabolic activity. Lengths of the seedlings were 20 to 30 cm. in 10 to 13 days after sowing.

CHAPTER III

GENERAL ANALYSIS OF NITROGENOUS COMPOUNDS OF THE SEEDS AND THE SEEDLINGS

Since the isolation by PASTEUR(6) of asparagine in leguninous seedlings germinated in the dark, the results of many investigations concerning the metabolism of nitrogenous compounds in the course of germination have been reported and including those on the soy-beans; nevertheless, many phenomena are still unexplained.

Experimental

The kind of specimen used was the "Yellow Autumn Seed No. 34" produced in Fukuoka, Japan. Seedlings were cultivated by the small scale method. Aliquot parts of the seeds and seedlings were taken to determine the total nitrogen by theKJELDAHL method. The other parts were ground with silica sand, and boiled to coagulate most of the protein with the addition of distilled water and acetic acid. The acidity of the solution was PH 3～4 which is near to the iso-electric point of soy-bean protein. The precipitates were filtered off and washed well with hot water. The nitrogens of the precipitates were determined. The filtrates were treated by the STUTZER method, and the nitrogens in the proteins determined. The filtrates of the precipitates by the STUTZER method were treated with sulfuric acid and phosphotungstic acid, and the nitrogens of the organic bases and the residues determined. Ammonical nitrogens in the samples were also determined.

Results and Discuision

The following table shows the nitrogen distribution of the seeds and seedlings cultivated in the light and in the dark.

Table I

Distribution of the Nitrogen of the Seeds and Seedlings by General Analysis

(Results expressed as percentage of the weight of original seeds used)

	Seeds	Seedlings			
		5 days after sowing		10 days after sowing	
		In light	In dark	In light	In dark
Total N	6.52	6.53	6.56	6.54	6.50
Protein N	5.68	4.50	4.49	3.72	3.60
Coagulable N	5.68	4.50	4.49	3.72	3.60
N precipitated by the STUTZER method	0	0	0	0	0
Non-protein N	0.75	1.93	2.04	2.72	2.83
Ammoniacal N	trace	—	—	0.014	0.008
N precipitated by phosphotungstic acid	0.48	0.84	0.90	1.19	1.21
Residual N	0.27	1.09	1.14	1.52	1.61

The amounts of total nitrogens did not change during germination either in the light and or in the dark.

Protein was gradually decomposed by germination, and the degree of the decomposition in the dark was slightly more than that in the light under the same condition.

After the coagulation of protein by heat at an adequate acidity, there occurred no precipitate of protein by the STUTZER method from the filtrate in any of the samples.

Free ammonia was scarcely detected either in the seeds or seedlings: this means that the urease which is contained in quantity in the soy-bean seeds does not act during germination, or that ammonia which is produced by the urease is soon converted into other compounds such as amide of aspartic acid.

In the seeds the amount of the organic base nitrogen was almost twice as much as that of the residual nitrogen, but in the seedlings this proportion was reversed, although the amount was increased.

CHAPTER IV

QUANTITATIVE DETERMINATION OF THE AMINO-ACIDS IN THE PROTEINS OF THE SEEDS AND SEEDLINGS BY THE VAN SLYKE METHOD

The object of this investigation was to determine the nitrogen distribution of the proteins of the seeds and the seedlings, and to examine whether the protein in the seeds, when germinated, makes homogeneous decomposition or not; that is to say some amino-acids in the protein may be broken down more rapidly than others.

To estimate the amino-acids in protein, the VAN SLYKE methods(7) (8) has been used by very many workers on account of its simplicity and accuracy, and even with regard to the protein of soy-beans this method has been used in more than ten instances as far as the author knows.

The results obtained by various workers do not always agree closely. The discrepancy may be due to the purity of protein, method of analysis, or individual errors: nevertheless these variations in results may be largely eliminated by the same conditions of analysis.

The results of the experiments of the several investigators will be shown on page 76.

Experimental

Definite quantities of the seeds and the seedlings were ground and boiled to coagulate the proteins at PH 3 ~ 4 with dilute acetic acid solution. The precipitates were filtered washed with boiling water, and extracted with alcohol and ether. The amino-acids were determined by the VAN SLYKE method as modified by PRIMMER and his co-workers(9) (10) (11).

Result and Discussion

Table II

Distribution of the Nitrogen of the Seeds and the Seedlings by the VAN SLYKE Method

(Results expressed as percentage of total nitrogen)

	Seeds	Seedlings			
		5 days after sowing		10 days after sowing	
		In light	In dark	In light	In dark
Insoluble humin N	2.9	3.2	3.3	4.3	4.0
Soluble humin N	0.8	1.1	1.0	1.1	0.9
Acid amide N	11.3	10.7	10.8	9.2	9.8
Diamino N	26.3	25.8	26.1	25.9	26.1
Arginine N	13.0	12.5	12.8	13.0	12.7
Histidine N	4.9	4.6	4.6	4.6	4.8
Cystine N	0.7	0.8	0.8	0.9	0.9
Lysine N	7.6	7.7	7.9	7.4	7.7
Manoamino N	57.4	57.5	58.1	58.1	57.7
Amino N	53.2	53.0	53.3	54.3	53.6
Non-amino N	4.2	4.5	4.8	3.8	4.1

High percentage of the nitrogens both in acid insoluble and soluble humin, and the increase of the former in proportion as the seedlings grow older are probably due to the amount of impurity mingled with the proteins.

There was a decrease of the amide nitrogen in the protein of the seedlings, but the difference was so little that it could not be decided whether this decrease came from the experimental error, the content of impurity, or the partial decomposition of the protein.

The previous experiments (Table I) showed that the nitrogen precipitated by phosphotungstic acid in the protein-free solution in the seeds had higher value than that of the non-precipitable nitrogen, and this relation was reversed in the seedlings. The explanation of this fact may be given from the results of the present experiment (Table II) as follows. If the protein is hydrolysed by germination and the basic substance of the hydrolysate produces only the basic substance in the solution, the amount of the nitrogen of non-basic compounds is naturally more than that of the basic compounds in the protein-free solution of the seedlings, as the amount of the nitrogen of the basic compounds, which is precipitated by phosphotungstic acid, is less than that of non-basic compounds in the protein of the seeds.

CHAPTER V

AMINO-ACIDS ISOLATED FROM THE HYDROLYSATE OF THE PROTEIN OF THE SEEDS

The investigations of Osborne and Campbell(12), and Muramatsu(13) show that the protein of soy-beans consists mainly of glycinin, a sort of globulin, associated with small amounts of phaseolin, a sort of globulin, legumelin, and isolegumelin, sorts of albumin, and proteose.

Osborne and Clap(14) hydrolysed glycinin, and isolated or detected glycine, valine, leucine, proline, phenylalanine, tyrosine, aspartic acid, glutamic acid, arginine, histidine and lysine, but as they worked by the old Fisher's ester method(15), and techniques of isolation were incomplete, they did not isolate arginine and histidine in crystalline forms from the hexone base fraction. Shimo and Yanagikawa(16) isolated leucine, proline, phenylalanine, aspartic acid and glutamic acid in the pressed cake of soy-beans, and Masuno and his co-workers(17)(18) leucine, valine, proline, phenylalanine, tyrosine, aspartic acid and glutamic acid in the residue of the methyl alcohol extract from soy-bean meal, but their work was rather qualitative, and the methods of identification not very strict.

Since the epoch-making work of the separation of protein hydrlysate by Fischer(15) some thirty years ago, many devices have been propossed and the study along this line has been greatly advanced.

Among these devices the following are noted.

Buthyl alcohol extraction method by Dakin(19)(20), and its modified method by Osborne, Leaveworth and Noran(21):—These methods are based on the fact that monoamino monocarboxylic acids only are extracted by butyl alcohol from the protein hydrolyate.

Lead salts method by Foreman(22):—This method is an improvement of Fischer's; i.e. a method by which the amino-acids of the hydrolysate are coverted into their dry lead salts and then esterified.

Copper salts method by Brazier(23) and Damodran(24), and its modified method by JOKES(25):—BRAZIER and DAMODARAN's method is one in which the amino-acids of the hydrolysate are converted into their copper salts and divided by means of their solubilities; JOKES' method is one in which the hexone bases of the hydrolysate are previously removed as their silver salts and phosphotungstates according to the method of VICKERY and LEAVENWORTH(26) and then the remaining amino-acids are isolated according the BRAZIER and DAMONARAN's copper salts method.

Carbamido-acids and hydantoins method by BOYD(27):—This method is one in which the amino-acids of the hydrolysate are isolated in the form of their corresponding carbamido-acids and hydantoins.

In addition to these methods there are several methods for isolating single amino-acid or a group of amino-amids from the hydrolysate of protein.

Experimental

(I) Preparation of Protein

The kind of specimen used was the "White Autumn Seeds" produced in Korea.

Preliminary experiments were carried out to find whether or not the total protein could be isolated in a pure state from soybean seeds. Nitrogen was estimated by the micro-KJELNAHL method.

Table III

Result of Separation of Protein in Seeds

(Figures represent per cent of total nitrogen.)

Form of nitrogen	
Total	100.0
Cotyledon freed from oil	98.0
Seed coat and oil	2.0
In cotyledon freed from oil.	
Substance precipitated by the STUTZER method	84.7
Substance not precipitated by the STUTZER method	13.3
In cotyledon freed from oil.	
Substance, neutralised with dilute alkali and extracted with 5% sodium chloride solution	92.5
Substance, neutralised with dilute alkali and not extracted with 5% sodium chloride solution	5.5
In the substance extracted with 5% sodium chloride solution.	

Substance coagulated by boiling at PH 3 ~ 4	77.9
Substance not coagulated by boiling at PH 3 ~ 4	14.7
In the substance extracted with 5% sodium chloride solution.	
Substance precipitated by the Stutzer method	79.2
Substance not precipated by the Stutzer method	13.3

From the above figures it was known that the protein in the the seeds was almost entirely extracted with 5 per cent saline water containing alkali sufficient to neutralise the acidity of seed, and coagulated by boilling at PH 3 ~ 4.

The protein used for the isolation of amino-acids was prepared by the following procedure.

The seeds were pressed, coarsely ground, extracted with petroleum ether, freed from outer seed coats, finely ground again, and extracted with petroleum ether until oil and allied substances were entirely removed. The powder was extracted with a large quantity of 5 per cent saline water containing alkali sufficient to neutralise the acidity of the powder. The insoluble matter was separated by centrifugation, and the precipitate extracted two more times with 5 per cent saline water as before. The supernatant was filtered through a layer of filter paper pulp. The filtrate was a clear yellowish solution. The solution was acidfied with dilute acetic acid to PH 3 ~ 4, and boiled for 20 minutes. After standing for a short time the supernatant liquid was syphoned out. The precipitate of protein was agitated by adding with boiling water and the supernatant was again syphoned out. After repeating this process seven times, the precipitate was filtered off by a Buchner funnel, and washed thoroughly with water, alcohol and ether, and dried over sulfuric acid. The protein, thus prepared, contained 2.7 per cent water, 0.09 per cent ash and 16.24 per cent introgen (16.60 per cent nitrogen as ash and and moisture free basis).

(II) Dicarboxylic Amino-Acid

(Aspartic acid, glutamic acid and oxyglutamic acid)

These amino-acids were isolated by modifying the method of Jones and Moller.

(1) From the hydrolysate by hydrochloric acid:—

50 g. of the protein (ash and moisture free basis) were hydrolysed with 250 c.c. of 20 per cent hydrochloric acid under reflux

condenser. Boiling was discontinued after 32 hours. The acid insoluble substance was removed by filtration, the filtrate concentrated to a small volume, and after saturating with hydrochloric acid gas it was allowed to stand for 5 days at – 10° ~ – 15°. The crude glutamic acid hydrochloride was separated, redissolved in a small volume of water, and recrystallised as before. The hydrochloride had a characteristic taste, and melted at 194°. After drying in a vacuum desiccator, it weighed 7.11 g.

Analysis :— 12.54 mg. substance : 3.45 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_5H_9NO_4 \cdot HCl$. Calculated. N 7.64
Found. 〃 7.70

Glutmatic acid was recovered from the hydrochloride and identified.

The combined filtrates and washings from the glutamic acid hydrochloride were diluted with water to make a volume of about 2 l., and hydrochloric acid was added till the concentration of the acid was 3.5 per cent. Diamino-acids were removed from the solution in the usual way by means of phosphotungstic acid, and the excess of the phosphotungstic acid of the filtrate removed quantitatively by shaking with a mixture of equal parts of amyl alcohol and ether. The aqueous phase was evaporated to syrup to remove hydrochloric acid as much as possible, and the residue taken up with water. Dicarboxylic amino-acids in the solution were precipitated with baryta and alcohol according to the direction of JONES and MOELLER(24). The precipitated barium dicarboxylates were decomposed with sulfuric acid and the excess of the sulfuric acid was removed by quantitative addition barium chloride. The solution was concentrated to about 50 cc., saturated with hydrochloric acid gas, and allowed to stand for a week at – 10° ~ – 15°. Glutamic acid hydrochloride was separated and dried. 2.55 g. of the hydrochloride were thus obtained, which, together with the 7.11 g. of hydrochloride directly separated from the hydrolysate of the protein, was equivalent to 7.83 g. of the free acid, or 15.7 per cent of the protein. The description of the identifications of the hydrochloride is omitted.

The filtrate from the glutamic acid hydrochloride was concentrated to a syrup, and taken up in water. Silver carbonate was added to a slight excess, the silver chloride which was precipipitated removed, and the excess of siver ion in the solution removed as silver sulfide. The solution was concentrated, tyrosine being crystallised out filtered off, and the filtrate treated with copper carbonate. 4.81 g. of copper aspartate were thus obtained corresponding to 2.30 g. of the free acid, or 4.60 cent of the protein.

The hydrate of copper aspartate crystallised in characteristic long needles of pale blue colour.

Analysis :— 1.0801 g. substance : 0.3208 g. H_2O
$C_4H_5NO_4Cu \cdot 4\frac{1}{2} H_2O$. Calculated. H_2O 29.4
Found. 〃 29.7

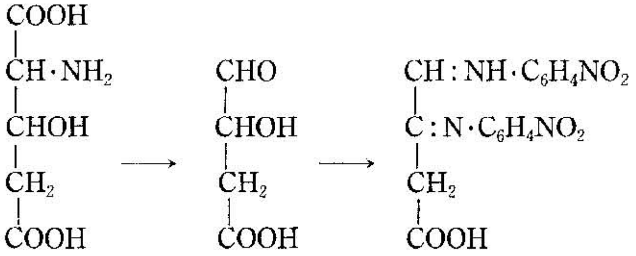
15.02 mg. substance : 3.93 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_4H_5NO_4Cu$. Calculated. N 7.19
Found, 〃 7.04

The filtrate from the copper aspartate, removing the copper by hydrogen sulfide, was concentrated to dryness in a vacuum desiccator, and the soluble matter extracted with 95 per cent alcohol. The residue was dissolved in a small quantity of water and recrystallised with absolute alcohol. It weighed 5.99 g., and contained 0.497 g. of nitrogen. If this amount of nitrogen was entirely due to oxyglutamic acid, it was equivalent to 12.6 per cent of the protein.

The following examinations were performed for the purpose of identification ;—

a) When the solution was evaporated to dryness under diminished pressure at ordinary temperature, a white crystalline magma was precipitated. It contained a trace of tyrosine according to MILLON'S test, but the crystals of tyrosine could not be separated. It was entirely extracted with acetic acid, but practically insoluble in ethyl alcohol, either, or ethyl acetate

b) According to DAKIN[19] oxyglutamic acid reacts with chloramine T and p-nitrophenyl-hydrazine, and forms a sparingly soluble osazone.



$$\begin{array}{l} COOH \\ | \\ CH \cdot NH_2 \\ | \\ CHOH \\ | \\ CH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CHO \\ | \\ CHOH \\ | \\ CH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} CH : NH \cdot C_6H_4NO_2 \\ | \\ C : N \cdot C_6H_4NO_2 \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

The yield of the osazone was almost as much as that given in the description by DAKIN. It was recrystallised by nitrobenzen and separated out as red-brown needles decomposing at 297° ~ 299°. On adding sodium hydroxide to an alcoholic solution containing a trace of this substance, a deep blue colour was produced.

c) Precipitation test :—Silver nitrate test and mercuric acetate test according to DAKIN(19) were positive for this substance, but these tests are also positive for other amino-acids especially for aspartic and glutamic acids, and not characteristic of oxyglutamic acid only.

d) Colour test :—Resorcinol test, α-naphthol test and β-naphthol test according to DARKIN were positive, but the colours developed were not so intensive.

e) Oxyglutamic acid was prepared from HAMMERSTEN casein and indentified with the substance prepared from the soy-bean protein.

(2) From the hydrolysate by sulfuric acid :—

200 g. of the protein (ash and moisture free basis) were hydrolysed by boiling with 1100 cc. of 25 per cent sulfuric acid for 32 hours. The cooled solution was diluted to 4 l. Humin was filtered out, thoroghly washed with hot water and the nitrogen content determined. Ammonia and melanin nitrogen were determined in a small aliquot of the solution by adding an excess of baryta. The main solution was diluted to about 5.5 l., and treated with phosphotungstic acid. The precipitate of basic amino-acids was filtered off, and dissolved in a mixture of acetone and water. The solution was treated with baryta to remove the reagents, concentrated to about 5 l., and again treated with sulfuric acid and phosphotungstic acid. The precipitate was filtered off and washed with 5 per cent sulfuric acid.

The combined filtrates and washings freed from basic amino-acids were neutralised with baryta, and the precipitate of the barium compounds was filtered. The precipitate was ground with water, warmed in boiling water, and filtered off to separate the adhering amino-acids from the precipitate. The extraction was repeated 6 times. A large amount of tyrosine was crystalled out from the combined filtrate and extract by concentration. 7.05 g.

of pure tyrosine were obtained by recrystallisation. From the filtrate of the precipitate of tyrosine, 30.83 g. of glutamic acid, 10.17 g. of aspartic acid, 20.18 g. of oxyglutamic acid and 0.32 g. of tyrosine were isolated by the same procedure as described in the case of the " hydrolysate by hydrochloric acid ". The description of the methods of separations and identifications is omitted.

The alcoholic filtrates from the precipitate of the barium salts of dicarboxylic amino-acids were freed from alcohol and barium, and the amino-acids in the solution converted into their ethyl esters according to the method of FOREMAN(22). The barium residue obtained by filtering off the second chloroform solution of esters and the distillation residue of the esters (of which detailed descriptions will be given in the following pages) were combined and again treated with an excess of baryta and alcohol to separate dicarboxylic amino-acids. 2.16 g. of glutamic acid, which together with the first crop was equivalent to 16.50 per cent of the protein, 6.22 g. of oxyglutamic acid, which together with the first crop was equivalent to 13.20 per cent of the protein, and 0.02 g. of tyrosines were further obtained. The description of the methods of separations and identifications is omitted.

(III) **Monocarboxylic Monoamino-Acids**

(Glycine, alanine, valine, leucine, isoleucine,
phenylalanine and tyrosine)

The alcoholic filtrate, described above, from the precipitate of the barium dicarboxylate, was used for this purpose (the protein used for analysis was 200 g. as ash and moisture free basis).

After adding a large quantity of water to the solution, the alcohol was distilled off, and the barium removed as barium sulfate. The amino-acids in the solution were converted into their ethyl esters according to the method of FOREMAN(22). For this purpose an excess of litharge was added to the hot solution of amino-acids and steam passed in for 45 minutes. After filtering off and washing the excess of the litharge the solution was ovaporated to dryness. The dry lead lead salts were supeneded in absolute alcohol and the mixture was saturated with dry hydrochloric acid gas. After standing for a short time the supernatant was decanted off and replaced by fresh absolute alcohol. The precipitate of lead chloride was then centrifuged off from the alcoholic solution and washed several times with absolute alcohol. The greater parts of the hydrochloric acid and alcohol were removed by destillation under reduced pressure from the ethyl ester hydrochlorides. The residue of the distitillation was dissolved in dry chloroform, and esters were liberated by means of an excess of anhydrous baryta. After the removal of barium compounds by filtration and of chloroform by distillation, the residue was entirely dissolved in anhydrous ether and fractionally distilled in the usual manner by an apparatus described by FISCHER and HARRIES(29).

The barium residue remaining after filtering the chloroform solution of esters was decomposed with warm dilute sulfuric acid, the barium sulfate filtered off, and the most part of the free hydrochloric acid removed by distillation. The residue was again converted into lead salts and then ethyl esters, and the esters were fractionally distilled as in the manner already described.

Unesterified residue

The barium residue filtering the second chloroform solution of esters was decomposed with sulfuric acid, barium sulfate removed, and hydrochochloric acid quantitatively removed by means of silver carbonate. The solution was again treated with phosphotungstic acid and sulfuric acid. A small amount of brown precipitate was filtered off and the reagents were completely removed from the filtrate by means of barium hydroxide solution. The solution was neutralised with dilute ammonia and evaporated to a small volume. 0.21 g. of tyrosine were crystallised out. The filtrate from the tyrosine was further evaporated, but it was impossible to separate the crystals of serine. The solution was combined with the distillation residue of esters, which will be mentioned later, and used for further researches on dicarboxylic amino-acids.

Distillation of esters

The two lots of esters dissolved in ether were separately distilled, and then the fractions obtained at the same temperature and pressure were combined.

Table IV

Fractional Distillation of Esters

Fraction No	Temperature of the vapours up to	Pressure
I (Glycine fraction)	70°C	16
II (Leucine fraction)	70°C	3
Distillation residue	—	—

The esters of the "glycine fraction" and the "leucine fraction" were separately hydrolysed by boilling with water for 7 ~ 8 hours, and the hydrolysates evaporated to dryness. The dry residues were extracted by boiling with absolute alcohol, and the extracts evaporated to dryness and re-extracted with absolute alcohol.

The alcoholic extracts of the two fractions were combined and amino-nitrogen, total nitrogen and rotation were examined showing that the solution contained not only active proline, but also a considerable amount of racemic proline and a little of other amino-acids.

The residue of the "leucine fraction" insoluble in boiling absolute alcohol was fractionally crystallised with water. The first 1 ~ 3 crops, which, by determing the nitrogen contents, seemed to contain some glycine or alanine, were combined to the residue insoluble in boiling absolute alcohol of the "glycine fraction" of the ester distillates. The next 4 ~ 8 crops seemed to contain valine, leucine and isoleucine, and the last 9 ~ II crops seemed to contain leucine and isoleucine,

Glycine:—OSBORNE and CLAP(18) had isolated glycine as its ester hydrochloride from the glycinin hydrolysate, but in the present experiment the isolation of glycine was performed by LEVENE and VAN SLYKE's picrate method(30). 0.76 g. of glycine picrate were obtained from the combined substances resulting from the alcohol insoluble residue of the "glycine fraction" and from the 1 ~ 3 crops of the fractional crystallisation of the "leucine fraction". The filtrate from the precipitate of the glycine picrate was freed from picric acid in the usual way, and the amino-acids in the solution were converted into their copper salts to remove alanine copper by a method which will be described later. From the easily soluble and non-crystallisable fraction of the copper salts, the copper was removed as copper sulfide, and 0.45 g. of glycine picrate were further obtained, which together with the 0.76 g. of the picrate of the first crop was equivalent to 0.46 g. of free glycine or 0.23 per cent of the protein. The glycine picrate was in the form of yellow long plates or prisms decomposing at 200 ~ 202°.

Analysis :— 0.3125 g. substance : 0.1879 g. Picric acid
$(C_2H_5NO_2)_2\,C_6H_3N_3O_7$. Calculated. Picric acid 60.26
Found. 〃 〃 60.14

Free glycine was obtained from the picrate by treating with ether and sulfuric acid. It crystallised in clear long needles decomposing at 230°.

Analysis :— 5.01 g. substance : 3.28 cc. $N/50\ H_2SO_4$ (Micro-KJELDAHL)
$C_2H_5NO_2$. Calculated. N 18.7
Found. 〃 18.3

Alanine :—The filtrate from the first crop of the glycine picrate was freed from picric acid, and the amino-acid in the solution were converted into their copper salts by copper carbonate. The solution of copper salts was evaporated to dryness and the residue extracted with methyl alcohol. The extract consisting of copper salts of valine and isoleucine was freed from copper by hydrogen sulfide, and combined with the 4 ~ 8 crops of the fractional crystalisation of the " leucine fraction " of the ester distillates.

The copper salts insoluble in methyl alcohol were fractionally crystallised several times and divided into the following 3 fractions.

1) Copper salts hardly soluble in water :—This fraction, containing leucine copper only, was combined with the copper salt insoluble in methyl alcohol from the " leucine fraction " and decomposed with hydrogen sulfide.

2) Copper salt easily soluble in water and crystallisable in deep purplish blue prisms :—This fraction contained alanine copper only weighing 10.45 g.

3) Copper salts easily soluble in water and non-crystallisable :—This fraction, consisting of copper salts of glycine and

alanine, was decomposed with hydrogen sulfide, and treated with picric acid by the method of LEVENE and VAN SLYKE(30) to obtain the second crop of glycine picrate weighing 0.45 g. The filtrate from the glycine picrate was freed from picric acid, and pure alanine copper recovered weighing 1.46 g. which together with 10.45 g. of the first crop was equivalent to 8.24 g. of free alamine or 4.12 per cent of the protein. The copper salt decomposed at 255°.

Analysis :— 20.0 mg. substance : 4.12 cc. N, 27°, 752 mm. (VAN SLYKE)
$(C_3H_6NO_2)_2Cu \cdot H_2O$. Calculated. N 10.9
Found. 〃 11.1

The free alanine crystallised in beautiful large needless.

Analysis :— 6.39 mg. substance : 3.56 cc. N/50 H_2SO_4 (Micro-KJELDHAL)
$C_3H_7NO_2$. Calculated. N 15.73
Found. 〃 15.61

The rotation and melting point of this substance were much lower than those of the ordinary active alanine. It was identified further by converting it into bezoyl-alanine.

Valine :—The copper salts of the "glycine fraction" soluble in methyl alcohol were freed from copper by hydrogen sulfide, and combined with the 4 ~ 8 crops of the fractional crystallisation of the "leucine fraction". The combined solution was treated with ammonia and lead acetate according to the method of LEVENE and VAN SLYKE(31). The filtrate of the precipitate of lead salts was freed from lead by hydrogen sulfide, and 5.11 g. of valine were obtained. The valine crystallised in silverly lustred plates.

Analysis :— 8.91 mg. substance : 3.82 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_5H_7NO_2$. Calculated. N 11.96
Found. 〃 12.01

Rotation :— 0.820 g. substance in 13 cc. of 20% HCl
Rotation in 2 dm. tube, + 3.55°. ∴ $(\alpha)_D^{20} = + 28.2°$

The copper salt was easily soluble in methyl alcohol.

Isoleucine :—The precipitate of the lead salts was freed from lead by hydrogen sulfide, combined with the last 9 ~ 11 crops of the fractional crystallisation of the "leucine fraction", and converted into copper salts by copper carbonate. The solution was evaporated to dryness and the residue extracted with methyl alcohol.

The extract was evaporated to dryness. The isoleucine copper thus obtained crystallised in blue lustred plates weighing 5.92 g. which was equivalent to 4.76 g. of isoleucine or 2.38 per cent of the protein.

Analysis :— 0.3653 g. substance : 0.0889 g. CuO
$(C_7H_{12}NO_2)_2$ Cu. Calculated. Cu 19.64
Found. 〃 19.48

The copper salt was decomposed with hydrogen sulfide to free from copper.

Analysis :— 9.64 mg. substance : 3.69 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_6H_{13}NO_2$. Calculated N 10.69
Found 〃 10.72

Rotation :— 0.7681 g. substance in 10 cc. of 20% HCl.
Rotatation in 2 dm. tube, + 5.67°. $\therefore [\alpha]_D^{20} = +36.90°$

Leucine :—The copper salt of the alanine fraction hardly soluble in water was combined with the residue remaining after extracting the copper salt of the "isoleucine fraction" with methyl alcohol, and freed from copper by hydrogen sulfide. On evaporation 20.04 g. of leucine were obtained. The leucine crystallised in lustred thin plates and had a bitter taste.

Rotation :— 0.4986 g. substance in 10 cc. of 20% HCl
Rotation in 2 dm. tube, + 1.69°. $\therefore [\alpha]_D^{20} = +16.9°$

The lead salt obtained from the leucine by the method of LEVENE and VAN SLYKE(31) gave the following result on analysis.

Analysis :— 0.3713 g. substance : 0.2418 g. $PbSO_4$
$(C_7H_{12}NO_2)_2$Pb. Calculated. Pb 44.3
Found 〃 44.2

The distillation residue

The residue remaining after the distillation of the esters was shaken with water and ether in the usual way in order to remove the phenylalanine ester. The water phase was boiled with 25 per cent sulfuric acid to hydrolyse the esters, and the pyrrolidonecarboxylic acids which might be formed. The hydrolysates were diluted and treated with phosphotungstic acid in the usual way, and the filtrate from a small brown precipitate was freed from reagents quantitatively by baryta. The solution was combined with the substance from the unesterified residue remaining after filtering the chloroform solution of esters, and used for the further researches of dicarboxylic amino-acids according to the method of JONES and MOELLER(28). The yields of the amino-acids have already been described.

Phenylalanine: The ester extracted by ether from the distillation residue of the esters was dissolved in concentrated hydrochloric acid, and the solution evaporated to a small volume. From this solution there were obtained 8.90 g. of phenylalanine hydrochloride. The hydrochloride crystallised in clear prisms hardly soluble in concentrated hydrochloric acid.

Analysis :— 12.61 mg. substance : 3.04 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_9H_{11}NO_2 \cdot HCl$. Calculated. N 6.95
Found. 〃 6.74.

The free phenylalanine obtained by decomposition of the hydrochloride with ammonium hydroxide gave the following results on analysis.

Analysis :— 11.75 mg. substance : 3.53 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_9H_{11}NO_2$. Calculated. N 8.49
Found. 〃 8.40

As it was impossible to separate the phenylalanine hydrochloride further by crystallisation, the phenylalanine in the filtrate was detetermined indirectly by converting it into benzoic acid according to the methods of KOLLMANN(32). The solution was oxidised by boiling for 6 hours with BECKMANN's mixture(33); the benzoic acid, together with the fatty acids formed, were extracted with ether in a continuous extractor. On evaporating the ether solution to dryness the benzoic acid was separated from the fatty acids by washing with water previously saturated with benzoic acid, and dried to constant weight at 60°. It was compared with the benzoic acid prepared from pure phenylalanine. By this method 3.21 g. of phenylalanine were further obtained which together with 8.90 g. of the phenylalanine hydrochloride previously obtained was equivalent to 10.42 g. of phenylalanine or 5.21 per cent of the protein.

Tyrosine :—A complete separation of tyrosine from the other products of protein hydrolysis is very difficult to accomplish. It was shown in this experiment that the tyrosine was either isolated by crystallisation, or detected by MILLON's test in various fractions. The main fractions from which the tyrosine was recovered were as follows:—

1) When the protein hydrolysate was treated with phosphotungstic acid, and the filtrate from the precipitate of the basic amino-acids was neutralised with baryta, tyrosine was isolated weighing 7.05 g.

2) When the first crop of the precipitate of barium salts by the method of JONES and MOELLER(28) was decomposed with sulfric acid, tyrosine was isolated weighing 0.32 g. (if the solution is acidic, tyrosine is not crystallised).

3) When the first crop of glutamic and aspartic acids was removed from the filtrate of the precipitate of the tyrosine of 2), tyrosine was isolated weighing 0.03 g.

4) When the barium residue remaining after filtering the chloroform solution of esters was decomposed with sulfuric acid and treated with phosphotungstic acid, and the solution neutralised with baryta, tyrosine was isolated weighing 0.21 g.

5) When the second crop of barium dicarboxylates was precipitated from the combined solution of unesterified residue and undistilled residue of the esters and the precipitate decomposed with sulfuric acid, tyrosine was isolated weighing 0.02 g.

The total yield of tyrosine weighed 7.63 g. corresponding to 3.82 per cent of the protein. A representative sample of the tyrosine had the following properties.

Analysis:— 11.36 mg. substance: 3.20 cc. N/50 H_2SO_4 (Micro-KJELDAHL).
$C_9H_{11}NO_3$. Calculated. N 7.74
Found. 〃 7.70

Rotation:— 0.7325 g. substance in 10 cc. of 10% HCl
Rotatian in 2 dm. tube, +2.01°. $\therefore (\alpha)_D^{20} = +13.7°$

(IV) Proline

At it was impoesible to isolate active proline quantitatively by the ester method, another 100 g. of the protein (ash and moisture free basis) were hydrolysed with 600 cc. of 25 per cent sulfuric acid. After the hydrolysis was complete the solution was diluted with 2 l. of water, and basic amino-acids were precipitated with phosphotungstic acid. The filtrate from the precipitate of phosphotungstate was treated with baryta to remove the phosphotungstic acid and sulfuric acid. Amino-acids in the solution were converted into their copper salts by adding copper carbonate to the boiling solution until there was no further effervescence; after boilling for a short time it was evporated to dryness with an excess of the carbonate; the solid was extracted with water and the extract again evaporated to dryness with more copper carbonate; the residue was granulated by stirring with dry acetone to aborb the last trace of moisture according to the method of TOWN(34). The pewder of dry copper salts was stirred with 100 cc. of anhydrous methyl alcohol for one hour, the alcohol filtered off, and the residue again treated with methyl alcohol as before. This extraction was repeated six times. The combined solution of methyl alcohol was freed from the solvent and dried with aceton as before. The powder was re-extracted with anhydrous methyl alcohol, and the extract evaporated to dryness. The dry residue of the copper salts was taken in water and freed from copper by hydrogen sulfide. The solution was evaporated to dryness and the residue extracted with absolute alcohol. The alcohol was distilled off from the extract and the residue taken up in water. Non-amino-nitrogen was estimated, and the solution treated with a sufficient quantity of picric acid to combine with the non-amino-nitrogen according to the method of TOWN(34). After evaporating to a small volume and cooling pure proline picrate crystallised out. On further evaporation of the mother-liquor a second small crop of somewhat impure proline picrate was obtained. The picrate was washed with cold water, and, after drying in a desicator, extracted with ether to remove an excess of picric acid. The yield of proline picrate weighed 11.90 g.

The aqueous and ethereal filtrates from the proline picrate were freed from picric acid by means of sulfuric acid and ether in a continuous extractor, and were boiled for 15 hours with 20 per cent sulfuric acid to hydrolyse any proline peptides. After the quantitative removal of sulfuric acid the solution was evaporated to a syrup, taken up in 95 per cent alcohol, and treated with a saturated solution of cadmium chloride according to the method of KAPFHAMMER and ECK(35). The precipitate was filtered off and washed with cold 95 per cent alcohol, re-dissolved in water and freed from cadmium chloride by silver carbonate and hydrogen sulfide. The solution was once more treated with picric acid. The yield of proline picrate weighed 0.82 g., which together with 11.90 g. of the first crop was equivalent to 4.21 g. of proline.

The proline picrate crystallised in golden yellow needles, and melted sharply at 147°.

Analysis :— 1.3682 g. substance : 0.9216 g. Picric acid
$C_5H_9NO_2 \cdot C_6H_3N_3O_7$. Calculated. Picric acid 66.9
Found. 〃 〃 67.3

The free proline contained no amino-nitrogen by the VAN SLYKE method(36) and had the following optical rotation.

Rotation :— 0.4865 g. substance in 10 cc. of water.
Rotation in 2 dm. tube, $-6.38°$. $\therefore [\alpha]_D^{20} = -83.1°$

(V) Basic Amino-Acid *

(Arginine, histidine and lysine)

The basic amino-acids of the protein hydrolysate were isolated by comparing and choosing the methods KOSSEEL and STAUDT(37), VICKERY and LEAVENWORTH(38)(39), CALVERY(40), VICKERY and BLOCK(41), and VICKERY and SHORE(42). 70 g. of the protein (ash and moisture free basis) were hydrolysed by boilling with 350 cc. of 20 per cent hydrochloric acid. After the hydrolysis was complete the solution was evaporated to a syrup to remove most of the hydrochloric acid, and the syrup taken up in water. The solution was decolorised by charcoal, and glutamic acid removed as hydrochloride by saturating with hydrochloric acid gas in the usual way. The crude glutamic acid hydrochloride was dissolved in a small volume of water and re-crystallised as hydrochloride to recover any basic amino-acids adhering to the crude hydrochloride. The combined filtrate from the glutamic acid hydrochlorides was evaporated to a syrup to remove as much hydrochloric acid as possible. The residual syrup was dissolved in water. Keeping

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* The investigation of the basic amino-acids in the soy-bean protein was carried out with the collaboration of Mr. AKINE, to whom the author wishes to express his thanks.

the solution distinctly acid to congo-red with sulfuric acid, silver oxide was added until all the chlorides had been precipitated. The precipitate was filtered and suspended in dilute hydrochloric acid; the mixture was boiled to dissolve any basic amino-acids which might adhere to the precipitate. The filtrate from the precipitate was freed from chlorine as before and combined with the main solution of monoamino-acids.

Keeping the solution acid with sulfuric acid as before, silver oxide was added until silver ion had been excess; then hot saturated baryta solution was added until the solution had been brought to PH 7.0 ~ 7.2 The precipitate (I) was filtered off and washed once with cold water. The combined filtrate and washing were concentrated to a small volume, and hot saturated baryta solution was again added until the solution had been brought to PH 11 ~ 12. The precipitate (II) was filtered off and washed once with cold water made alkaline with baryta. The combined filtrate and washing were acidified and set aside (solution (I)).

The precipitate (I) and the precipitate (II) were separately taken up in dilute sulfuric acid, and treated with hydrogen sulfide. To the solutions free from barium and silver was added silver oxide in excess keeping the solutions acid as before. Then baryta was added until the solutions had been brought to PH 7.0 ~ 7.2. The precipitates resulting from the precipitate (I) and the precipitate (II) were combined and decomposed with the sulfuric acid and hydrogen sulfide as before (crude histidine fraction). The filtrates from the precipitates of the "crude histidine fraction" combined, concentrated to a small volume, and treated with baryta until the solution had been brought to PH 11 ~ 12. The precipitate was filtered and decomposed with sulfuric acid and hydrogen sulfide as before (First crude arginine fraction). The filtrate from the precipitate of the "First crude arginine fraction" was acidified and combined with the solution (I) (Crude lysine fraction).

Histidine:—To the "crude histidine fraction", free from barium and silver, was added sulfuric acid to make 5 per cent, and histidine precipitated by addition of HOPKINS' reagent(43) according to the method of CALVERY(40). The precipitate was decomposed with hydrogen sulfide, the filtrate from the mercuric sulfide brought to PH 5 ~ 6 by means of baryta, and the precipitate of barium sulfate filtered. The solution was treated with a sufficient amount of flavianic acid to make histidine into its difravianate, assuming that all the nitrogen in the solution was due to histidene. The fravianate crystallised in pale yellow needles and decomposed at 240° by effervescence.

Analysis :— 0.2614 g. substance : 0.1495 g. $BaSO_4$
$C_6H_9N_3O_2 \cdot 2C_{10}H_6O_8SN_2$. Calculated. S 8.14
$C_6H_9N_3O_2 \cdot C_{10}H_6O_8SN_2$. 〃 〃 6.83
Found. 〃 7.92

As shown above histidine diflavianate contains 8.14 per cent S., and histidine monoflavianate 6.83 per cent S.; consequently this flavianate was a mixture of two flavianates in a ratio of 83 to 17 according to the description of VICKERY(44). The yield of the flavianate weight 8.08 g. which was equivalent to 1.78 g. of histidine or 2.55 per cent of the protein. It was further verified by converting the flavianate into histidine dihydrochloride.

As the filtrate from the precipitate by HOPKINS' reagent(43) contained much nitrogen, it was freed from mercury and the greater part of sulfuric acid, treated with an excess of silver oxide, and divided into histidine fraction and arginine fraction by varying PH with baryta as before. The precipitate of histidine fraction was decomposed and treated with HOPKINS' reagent(43), but no precipitate of histidine occurred. The precipitate of argine fraction was decomposed with sulfuric acid and hydrogen sulfide (Second crude arginine fraction).

Arginine:—The "First crude arginine fraction" and the "Second crude arginine fraction" free from barium and silver were treated with flavianic acid according to the method of KOSSEL and STAUDT(37). 12.45 g. and 0.80 g. of arginine monoflavianate were thus obtained from the "First crude arginine fraction" and the "Second crude arginine fraction" respectively, which was equivalent to 4.70 g. of arginine or 6.71 per cent of the protein. The flavianate crystallised in brownish yellow microscopic plates and decomposed at 260°.

Analysis :— 0.3115 g. substance : 0.1425 g. $BaSO_4$
$C_6H_{14}N_4O_2 \cdot C_{10}H_6O_8SN_2$. Calculated. S 6.53
Found. 〃 6.34

Lysine :—The "Crude lysine fraction" was freed from barium and silver, and concentrated to about 700 cc. Lysine was precipitated as phosphotungstate by phosphotungstic acid and sulfuric acid in the usual way. After standing for 3 days at 0° the precipitate was filtered off and not washed. The precipitate was then dissolved in a mixture of acetone and water, and freed from phosphotungstic acid and sulfuric acid by baryta. The solution was concentrated, and the lysine reprecipitated by phosphotungstic acid and sulfuric acid. The precipitate was filtered and decomposed by baryta; the solution free from the reagent was evaporated to a syrup, and about two-thirds of the calculated amount of a saturated solution of picric acid in absolute alcohol required to form the lysine monopicrate added. After standing over night, the precipitate of lysine monopicrate was filtered off; the filtreate was again treated with one half of the remainder of the calculated amount of the picric acid solution, and a small crop of the picrate further obtained. The combined yield of the first and the second crops weighed 9.84 g. which was equivalent to 3.80 g. of lysine or 5.43 per cent of the protein. The picrate crystallised in golden yellow needles and decomposed at 250 ~ 252° with a slight explosion.

Analysis :— 1.007 g. substance : 0.6235 g. Picric acid.
$C_6H_{14}N_2O_2 \cdot C_6H_3N_3O_7$. Calculated. Picric acid. 61.45
Found. 〃 〃 61.97

The lysine picrate was treated with hydrochloric acid and ether to free from picric acid in the usual way, and lysine dihydrochloride obtained by evaporation. The hydrochride crystallied in transparent prisms and melted at 215°.

Analysis :— 8.04 mg. substance : 3.65 cc. $N/50\ H_2SO_4$ (Micro-Kjeldahl)
$C_6H_{14}N_2O_2 \cdot 2\,HCl$. Calculsted. N 12.78
Found. 〃 12.71

(VI) Tryptophane

The tryptophane in the protein was calorimetrically determined by the method Folin and Marenzi⁽⁴⁵⁾ showing the tryptophane to be 1.4 per cent.

(VII) Cystine and Methionine

These substances were not determined by the author. Results worked out by TOMIYAMA and HANADA(46) in this laboratory have shown that the protein of soy-bean seeds contains 1.05 per cent of cystine and 1.96 per cent of Methionine.

Results and Discussion

The pretein in soy-bean seeds could be isolated almost quantitatively in a pure state.

The protein was hydrolysed by mineral acid, and the amino-acids in the hydrolysate were isolated in the form of crystals except tryptophane, cystine and methionine which were determined calorimetrically. The results are shown as follows.

Table V

Results of Seperation of Amino-Acids of the Protein Hydrolysate

Amino-acid	Form in which separated	% of the weight of the protein	% of total nitrogen
Melanin	Insoluble in acid		1.67
	Soluble in acid		1.93
Ammonia			9.50
Glutamic acid	Hydrochloride	16.50	9.44
Aspartic acid	Copper salt	5.19	3.29
Oxyglutamic acid		13.20	6.78
Glycine	Picrate	0.23	0.26
Alanine	Copper salt	4.12	3.09
Valine		2.56	1.84
Isoleucine	Copper salt	2.38	1.53
Leucine		10.02	6.45
Phenylalanine	Hydrochloride Benzoic acid	5.21	2.66
Tyrosine		3.82	1.78
Proline	Picrate	4.21	3.09
Arginine	Flavianate	6.71	12.93
Histidine	Flavianate	2.55	3.99
Lysine	Picrate	5.43	6.27
Tryptophane	Calorimetric	1.3	1.07
Cystine*	〃	1.05*	0.74*
Methionine*	〃	1.96*	1.11*
Total		86.44	79.42

* TOMIYA, T. and HANADA. M., J. Agr. Chem. Soc. Japan, 10, 53 (1934).

The main source of the loss of amino-acids of the protein hydrolysate occurs, as Jokes(27) pointed out, in the filtration and the washing of the rejected precipitate; hence before and after the filtration nitrogen was always determined by Micro-Kjeldahl method, and the filtration and the washing were carried out as completely as possible. Concentration was generally performed at a temperature not over 40° under reduced pressure in a Cleisen flask or at a ordinary temperature in a vacuum desiccator in order to avoid the loss of amino-acids by passing into peptides, racemic compounds and pyrrolydone-carboxylic acids. When a comparatively soluble compound was separated from its mother liquor, the filtrate from the precipitate was concentrated; second or third crops, if necessary, were separated, all crops recrystallised, and the last filtrate of recrystallisation was combined with the last mother liquor.

Although aspartic acid was perfectly precipitated with baryta and alcohol by the method of Jones and Moeller(28), glutamic and oxyglutamic acids were partially soluble by the same method; these soluble parts were recovered further by the second crop of barium salts from the combined solution of unesterified and undistilled residues by the ester methods. The yield of glutamic acid in this experiment was much less than those of Osborne and Gilbert(47) (19.46 %) and Jones and Moeller(28) (18.5 %), and the yield of aspartic acid than that of Jones and Moeller(28) (9.4 %). An extraordinary high yield of oxyglutamic acid was obtained as compared with that of the experiment of Onuki.(48) Loss of amino-acids occured scarcely in the esterification and destillation of esters by the method of Foreman(22), and satisfactory results were obtained for the separation and purification of individual monoamino-acids.

Alanine and isoleucine were isolated for the first time, as far as the authors knows, from the hydrolysate of soy-bean protein.

As it was impossible to separate active proline quantitatively by the ester method, another hydrolysate was used for the isolation: the first crop of the proline was obtained as picrate by the method of Town(34), and the second crop obtained as cadmium chloride compound from the filtrate of the picrate. The yield of the proline thus obtained was nevertheless much less than that of Spörer and Kapfhammer(49) (4.29 % of the protein N).

Although many studies of the basic amino-acids of soy-bean protein have been made by the VAN SLYKE method, examination of glycinine of OSBORNE and CLAPP(14) was probably the only one based on gravimetry: even in their work, arginine and histidine were not actually isolated as crystallised compounds. The present results are in close agreement with that of the author's VAN SLYKE method, and represent at least the minimum value of the basic amino-acids of the protein. In the following table the auther's results are compared with those of many other investigators.

Determination of tryptophane was performed by the method of FOLIN and MARENZI(45).

Values of cystine and methionine are quoted from the results of TOMIYAMA and HANADA(46) obtained in our laboratory.

CHAPTER VI

ORGANIC BASES OF THE SEEDS

A large proportion of the nitrogen of the protein free compounds of the seeds originates from organic bases. SCHULTZE(3) detected arginine and choline, and YOSHIMURA(5) detected adenine, arginine, choline and trigonelline, but as their studies had not been exhaustive, several further organic bases were found in this experiment.

Detection of the free monoamino-acids was abandoned, since it was very difficult to separate a small quantity of monoamino-acids from the mixture containing carbohydrates and other impurities.

Experimental

The kind of specimen used was the "White autumn seeds" produced in Korea. The results of the general analysis are shown as follows.

Table VII

Distribution of the Seeds by General Analysis

(Results expressed as percentage of the weight of seeds)

Protein N	6.48
Non-protein N	0.55
A. N. precipitated by basic lead acetate	0.16
B. N. precipitated by phosphotungstic acid after the removal of the precipitate by basic lead acetate	0.24
Residual N	0.15

Table VI

Basic Amino-Acids of Soy-Bean Protein

(Results expressed as percentage of total nitrogen)

Investigator	Ammonia	Melanin	Arginine	Histidine	Lysine	Cystine	Mono-amino	Non-amino	Remarks
NOLLAU[50] *	12.97	3.69	15.52	2.60	7.07	1.52	48.76	7.12	Crude protein
GRINDLAY & SLATER[51] *	10.12	6.63	12.67	5.77	6.14	0.67	49.76	8.56	〃 〃
JONES & WATERMAN[52] *	12.19	0.97	15.35	2.38	10.27	0.80	55.18	2.93	Glycinin
FRIEDMANN[53] *	11.31	1.84	14.57	5.92	8.26	1.04	54.32	2.71	Protein soluble in 0.2% NaOH
HAMILTON & Associates[54] *	9.38	2.87	15.70	5.60	6.18	1.46	48.28	2.43	Crude protein
MASUNO & NISHIMURA[55] *	9.58	6.14	15.55	7.03	6.08	1.74	49.76	5.19	Crude protein, mean of 4 species
TADOKORO & YOSHIMURA[56] *	7.60	1.47	16.04	10.69	5.90	0.78	59.19	5.90	Legumelin, mean of 4 species
〃 〃 *	9.87	1.72	16.31	7.83	7.74	0.46	56.08	7.74	Glycinin A, mean of 4 species
〃 〃 *	8.70	2.69	13.86	7.66	7.64	0.63	58.83	7.64	Glycinin B, mean of 4 species
〃 〃 *	7.73	1.64	12.91	8.31	9.54	0.50	59.49	9.54	Glycinin, mean of 4 species
TOMIYAMA & Associates[57] *	10.39	2.32	16.30	4.81	7.99	1.50	52.95	2.60	Total protein
SASAKI *	11.3	3.7	13.0	4.9	7.6	0.7	53.2	4.2	Total protein
OSBORNE & CLAPP[14] *			9.39	2.15	3.10				Glycinin
SASAKI *			12.93	3.99	6.27				Total protein

* VAN SLYKE Method
** Gravimetry

5 kg. of well selected seeds were pressed and coarsely ground, extracted with petroleum ether, freed from outer seed coats, finely ground again, and extracted with petroleum ether until oil and allied substances were entirely removed. The powder was extracted with 15 l. of boiling water: this extraction was repeated five times: at the first extraction a little acetic acid was added to the extracting water to coagulate the protein. The extracts were treated with neutral and basic acetates successively, and the clarified solution was freed from lead by hydrogen sulfide. The bases were precipitated with sulfuric acid and phosphotungstic acid in the usual way. The liquid was filtered off, and the precipitate thoroughly decomposed with baryta: the excess of barium was removed as carbonate.

(1) *Precipitate by Silver Nitrate (Adenine and Guanine)*

The solution of bases thus obtained was neutralised with nitric acid and concentrated to a small volume. A large amount of well crystallised potassium nitrate was removed by successive crystallisation with water and alcohol, and then purine bases were precipitated from the solution by the addition of concentrated solution of silver nitrate. The precipitate was decomposed with hydrochloric acid and then subjected to the phosphotungstate method in the usual way. Two kinds of hydrochloride resulted by the addition of hydrochloric acid to the solution of free bases, namely, adenine and guanine hydrochlorides; and these were separated by means of fractional crystalliration.

a) Adenine:—Adenine picrate was obtained from the soluble part of the hydrochlorides by treating with sodium picrate. Neither xanthine nor hopoxanthine picrate was obtained. The picrate crystallised in hardly soluble needles and decomposed at 283° ~ 284° weighing 1.40 g. which was equivalent to 0.52 g. of adenine. The adenine chloroplatinate crystallised in yellow needles and did not decompose until 285°.

Analysis:— 0.2143 g. substance: 0.0613 g. Pt
$(C_5H_5N_5 \cdot HCl)_2\,PtCl_4$. Calculated. Pt. 2.87
Found. 〃 2.86

b) Guanine:—Free guanine was obtained from the insoluble part of the hydrochlorides by treating with dilute ammonia. The yield of guanine weighed 0.31 g. This substance gave nitric acid test, but did not give KOSSEL's reaction, while the adenine gave KOSSEL's reaction, but did not give nitric acid test. The guanine chloroplatinate crystallised in hardly soluble yellow particles.

Analysis:— 0.2261 g. substance: 0.0834 g. Pt
$C_5H_5N_5O \cdot HCl \cdot PtCl_4$. Calculated. Pt 37.2
Found. 〃 35.9

(2) *Pecipitate by Silver Nitrate and Baryta (Histidine and Arginine)*

To the filtrate from the precipitate formed by silver nitrate were added silver nitrate and baryta alternatively until a brown precipitate had been formed. This precipitate was filtered, washed with cold water, made alkaline with baryta, decomposed by hydrogen sulfide, and then subjected to the phosphotungstate method in the usual way.

a) Histidine:—The solution of free bases thus obtained was saturated with carbon dioxide and treated with mercuric chloride. The precipitate was filtered and decomposed by hydrogen sulfide. The solution was neutralised with sodium hydroxide, clarified with tannic acid, and again subjected to the phosphotungstate method. The solution of the free base was strongly acidified by hydrochloric acid, and 0.58 g. of histidine dihydrochloride were obtained by concentration which was equivalent to 0.40 g. histidine. The dihydrochloride crystallised in transparent platees and decomposed at 240°.

Analysis:— 0.1983 g. substance: 0.2467 g. AgCl
$C_6H_9O_2N_3 \cdot 2HCl$. Calculated. Cl 31.1
Found. 〃 30.8

b) Arginine:—The filtrate from the precipitate by mercuric chloride was freed from mercury by hydrogen sulfide, and arginine flavianate obtained by the method of KOSSEL and GROSS(38). Flavianic acid was freed from the flavianate by treating with baryta, excess of the barium removed as carbonate, and arginine nitrate obtained by neutralising the solution with nitric acid. The yield of arginine nitrate (crystal water free) weighed 9.55 g. which was equivalent to 7.02 g. of arginine. The nitrate crystallised in milky white microscopic needles and decomposed at 130°.

Analysis :— 0.1125 g. substance : 0.1911 g. $C_{20}H_{16}N_4 \cdot HNO_3$ (Nitron method)
$C_6H_{14}N_4O_2 \cdot HNO_3$. Calculated. HNO_3 26.6
Found. 〃 2.62

(3) *Filtrate from the Precipitate by Silver Nitrate and Baryta (Choline and Trigonelline)*

The filtrate was freed from silver and barium, and subjected to the phosphotungstate method. The solution of free bases was neutralised by hydrochloric acid and evaporated to dryness. The dry residue was extracted with methyl alcohol to remove potassium chloride. The methyl alcoholic solution was again evaporated to dryness; the residue was divided into two parts by means of fractional crystallisation with absolute alcohol.

The soluble part was treated with alcoholic solution of mercuric chloride. After standing several days the precipitate was filtered and divided into two part by means of fractional crystallisation with water.

a) Choline :—The insoluble part of the compounds of mercuric chloride was taken up in water, and freed from mercury by hydrogen sulfide; choline hydrochloride was obtained from the solution by evaporation. The hydrochloride crystallised in hygroscopic transparent needless. The hydrochloride was treated with chloroauric acid, and choline chloroaurate obtained, weighing 24.36 g. which was equivalent to 6.66 g. choline. The chloroaurate was in the form of yellow leaf-like crystals and decomposed at 251°.

Analysis :— 0.2149 g. substance : 0.0958 g. Au
$C_5H_{14}NOCl_3 \cdot AuCl$. Calculated. Au 44.5
Found. 〃 44.4

The chloroplatinate was in the form of orange-yellow prisms and decomposed at 238°.

Analysis :— 0.2571 g. substance : 0.0821 g. Pt
$(C_5H_{14}NOCl)_2$ PtCl. Calculated. Pt 31.6
Found. 〃 31.9

b) Trigonelline :—The soluble part of the compound of mercuric chloride was freed from mercury, evaporated to dryness, and combined with the hydrochloride insoluble in absolute alcohol. The dry substance was again treated with methyl alcohol: the soluble part was trigonelline hydrochloride which crystallised in silverly lustred prisms and decomposed at 258°. The yield of the hydrochloride was 0.33 g. which was equivalent to 0.25 g. of trigonelline. The normal trigonelline chloroaurate crystallised in plates and decomposed at 299°.

Analysis:— 0.2011 g. substance: 0.0845 g. Au
$C_7H_7NO_2 \cdot HCl \cdot AuCl_3$. Calculated. Au 41.3
Found. 〃 42.2

The basic chlorourate crystallised in pale yellow prisms and decomposed at 187°.

Analysis:— 0.1658 g. substance: 0.0620 g. Au
$(C_7H_7NO_2)_4 (HCl)_3 (AuCl_3)_3$. Calculated. Au 37.7
Found. 〃 37.4

Results and Discussion

The experiment was performed as quantitatively as possible for the purpose of examining what variation of the bases might occur during germination.

The results of the experiment are compared with those of SCHULZE(3) and YOSHIMURA(5) in the following table:

Table VIII

Organic Bases in the Water Extract of the Seeds
(Figures represent g. of bases isolated from one kg. of the seeds)

	SCHULZE	YOSHIMURA	SASAKI
Adenine	—	0.02	0.10
Guanine	—	—	0.06
Histidine	—	present	0.08
Arginine	0.33	0.07	1.40
Choline	0.61	0.08	1.33
Trigonelline	—	0.01	0.05
Betaine	present?	—	—

CHAPTER VII

MONOAMINO-ACIDS AND A ACID-AMIDE IN THE JUICE OF THE ETIOLATED SEEDLINGS

In spite of the interest of the problem, of researching the distribution of amino-acids in the plant tissue, the works have been comparatively little reported. This is partly due to the lack of adequate methods of separation; nevertheless, much progress has been made in recent years in our knowledge of the analysis of the protein hydrolysate as well as the derivatives of the amino-acids, and a summary of this development will throw some light on the method of separation and identification of amino-acids contained in impure solution such as plant tissue.

Very many investigations have been reported on the asparagine in the seedlings of several species, among which soy-bean seedlings have been investigated by SCHULZE(2), SUZUKI(59), KOJIMA(60) and many others: however, concerning to amino-acids in the juice of soy-bean seedlings only one investigation was carried out, and that by SCHULZE(2), who dectected phenylalanine.

Experimental

The kind of specimen used was the "Yellow Autumn Seeds" produced in Kagoshima, Japan. Well selected seeds were sowed in a dark room. After 12 days the etiolated seedlings, having stems and roots 12 ~ 18 cm., were plucked out. There were almost no decayed plants, and furthermore the seedlings were selected with strict care. The results of the general analysis are shown as follows:

Table IX

Distribution of the Nitrogen in the Seedlings by General Analysis

(Results expressed as percentage of the total nitrogen)

Protein N	53.7
Non-protein N	46.3
N. precipitated by basic lead acetate	4.0
N. precipitated by phosphotungstic acid after the removal of the precipitate by basic lead acetate	16.6
Residual N	25.7

(I) Asparagine

The seedlings germinated from 4 kg. of the seeds were used for analysis. The yield weighed about 49 kg. They were comminuted and extracted with 15 l. of boiling water: this extraction was repeated five times: at the first extraction a little acetic acid was added to the extracting water to coagulate the protein. The extract was treated with neutral and basic lead acetates successively, and the clarified solution freed from lead by hydrogen sulfide. The bases in the solution were precipitated with sulfuric acid and phosphotungstic acid in the usual way; the precipitate was filtered and washed with cold 5 per cent sulfuric acid. The combined filtrate and washing were treated with baryta to free from reagent: excess of barium was removed as sulfate, and the solution concentrated to a small volume.

After standing for two days at 0° a large amount of asparagine, which crystallised out, was separated and recrystallised weighing 190.31 g. The asparagine was in the form of large transparent rhombic crystals and decomposed at 225°.

Analysis:— 4.89 mg. substance: 2.89 cc. N/50 H_2SO_4 (Micro-Kjeldahl.)
$C_4H_8N_2O_3 \cdot H_2O$. Calculated. N 18.59
Found. 〃 18.62

The copper salt crystallised in insoluble microscopic prisms.

Analysis: — 0.3608 g. substance: 0.0700 g. Cu
$(C_2H_7N_2O_3)_2$ Cu. Calculated. Cu 19.52
Found. 〃 19.40

After the removal of the first crop of asparagine the solution was treated with Neuberg's reagent(61), which consists of mercuric acetate, sodium carbonate and alcohol. The precipitate was filtered, washed with 80 per cent alcohol, taken up in water, and decomposed by hydrogen sulfide. 7.83 g. of asparagine were obtained from the solution by concentration.

Analysis: 0.4982 g. substance: 0.0595 g. H_2O
$C_4H_8N_2O_3 \cdot H_2O$. Calculated. H_2O 12.01
Found. 〃 11.96

4.13 mg. substance: 2.68 cc. N/50 H_2SO_4 (Micro-Kjeldahl)
$C_4H_8N_2O_3$. Calculated. N 21.22
Found. 〃 21.26

The filtrate from the second crop of asparagine was again treated with sulfuric acid and phosphotungstic acid, and a small volume of brown precipitate removed. The filtrate from the precipitate was neutralised with baryta to free from reagents and divided into two parts of equal volume.

a) One part of the solution was made up to 500 cc. with water and 100 cc. of 15 per cent sulfuric acid, and boiled for 2.5 hours under a reflux condenser to hydrolyse acid-amide into the corresponding ammonia and dicarboxylic amino-acid. After cooling, the sulfuric acid was removed with an excess of baryta, and the dicarboxylic amino-acid was precipitated from the solution with baryta and alcohol by the method of JONES and MOELLER(28). The precipitate was decomposed with sulfuric acid, and the filtrate from the barium sulfate concentrated to a small volume. The solution was saturated with hydrochloric acid gas, seeded with crystals of glutamic acid hydrochloride, and stood for a week at $-10^\circ \sim -15^\circ$: but no crystals were separated: then the solution was freed from hydrochloric acid by means of silver carbonate, and treated with copper carbonate to make the amino-acid in the solution into copper salt. 10.46 g. of characteristic crystals of copper aspartate were separated out, which was equivalent to 5.05 g. of aspartic acid or 5.68 g. of hydrous asparagine.

Analysis :— 0.3148 g. substance : 0.0638g. Cu
$C_4H_7NO_4Cu \cdot 4\frac{1}{2} H_3O$. Calculated. Cu 23.08
Found. 〃 22.81

By decomposing the copper salt free aspartic acid was recovered. The aspartic acid crystallised in prisms and decomposed at 269° ~ 270°.

Analysis :— 10.94 mg. substance : 4.05 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_4H_7NO_4$. Calculated. N 10.53
Found. 〃 10.36

The filtrate from the copper aspartate was treated with hydrogen sulfide to free from copper. The solution constained 0.228 g. of nitrogen and reacted as sttrong acid, but neither glutamic acid nor oxyglutamic acid could be detected.

b) Another part of the solution was directly treated with baryta and alcohol by the method of JONES and MOELLER(28). The precipitate was decomposed with sulfuric acid, and the filtrate from the precipitate of barium sulfate treated with copper carbonate to make the amino-acid and acid-amide in the solution into copper salts. The precipitate, which consisted of a mixture of cobalt-coloured prisms (copper aspartate) and pale blue long needles (copper asparaginate), was filtered off, taken up in water, and decomposed by hydrogen sulfide to free from copper. 513 g. of dry substance were obtained by evaporating the solution. It was hydrolysed by boiling for 2.5 hours with 200 cc. of 3 per cent sulfuric acid, and 0.304 g. of free ammonia were obtained from the hydrolysate. If one molecule of asparagine liberates one molecule of ammonia and one molecule of aspartic acid by hydrolysis, 51.3 g. substance must contain 2.46 g. of aspartic acid and 2.67 g. hydrous asparagine. The sulfuric acid used for the hydrolysis was removed quantitatily with baryta, and pure aspartic acid obtained.

Analysis :— 9.30 mg. substance : 3.52 cc. N/50 H_2SO_4 (Micro-Kjedahl)
$C_4H_7NO_4$. Calculated. N 10.53
Found. 〃 10.61

The aspartic acid was further verified as copper salt.

The aspartic acid which was found in the mixture with asparagine at b) seemed to be the decomposition product of asparagine; decomposition having taken place in the course of analysis in spite of sufficient care. If this is true, the yield of hydrous asparagine at b) must weigh 5.39 g. by calculation which is a little less than that at a) weighing 5.68 g.; this discrepancy may be explained by the fact that although aspartic acid is completely precipitated with baryta and alcohol by the method of Jones and Moeller(28), asparagine is incompletely precipitated by the same method, and the escaped asparagine recovered from the distillation residue of esters: this experiment will be described later.

The total yield of anhydrous asparagine weighed 184.93 g. which was equivalent of 4.62 per cent of the seeds.

(II) Monoamino-Acids

The seedlings germinated from 7 kg. of the seeds in the dark were treated as in the case of the isolation of asparagine. Most of the monoamino-acids in the juice of the seedlings were contained in the alcoholic filtrate from the precpitate of barium salt obtained by the method of JONES and MOELLER(28).

The alcoholic filtrate was concentrated, and barium removed quantitatively with sulfuric acid. For the purpose of isolating monoamino-acids, they were converted into their ethyl ester according to the method of FOREMAN(22). The solution was heated, and, under the passing of steam, an excess of litharge added. After filtering off and washing the excess of litharge the filtrate was evaporated to dryness, but contrary to the case of the hydrolysis of the protein, it was somewhat viscous.

Since the process of preparation and distillation of ethyl ester was much the same as that of the hydrolysis of the protein already described, the description is omitted.

Unesterified Residue

The barium residue remaing after filtering the second chloroform solution of esters was decomposed with sulfuric acid, and the precipitate of barium sulfate removed. The remaining hydrochloric acid was quantitatively removed by silver carbonate, and the solution concentrated. It was a slightly acidic syrup, and neither serine nor any other amino-acids could be separated.

Distillate of Esters (Alanine, Leucine and Isoleucine)

The two fractions of the distillate, i. e. the distillate at 70° and 16 mm., and the distillate at 70° and 3 mm. were hydrolysed separately by boiling with water for 7 ~ 8 hours, and the solution evporated to dryness. The dry residue was extracted by boiling with absolute alcohol, and a very little quantity of yellowish magma resulted from the extracts by evaporation: proline was not isolated.

The residue insoluble in absolute alcohol from the distillate at 70° and 16 mm. was fractionally crystallised by water, but no pure amino-acids were obtained: then all the crops were combined and converted into copper salts. The copper salts were dried and extracted with methyl alcohol. The residue insoluble in methyl alcohol was fractionally crystallised by water.

Alanine:—The copper salt insoluble in methyl alcohol, but easily soluble in water, was freed from copper by hydrogen sulfide.

As this substance was in the form of white powder containing 15.01 % N (pure alanine crystallises in needles containing 15.73 % N), it was coverted into benzoyl-alanine by the method of FISCHER(62). The benzoyl-alanine crystallised in clear prisms and melted sharply at 158°.

Analysis :— 13.55 mg. substance : 3.45cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_{10}H_{11}NO_3$. Calculated. N 7.25
Found. 〃 7.21

The yield of benzoyl-alanine weighed 5.2 g. which was equivalent to 2.4 g. of alanine. Alanine was recovered from the benzoyl-alanine by the method of FISCHER(62), and α-methyl-hydantoic acid prepared from the alanine by the method of LIPPICH(63). The α-methyl-hydantoic acid crystallised in prismatic needles and melted at 157°.

Analysis :— 5.26 mg. substance : 4.02 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_4H_8N_2O_3$. Calculated. N 21.22
Found. 〃 21.40

Glycine was not isolated.

Leucine :—The residue insoluble in absolute alcohol from the distillate at 70° and 3 mm. was coverted into copper salt and extracted with methyl alcohol. The residue was combined with the fraction of copper salt insoluble in water which was derived from the distillate at 70° and 16 mm., and freed from copper by hydrogen sulfide. The yield of leucine weighed 11.6 g. It crystallised in lustred white thin plates with a slightly bitter taste.

Analysis :— 10.49 mg. substance : 4.12 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_6H_{13}NO_2$. Calculated. N 10.69
Found. 〃 11.00

Rotation :— 0.483 g. substance in 10cc. of 20 % HCl
Rotation in 2 dm. tube, + 1.66°. $\therefore (\alpha)_D^{20} = +17.2°$

Carbamido-acid, i.e. α-uramido-isobutyl-acetic acid, was prepared from the leucine by the method of BOYD(27). It crystallised in microscopic needles almost insoluble in water and melted at 208°.

Analysis :— 5.91 mg. substance : 3.39 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_7H_{14}N_2O_3$. Calculated. N 16.09
Found. 〃 16.05

The yield was 95 per cent of the theory. Hydantoin i.e., isobutyl hydantoin, prepared from the carbamido-acid crystallised in thin plates resembling leucine and melted at 215°. It was almost insoluble in either cold or hot water.

Isoleucine:—Two copper salts soluble in methyl alcohol which were derived from the distillate at 70° and 16 mm., and from the distillate at 70° and 3 mm., crystallised in lustred purplish-blue thin plates. They were combined and freed from copper by hydrogen sulfide. The yield of isoleucine weighed 7.1 g.

Analysis:— 9.04 mg. substance: 3.51 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_6H_{13}NO_2$. Calculated. N 10.69
Found. 〃 10.87

Rotation:— 0.457 g. substane in 10 cc. of 20% HCl
Rotation in 2 dm. tube, +2.85. $\therefore [\alpha]_D^{20} = +30.5°$

The rotation was much less than that of active isoleucine ($[\alpha]_D^{20} = +37.4°$ in 20 per cent hydrochloric acid according to LEVENE and VAN SIYKE(31)). Further verification of this substance was performed by preparing carbamido-acid and hydantoin by the method BOYD(27). The carbamido-acid, i.e. α-uramido-secbutly-acetic acid, resembled α-uramido-isobutyl-acetic acid prepared from leucine as regards its melting point, solublity and crystalline form. The hydantoin, i.e. secbutyl-hydantoin, crystallised in long needles and melted at 95° by rapid heating. This substance was easily soluble in hot water, but comparatively insolubile in cold water.

Analysis:— 6.89 g. substance: 4.38 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_7H_{12}N_2O_2$. Calculated. N 17.95
Faund. 〃 17.78

Valine was not isolated.

Distillation Residue (Phenylanine and Aspartic Acid)

The disistillation residue of esters was taken up in water and extracted with ether in the ususal way to remove phenylalanine ester.

Phenylalanine:—The ester extracted with ether was dissolved in concentrated hydrochloric acid, and the solution evaporated to a small volume on a steam bath. Crude phenylalanine hydrochlochloride was separated and recrystallised by concentrated hydrochloric acid. The yield weighed 5.3 g., which was equivalent to 4.3 g. of phenylalanine.

Analysis :— 12.41 mg. substance : 3.07 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_9H_{11}NO_2 \cdot HCl$. Calculated. N 6.95
Found. 〃 6.93

Free phenylanine was prepared by decomposing the hydrochloride with ammonia. It crystallised in lustred thin plates and decomposed at 270°.

Analysis :— 10.44 mg. substance : 3.14 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_9H_{11}NO_2 \cdot HCl$. Calculated. N 8.49
Found. 〃 8.43

Aspartic acid :—The aqueous solution which remaining after extracting the phenylalanine ester with ether, was hydrolysed by boiling with concentrated hydrochloric acid, and the hydrochloric acid removed by silver carbonate. The solution was treated with copper carbonate, and 3.8 g. of copper aspartate obtained which was equivalent to 1.8 g. of aspartic acid. It was crystallised in pale blue characteristic needles almost insoluble in water.

Analysis :— 14.30 mg. substance : 2.95 cc. N/50 H_2SO_4 (Micro-KJELDAHL)
$C_4H_7NO_4 \cdot 4\frac{1}{2} H_2O$. Calculated. N. 5.09
Found. 〃 5.08

The filtrate from the precipitate of the copper aspartate was concentrated, but no substance remained other than a little syrup.

Tyrosine

Crystals of tyrosine were not isolated, and MILLON's test was negative in every fraction of the above analysis ; and moreover, tyrosine was not detected in the juice of the seedlings by the basic lead acetate method of SCHMALFUSS, HEIDLER and WINKELMANN(64).

Results and Discussion

4 kg. of soy-bean seeds were sowed in the dark, and 184.93 g. of asparagine which was equivalent to 4.62 per cent of the seeds was isolated from the juice of the seedlings.

2.4 g. of alanine (as benzoyl-alanine), 11.6 g. of leucine, 7.1 g. of isoleucine and 4.3 g. of phenylalanine (as hydrochloride) were isolated from the juice of the etiolated seedlings germinated from 7 kg. of the seeds: a little quantity of aspartic acid was also isolated, but this substance seemed to be produced by the decomposition of asparagine in the course of analysis in spite of great care.

All amino-acids isolated except asparagine were rather qualitative, but strictly verified.

As already stated in Chapter III, when the seeds are germinated, the protein is decomposed gradually, but the quantities of amino-acids which consist of the "building stones" of the protein molecule are not changed before and after germination; and as stated in Chapter IV, soy-bean protein contains a considerable amount of aspartic, glutamic and oxyglutamic acids; while in this experiment these discarboxylic amino-acids, except for a small amount of aspartic acid, were not dected in the juice of the seedlings. From these facts it is deduced that, as soon as the protein is hydrolysed by germination these dicarboxylic amino-acids are transformed into other substances, most probably such as asparagine which is plentifully contained in the juice.

Asparagine was incompletely precipitated by the baryta alcohol method of JONES and MOELLER(28), and the escaped asparagine was recovered as aspartic acid from the distillation residue of esters.

Asparagine was completely precipitated by NEUBERG's reagent(61) so that the mercuric nitrate used by SCHULZE(65) for the precipitant of asparagine may be replaced by this reagent.

Glycine was not detected in the juice. Although the glycine, which is produced by hydrolysing the protein, is not transformed into other substances, it is difficult to detect it, because the glycine content in the protein is very small; but it is certain that other amino-acids of higher molecules are not transformed into glycine which is the lowest homologue of all amino-acids.

Valine and proline, which are the constituents of soy-bean protein, were not dected in the juice, but the absence of these amino-acids is doubtful, because these two substance are, as NEUBERG and KERB(61) stated, not precipitated quantitatively by NEUBERG's reagent(61) which was used in this experiment.

Tyrosine was not detected in the juice at all. The fate of this amino-acid during germination is probably the same as that of the dicarboxylic amino-acids.

CHAPTER VIII

ORGANIC BASES IN THE JUICE OF THE ETIOLATED SEEDLINGS

SCHULZE(2)(3) reported that the juice of soy-bean seedlings contained histidine, arginine and choline, while YOSHIMURA(5) detected adenine, guanine, choline and betaine in the same material.

It has already been demonstrated in Chapter IV that 6.71 per cent of arginine, 2.55 per cent of histidine and 5.43 per cent of lysine were contained in the protein of the seeds, and in Chapter V that 0.010 per cent of adenine, 0.006 per cent of guanine, 0.140 per cent of arginine 0.008 per cent of histidine, 0.133 per cent of choline and 0.005 per cent of trigonelline were isolated from the water extract of the seeds.

Therefore the organic bases present in the juice of the seedlings were treated in order to determine, if possible, what variation of the organic bases in the seeds might occur during germination.

Experimental

In this experiment two specimens of soy-bean were used, namely, the "White Autumn Seeds" produced in Korea and the "Yellow Summer Seeds" produced in Kagoshima, but as the procedure of analysis was the same in both cases, the method of analysis of the former will be described here in detail together with the results of the latter only.

5 kg. of well selected seeds were sowed in the dark. After 12 days the seedlings were plucked out. The results of the general analysis of the seedlings are shown as follows:

Table X

Distribution of the Nitrogen in the Seedlings by General Analysis

(Results expressed as percentage of the total nitrogen)

Protein N	56.6
Non-protein N	43.4
N. precipitated by basic lead acetate	5.3
N. precipitated by phosphotungstic acid after the removal of the precipitate by basic lead acetate	12.8
Residual N	25.3

The seedlings plucked out were strictly selected, washed and ground immediately, and extracted with 18 l. of hot water: this extraction was repeated five times: at the first extraction a little acetic acid was added to the extracting water. The extracts were treated with neutral and basic lead acetates successively, and the clarified solution freed from lead by hydrogen sulfide and concentrated. Organic bases were precipitated with phosphotungstic acid and sulfuric acid in the usual way, and then the precipitate was thoroughly decomposed with baryta.

(1) *Precipitates by Silver Nitrate (Adenine and Guanine)*

The solution of bases was neutralised with nitric acid and concentrated to a small volume. A large amount of potassium nitrate was separated off, and purine bases were precipitated by the addition of concentrated silver nitrate solution. The precipitate was decomposed with hydrochloric acid, and free bases were obtained by the phosphotungstate method in the usual way.

a) Guanine:—Ammonia was added to the concentrated solution of the bases until its concentration had reached about 2 per cent. Guanine and a little adenine were precipitated in granular forms. The precipitate was filtered, washed with 1 per cent ammonia, and taken into dilute hydrochloric acid to convert the bases into hydrochlorides: the hydrochlorides were fractionally crystallised, and guanine hodrochloride obtained from the hardly soluble portion. The yield of guanine hydrochloride weighed 0.80 g. which was equivalent to 0.59 g. of guanine.

Analysis:— 0.1689 g. substance: 0.1200 g AgCl
$C_5H_5N_5O \cdot HCl \cdot H_2O$. Calculated. Cl 16.87
Found. 〃 17.25

The guanine chloroplatinate was hardly soluble in water and did not decomposed above 270°.

Analysis:— 0.1968 g. substance: 0.0724 g. Pt
$C_5H_5N_5O \cdot HCl \cdot PtCl_4$. Calculated. Pt 37.0
Found. 〃 36.8

b) Adenine:—Adenine was recovered from the filtrate of the ammoniacal solution and the souble portion of the hydrochloride.

The yield of adenine hydrochloride (anhydride) weighed 3.56 g. which was equivalent to 2.65 g. of adenine.

Analysis:— 0.2114 g. substance: 0.1569 g. AgCl
$C_5H_5N_5$. Calculated. Cl 20.67
Found. 〃 20.44

This substance gave KOSSEL's reaction, but did not give the nitric acid test. Its chloroplatinate crystallised in yellowish prisms and did not decompose above 280°.

Analysis:— 0.2516 g. substance: 0.0725 g. Pt
$(C_5H_5N \cdot HCl)_2PtCl_4$. Calculated. Pt 28.7
Found. 〃 28.8

(II) *Precipitate by Silver Nitrate and Baryta (Histidine and Arginine)*

The filtrate from the precipitate by silver nitrate was treated with more silver nitrate and baryta until a brown precipitate had been formed. The precipitate was filtered, washed with cold dilute baryta water, decomposed with sulfuric acid and hydrogen sulfide, and then subjected to the phosphotungstate method.

a) Histidine:—The solution of free bases was saturated with carbon dioxide, and treated with mercuric chloride. The precipitate was filtered, decomposed with hydrogen sulfide, neutralised with sodium hydroxide, clarified by tannic acid, and again subjected to the phosphotungstate method. The solution of free base was strongly acidified with hydrochloric acid, and 11.67 g. of histidine dihydrochloride equivalent to 7.99 g. of histidine were obtained. The dihydrochloride was in the form of transparent plates and decomposed at 245°.

Analysis:— 0.2108 g. substance: 0.1991 g. AgCl
$C_6H_9N_3O_2 \cdot 2\,HCl$. Calculated. Cl 31.1
Found. 〃 30.7

b) Arginine:—The filtrate from the precipitate by mercuric chloride was freed from mercury by hydrogen sulfide, and arginine flavianate obtained by the method of KOSSEL and GROSS(58). The yield of the flavianate weighed 46.90 g. which was equivalent to 16.64 g. of arginine.

Analysis :— 0.4063 g. substance : 0.1926 g. $BaSO_4$
$C_6H_{14}N_4O_2 \cdot C_{10}H_6N_2OS$. Calculated. S. 6.56
Found. 〃 6.51

The flavianate was decomposed with baryta, the excess of baryta removed as carbonate, and arginine nitrate obtained by acidifying the solution with nitric acid. The nitrate was in the form of milky white crystals.

Analysis :— 0.2276 g. substance : 0.5595 g. $C_{20}H_{16}N_4 \cdot HNO_3$ (Nitron method)
$C_6H_{14}N_4O_2 \cdot 2HNO_3$. Calculated. HNO_3 42.0
Found. 〃 41.3

(III) *Filtrate from the Percipitate by Silver Nitrate and Baryta (Choline and Cadaverine)*

The filtrate was freed from silver and barium as silver sulfide and barium sulfate, and subjected to the phosphotungstate method. The solution of free bases was neutralised with hydrochloric acid and evaporated to dryness. Potassium chloride was removed by extracting the dry residue with methyl alcohol. The solution of methyl alcohol was again evaporated to dryness, and the residue divided into the following two parts by means of cold anhydrous ethyl alcohol.

a) Part insoluble in cold alcohol (Cadaverine)

The insoluble part was dissolved in water, and the solution treated with sodium picrate. 3.10 g. of cadaverine picrate equivalent to 1.12 g. of cadaverine were obtained by recrystallisation. The picrate was in the form of prismatic needles and decomposed at 220° with effervescence.

Analysis :— 2.718 g. substance : 1.720 g. Picric acid
$C_5H_{14}N_2 \cdot 2\,C_6H_3N_3O_7$. Calculated. Picric acid 63.8
Found. 〃 〃 63.3

The chloroaurate crystallised in needles easily soluble in cold water and melted at 190°.

Analysis :— 0.3360 g. substance : 0.1699 g. Au
$C_5H_{14}N_2 \cdot 2\,HCl \cdot 2\,AuCl_3$. Calculated. Au 50.4
Found. 〃 50.6

From the filtrate of the cadaverine picrate, picric acid was removed by means of ether and hydrochloric acid. A small amount of hydrochloride of an unknown organic base, which seemed to be trigonelline hydrochloride, was obtained, but on account of its small yield accurate identification could not be made.

b) Part soluble in cold alcohol (Choline and Cadaverine)

The alcoholic solution of bases was treated with alcoholic solution of mercuric chloride. After standing for several days at 0° the precipitate was filtered, washed with absolute alcohol and decomposed with hydrogen sulfide. The solution free from mercury was treated with chloroplatinic acid. The precipitate of chloroplatinate was filtered off ; the filtrate was freed from platinum by hydrogen sulfide and treated with chloroauric acid. The precipitate of chloroaurate was filtered off ; the filttrate was freed from gold by hydrogen sulfide and again treated with chloroplatinic acid. By such alternative treatment with chloroplatinic acid and chloroauric acid, 20.55 g. of cadaverine chloroplatinate, which, together with 3.10 g. of the cadaverine picrate previously obtained, were equivalent to 6.08 g. of cadaverine, and 26.35 g. of choline chloroaurate, which were equivalent to 7.19 g. of choline, were obtained. The method of separation of these two substances was based on the properties that choline chloroplatinate is more soluble in water than cadaverine chloroplatinate, and cadaverine chloroaurate is more soluble in water than choline chloroaurate.

The cadaverine chloroplatinate crystallised in beautiful orange coloured prisms and decomposed at 224° with effervescence.

Analysis :— 0.2172 g. substance : 0.0827 g. Pt
$C_5H_{14}N_2 \cdot 2\,HCl \cdot PtCl_4$. Calculated. Pt 38.1
Found. 〃 38.1

The chloroaurate crystallised in needles and decomposed at 188°.

The choline chloroaurate was in the form leaf-shaped crystals and decomposed at 248° ~ 250° with effervescence.

Analysis :—0.3979 g. substance : 0.1770 g. Au
$C_5H_{14}NOCl \cdot AuCl_3$. Calculated. Au 44.5
Found. 〃 44.5

The chloroplatinate crystallised in orange coloured prisms and decomposed at 238° :

In this experiment it was acertained that Stanéck's reagent(66) for the isolation of choline could not be adapted for the mixture of choline and cadaverine, because cadaverine was also precipitated with this reagent.

Results and Discusssion

Adenine (as hydrochloride), guanine (as hydrochloride), histidine (as dihydrochloride), arginine (as flavianate), cadaverine (as picrate and chloroplatinate) and choline (as chloroaurate) were isolated from the juice of the etiolated seedlings. The results of the experiment are compared with those of Schulze and Yoshimura in the following table :

Table XI

Organic Bases in the Juice of the Seedlings

(Figures reprsent g. of bases isolated from the seedlings germinated from 1 kg. of the seeds)

	Schulze	Yoshimura	Sasaki	
			White Autumn Seeds	Yellow Summer Seeds
Adenine		present	0.53	0.42
Guanine		present	0.12	0.08
Arginine	increase*		3.33	3.09
Histidine	present		1.60	2.01
Choline	1.47	present	1.44	1.71
Trigonelline			present ?	
Betaine				
Cadaverine			1.22	1.16
Unknown base	present ?			

* Schulze stated " Arginine increased by germination ", but he did not given any figure indicating amount of the increase.

The isolation of the organic bases was performed as quantitatively as possible.

Adenine and guanine increased by germination: the increment seemed to be due to the decomposition of nucleic acid in the seeds. Xanthine and hypoxanthine were not detected.

Histidine and arginine increased remarkably, and that is quite natural if the amounts of the decreases of histidine and arginine by consumption during germination are less than their increases by the hydrolysis of the protein which contains histidine and arginine as "building stones."

Trigonelline which had been found in the water extract of the seeds was not ascertained to be present in the seedlings.

A large amount of cadaverine was isolated. This substance had been believed to be a putrefaction product by bacteria, and had not been found in higher plant. As lysine, a "building stone" of the protein of soy-bean seeds, was not detected in the juice of the seedlings, it is inferred that lysine was first produced by the decomposition of the protein in the seeds by a process much the same as that employed by the proteolytic enzymes, and soon further decomposed into cadaverine and carbon dioxide.

$$\underset{\text{Lysine}}{H_2N\cdot(CH_2)_4\cdot CH(NH_2)\cdot COOH} \longrightarrow \underset{\text{Cadaverine}}{H_2N\cdot(CH_2)_4\cdot NH_2} + \underset{\text{Carbon dioxide}}{CO_2}$$

It is of interest that putrescine $H_2N\cdot(CH_2)_3\cdot CH_2NH_2$, a homologue of cadaverine and a putrifaction product of arginine, was not detected in the seedlings.

STANĚCK's reagent(66) for the precipitant of choline does not meet the purpose when cadaverine is present, because cadaverine is also precipitated by this reagent.

Choline did not increase as much during germination as SCHULZE set forth: SCHULZE used STANĚCK's reagent for his experiment.

CHAPTER IX

TRANSFORMATION OF CARBOHYDRATES DURING GERMINATION IN THE DARK

There are many references in the literature as to the carbohydrates of soy-bean seeds since the work of LEVALLOIS(67) in 1880: first of all YUKAWA(68) made a very valuable investigation and found cane sugar, stachyose, araban, galactan and fiber in the matured seed.*

SCHULZE(69) investigated the metabolism of hemicellulose in the course of germination of leguminous plants, and KOMATSU and his co-workers(70) reported on the metabolism of carbohydrates of soy-bean seedlings.

The present paper is to treat briefly of the transformation of the substances detected by YUKAWA(67), and also of reducing sugar and starch, which are produced in the seedlings of soy-beans by germination.

Recently SUMIKI(71) isolated saponine in soy-bean seeds, WARZE(72) detected several glycosides in the same seeds, and the author detected also a sort of glucoside in the seedlings, but owing to their small quantity, their metabolisms were not considered in this experiment.

Experimental

" White Autumn Seeds " produced in Korea were used as the material for this experiment. The seeds, and the seedlings, plucked out every 2, 5, 7 and 10 days after sowing in the dark, were analysed.

1) Reducing sugar:—The sample was extracted with hot 90 per cent alcohol, and clarified by lead acetate: the reducing sugar was estimated directly by the BERTRAND method.

2) Cane sugar:—The sample was treated with alcohol and lead acetate as in the case of the reducing sugar, and the sugars in the solution were inverted by boiling with dilute hydrochloric acid; then the total reducing power in the solution, or the sum of reducing powers of reducing and non-reducing sugars in the sample, was determined. Cane sugar was calculated by subtracting the reducing powers of the reducing sugar estimated in 1) and of the stachyose estimated in 3) from the total reducing power. The reducing power of stachyose can be theoretically calculated.

3) Stachyose:—Stachyose is a tetrasaccharide having the following formula according to the investigafion of ONUKI(73).

Galactosido-galactosido-glucosido-fructose

Stachyose was indirectly estimated by calculation from the difference between the weight of mucic acid obtained from the sample and that obtained from the residue extracted from the sample with hot 90 per cent alcohol; because the mucic acid method for the estimation of galactose or galactan is not reliable in case that the percentage of those in a sample is small, and because stachyose is not extracted with hot 90 per cent alcohol while galactan is not extracted with it.

4) Starch and dextrin:—Starch and dextrin were estimated together because they were difficult to separate. The residue extracted with hot 90 per cent alcohol was digested with malt amylase by the method of PRINGSHEIM and THILO(64), and then the reducing power of the digested substances was determined in the usual way; starch was calculated from the reducing power.

5) Araban:—Araban was determined by the furfurol method of ÔSHIMA and KONDÔ(75). Pentose and methylpentosan were also estimated by the same auther's method, but the amounts being very small the figures were not described.

6) Galactan:—Galactan was determined by the mucic acid method of DORE(76) from the residue extracted from the sample with hot 90 per cent alcohol.

7) Crude fiber:—Crude fiber(77) and true cellulose(78) were estimated, but as the difference between them was not noticeable, the amount of the former only is described.

Results and Discussion

The results of the experiment are shown as follows:

Table XII

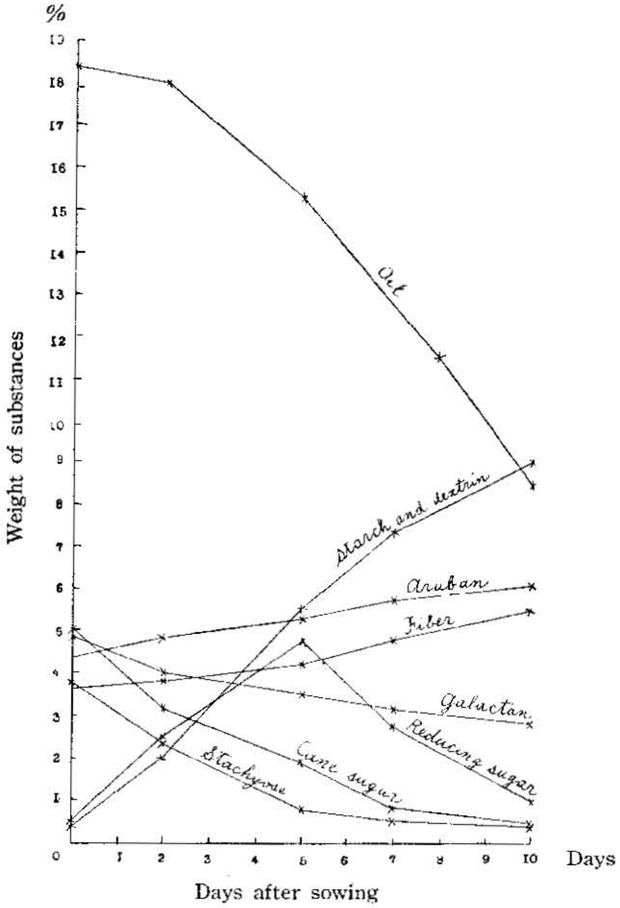
Carbohydrates of the Seeds and the etiolated Seedlings
(Resultss expressed as percentage of the weight
of the seeds used)

	Seeds	Seedlings (days after sowing)			
		2	5	7	10
Reducing sugar	0.5	2.5	4.7	2.8	1.0
Cane sugar	5.0	3.1	1.9	0.8	0.5
Stachyose	3.7	2.4	0.8	0.5	0.4
Starch and dextrin	0.4	2.0	5.5	7.3	9.0

Araban	4.5	4.9	5.2	5.7	6.1
Galactan	4.9	4.0	3.5	3.1	2.9
Crude fiber	3.6	3.8	4.2	4.8	5.2
Total	22.6	22.7	25.8	24.9	25.1

Fig. I

Carbohydrates and Oil in the Seeds and the Seedlings



Reducing sugar which was scarcely detected in the seeds increased in the early stage of germination and decreased afterwards. This fact indicates that some non-reducing substances are transformed into reducing sugar, and then consumed. It is a matter of course that the amount of real reducing sugar was smaller than the figures shown in the table.

Cane sugar and stachyose decreased gradually.

Starch and dextrin increased markedly: this phenomena will be discussed in the following chapter.

Galactan was consumed rapidly in the early stage and slowly afterwards.

Araban increased contrary to the author's expectation and also to the experimental result of SCHULZE(79) who concluded that the hemicellulose, such as araban, in oily seeds was consumed during germination and played a role such as starch do in starchy seeds. In this connection the works of SCHÖNE and TOLLENS,(80) and WINDISCH and HASSE(81) are interesting: these workers state that an increase of pentosan takes place during germination of cereals. The author is now in process investigating this problem.

Crude fiber increased.

The total amount of carbohydrates did not show any marked change during the growth of the seedlings in the period of the experiment. Consequently it is inferred that the origin of kinetic energy in the early stage of germination of oily seeds, such as soy-bean, is rather due to the decomposition of oil and protein than that of carbohydrates as a whole.

CHAPTER X

STARCH PRODUCED DURING GERMINATION OF THE SEEDS IN THE DARK

Many investigators have studied starch in soy-bean seeds. HARZ(82) stated that unripened seeds or seeds ripened after being picked off the twigs contained starch, but nomally ripened seeds did not contain it. STREET and BAILEY(83) found 0.5 per cent of starch and dextrin by the diastase method, but YUKAWA(68) found practically none. MURAMATSU(84) isolated a very little quantity of starch from the ripened seeds. It is certain, from the experiments of the investigators mentioned above, that starch content decreases gradually with the ripening process, and, finally, at the stage of complete ripening it is so small as to be impossible of detection.

SACHS(85) made a detailed study of the product of oily reserves during germination, and concluded that the oil of some seeds was transformed into starch. VON OHLEN(86) reported in his microchemical study that starch was produced by germination of soybean seeds in the dark.

Experimental

(I) *Detection and Separation of Starch in the Seedlings*

Tne section of the cotyledon of the seedling gave a blue colour on treatment with a drop of iodine solution, and many little starch granules were observed under a microscope: these phenomena were not observed in the seeds before germination.

For the purpose of isolating the starch, the following method was adopted.

The seedlings germinated in the dark were frozen soon after being plucked out, and the tissue was comminuted while still frozen. The powder was suspended in a large volume of 5 per cent sodium chloride solution with stirring. The suspension was filtered by suction through a thin layer of filter paper pulp. As the sizes of the starch granules were so minute they passed easily through the filter with sodium chloride solution. The residue on the filter was again ground, mixed with sodidum chloride solution, and filtered. This process was repeated till all the starch granules had passed completely through the filter. Isolation of the starch from the filtrate was now carried out by centrifugation. The precipitate was mixed with 0.2 per cent NaOH solution, and allowed to stand overnight at 0° to dissolve fine gray sludge. The alkaline liquid was centrifuged off, and the starch washed several times with distilled water, 90 per cent alcohol, and ether.

Table XIII

Contents of Starch in the Seedlings

(Results expressed as percentage of the weight of the seed used)

Seed	Seedlings (days after sowing)								
	1	2	3	4	5	6	7	8	9
Yellow Autumn Seed	0.4	1.7	3.2	4.4	5.1	4.8	5.0	4.7	4.8
White Summer Seed	0.5	2.8		5.8		7.5		7.7	

The difference of the yield of starch of the two species is due rather to the character of different species and to the period of storage than to the experimental error. In general the larger the grain and the shorter the period of storage of the seed, the more yield of starch seemed to be produced.

(II) *Properties of the Starch*

a) Size and Shape :—MUTER(87) has classified the starches into potato group, leguminous starches, wheat group, sago group and rice group. The author selected typical ones from each of the above 5 groups of starch, and compared them with that of the soy-bean seedling in their sizes and shapes.

Table XIV

Sizes and Shapes of the Granules of Several Starches

Name of species	Diameter	Shape	Remarks
Potato	μμ 40～100 variable	Small granules circular, large granules oyster shaped	Hilum and concentric rings clearly visible; very distinct cross with polarised light
Pea	20～60 variable	Circular or kidney shaped	Concentric ring become apparent when treated with chromic acid; hilum well marked
Wheat	4～15 20～40 very variable	Small granules circular, large granules ellipse	Hilum and rings invisible in the majority of the granules; cross visible in glycerine with polarised light
Sago	20～60 variable	Ellipse, crack at convex end	Faint rings; well defined cross and often colours with polarised light
Rice	5～8 nearly uniform	Aggrigated, pentagonal or hexagonal	Distinct hilum under a very high power; cross visible in larger granules with polarised light
Seedling of soy-bean	0.5～4	Circular	

The starch granules of the seedlings were so small that they made BROWN motion in water.

b) Optical properties :—A distinct hilum and faint concentric rings were visible with a very high magnifying power of microscope, and a black crosss appeared when the starch was examined with polarised light.

c) Ash and Moisture :—Ash and moisture in the starch of the seedling were estimated by the usual methods of analysis(88) and compared with those of potato made by the author in the usual way and those of wheat manufactured by E. MERK.

Table XV

Ash and Moisture of Several Starches
(Results expressed as percentage of the weight of the starch)

	Potato	Wheat	Seedling
Moisture	12.5~13.5	13.2	13.6~13.8
Ash	0.10	0.13	0.22

The relatively high content of ash in the starch of the seedling was due rather to the impurity which was mixed at the preparation.

d) Temperature of Gelatinisation :—The gelatinisation-temperatures of starches differ with different species. The starch of the seedling was gelatinised at 70° ± 2°, while the potato starch gelatinised at 83° ± 3°, and the wheat starch gelatinised at 83° ± 3°. After being gelatinised the solution of the starch of the seedling was somewhat turbid.

e) Hydrolysis :—Specimens were treated with the direct acid hydrolysis method(89), and with the diastase method with subsequent acid hydrolysis(90); reducing sugar (dextrose) in the hydrolysate was estimated by the BERTAND method, and the weight of the dextrose obtained multiplied by 0.9 gave the weight of starch.

Table XVI

Estimation of the Starch by Hydrolysis
(Figures represent starch per cent of dry material)

	Direct acid method	Diastase method
Potato starch	97.1	97.3
Wheat starch	96.7	94.0
Starch of seedling	96.9	96.3

The acid hydrolysed product of the starch of the seedling consisted only of dextrose by identifying for phenylhydrazine test and Seliwanoff test (negative).

f) Action of Diastase:—SHERMAN and his co-workers(91) (92) found that the velocity of enzyme action upon starch differed according to differing origins.

Exp. I—2 g. of starch was mixed with 100 cc. of cold water and gelantinised by boiling for 10 minutes with constant stirring; then 0.1 g. of sodium chloride, the necessary phosphate buffer to bring the mixture to PH = 5.6, and water to bring the total volume to 200 cc., were added. The mixture was placed in a 37° ± 0.1° thermostat water bath, and being shaken, to the mixture 10 cc. of 0.5 per cent taka-diastase solution was poured in. After maintaining a definite time, 10 cc. of the digestion mixture was taken up, and the action of enzyme stopped by the addition of sodium hydroxide solution. The reducing power was estimated by the BERTRAND method.

Exp. II—2 g. of starch was mixed with 100 cc. of cold water, and gelantinised by boiling for 10 minutes with constant stirring; then the starch was further liquified in an autoclave at 18 lbs. for 15 minutes. The following procedure was the same as in Exp. I.

Table XVII

Action of Enzyme upon Starch
(Figures represent mgs. of copper reduced by
the sugar from 10 mg. of starch)

Time	Potato starch		Wheat starch		Starch of seedling	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
10 mins.	43	44	40	41	41	41
30 mins.	55	57	51	53	53	55
1 hr.	66	67	60	62	61	65
2 hrs.	80	80	71	74	75	76
4 hrs.	86	88	82	82	84	86
24 hrs.	131	130	123	126	128	128

Discussion

Starch produced in the seedling of soy-bean germinated in the dark was isolated.

The size of the starch granules of the seedling seems to be the most minute of all the starches.

Several physical and chemical properties were examined, and it was shown that the starch of the seedling was not different from other common starches.

CHAPTER XI

TRANSFORMATION OF OIL DURING GERMINATION*

Owing to the high percentage of the content, and the usefulness as foodstuffs and industrial materials, many workers have investigated the physical and chemical properties of the oil of soy-bean seeds. The review of the literature will not be made here.

Several investigators have reported on the changes of contents and some properties of oils and fats for a number of species of the plant during germination. Miller(93) made a comparatively minute examination of the changes of the oil of *Helianthus annuus* during germination in the light, but, since he had prepared the sample in the air, the oil under investigation may have been oxidised to a certain extent. Iwanow(94) examined several plants, but his processs of the preparation of the oil was also unsatisfactory.

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* The work reported in this chapter was carried out with the collaboration of Mr. H. Katagiri, to whom the author wishes to express his thanks.

Experimental

(I) Contens of Oil

The specimen used for the experiment was the " Tetsugen Korea " produced in Kagoshima, Japan.

After drying the seeds in a vacuum $CaCl_2$ desiccator, they were ground and extracted with anhydrous ether by a SOXHLET apparatus.

The seedlings germinated in the dark were suspended in a large volume of a absolute alcohol for 2 days, and then the suspension was filtered. The alcoholic filtrate and the residue, drying separately in CO_2, were combined and extracted with ether by a SOXHLET apparatus.

(II) Properties of Oil

A. Preparation of oil

The seeds were ground and extracted with ether by a large SOXHLET apparatus as in the case of the examination of the oil content.

The seedlings were suspended in a large volume of absolute alcohol; the mixture was heated for 40 minutes, stood for 2 days, and then filtered. The percipitate was ground and again extracted with alcohol. The combined extracts were evaporated in vacuo at a low temperature with a fine, slow stream of CO_2 through a capillary tube beneath the surface in a CLEISEN flask; the residue was extracted with ether by a separating funnel. The residue, which was extracted with alcohol, was dried in vacuo with a fine stream of CO_2 and extracted with ether by a large SOXHLET appaarus.

The etheral solutions extracted from the seeds and seedlings were dehydrated by the addition of anhydrous sodium sulphate. The precipitates were filtered, and the filtrates evaporated as before. The oil thus prepared was stored in a $CaCl_2$ desiccator filled with CO_2 and placed in a dark room.

B. Technique of determination

1) Colour: –The colour was observed by the naked eye.

2) Refractive index :—The refractive index was determined in the direct reading ABBÉ refractometer at 20°.

3) Specific gravity :—

4) Acid value :—

5) Saponification value :—

6) Ester value :—

7) Free fatty acid :—

The values of 3 to 7 were determined in the usual way(95).

8) HEHNER values :—The HEHNER value was determined referring to the methods of DAVIDSOHN(96), and LEATH and RAPER(97) 3 to 4 g. of oil was taken into a ERLENMEYER flask and saponified by heating with 50 cc. of $\frac{1}{2}$ normal alcoholic potash. The alcohol was evaporated, and 150 cc. of water and dilute sulfuric acid were added to the residue. The flask was kept on a hot bath till the subjacent aqueous layer was clear, and then cooled to solidify the fatty acid. The acidic solution was poured through a wetted filter paper. Care was taken so as to insure rapid filtration and be half filled with water before the acidic solution was poured on. 150 cc. of boilling water was poured into the flask with shaking, then the flask cooled, and the acidic solution filtered off as before. This process was repeated till no acid reaction could be detected in the washing. The fatty acid in the flask was dissolved in hot alcohol and filtered into a weighing bottle through the fillter paper which had been used for the filtration of the acidic solution. The flask was washed with alcohol and ether. The solvent was evaporated, and the residue in the weighing bottle weighed.

9) REICHERT-MEISSL value :—The REICHERT-MEISSEL value was determined by the method of REICHERT-WOLLNY(98).

10) Iodine value :—The Iodine value was determined by the method of WIJS(99).

11) Rhodan value :—

12) Acetyl value :—

The values of 10 to 11 were determined in the usual way(95).

13) Unsaponifiable matter :—The oil was saponified by a excess of alcoholic potash, and then taken into water. The alcohol was evaporated off, and the unsaponified matter extracted by ether with a continuous extractor of modified SOXHLET apparatus.

Results and Discussion

Table XV

Contents of Oil in the Seeds and the Seedlings
(Results expressed as percentage of the weight of seeds used)

	Seeds*	Seedlings (Days after sowing)			
		2	5	8	10
Oil	18.41	18.11	15.31	11.54	8.41

* The seeds contained 10.35 per cent of moisture.

Table XVI

Properties of Oil of the Seeds and the Seedlings

Properties	Seeds	Seedlings (Days after sowing)	
		4	8
Colour	pale yellow	brownish yellow	dark brown
Refractive index (n_D^{20})	1.4744	1.4747	1.4763
Specific gravity (d_4^{15})	0.925	0.928	0.939
Acid value	1.30	2.30	7.47
Saponification value	192.03	190.01	189.11
Ester value	190.73	187.71	181.64
Free fatty acid (%)	0.68	1.21	3.93
HEHNER value	96.47	95.37	92.08
REICHERT-MEISSL value	0.44	0.88	1.46
Iodine value	139.22	136.24	132.18
Rhodan value	89.58	90.05	90.79
Diff. betw. Iodine V. and Rhodan V.	49.64	46.19	41.39
Acetyl value	10.81	19.23	33.42
Unsaponifiable matter	0.32	0.34	0.28

The oil of the seeds decreased during germination, slowly in the early stage and rapidly afterwards. This indicates that, in the early stage of germination, the main energy is produced by the consumption of cane sugar, stachyose, etc. (refer Figure I and Chapter IX), and later by that of oil. The oil seems to be consumed directly for the use of generating kinetic energy or is transformed into other substances such as carbohydrates.

The oil darkened by germination. This indicates that an ether soluble chromogenic compound or componds are produced by germination.

The acid value increased remarkably. As the acid value is a measure of the quantity of free fatty acid, the experiment indicates that the free fatty acid increases, in spite of the decrease of the total oil.

The saponification value decreased slightly. This indicates that the fatty acid, whether combined with glycerol or other alcohol or free, is more rapidly consumed than other substances in the sample. As the oil in the seeds decreased about 37 per cent in 8 days after sowing, the total fatty acid decreased, of course, more than the saponification value showed.

The free fatty acid means the rate of the amount of the free fatty acid to the total fatty acid free and combined, and is calculated from the following formula.

$$\% \doteqdot \frac{A.V}{S.V} \times 100$$

Here A. V denotes the acid value, and S. V denotes the saponification value. The trend of the value obtained indicates the increase of the free fatty acid in spite of the decrease of the total oil.

The HEHNER value decreased slight, while the REIHERT-MEISSEL value increased remarkably. These indicate that the higher fatty acids, which are yielded from the oil by saponification, decrease on the whole.

The decrease of the iodine value indicates that the unsaturated fatty acids entering into the composition of the oil are more rapidiy consumed than the satured ones.

The rhodan value recognised increased slightly ; and, therefore, the difference between the iodine value and the rhodan value increased remarkably. This indicates that either the high unsatured fatty acids entering into the composition of the oil are more rapidly consumed than the lower ones, or, more reasonably, the higher ones change at first to the lower ones.

The acetyl value increased remarkably. As the acetyl value is due to the $-OH$ radical of the oil and the allied substances, the increase of the value indicates that the oil oxidises to decompose during germination.

The unsaponifiable matter varied irregularly. The unsaponifiable matter includes all of those substances which are not saponified by alkali, but which are soluble in ether. This matter seems to decrease by germination.

There was no evidence of the synthesis oil during germination except the chromogenic compound or compounds soluble in ether.

SUMMARY

(1) Nitrogeous compounds of soy-bean seeds and their transformations during germination were investigated.

a) The amount of total nitrogen did not change during germination.

b) The amount of nitrogen of basic non-protein compounds in the seed is almost twice as much as that of non-basic non-protein compounds, but in the seedling this proportion reverses though both nitrogens increased.

c) The protein in the seeds was isolated almost exhaustively in a pure state; amino-acids of the protein hydrolysate were isolated as quantitatively as possible; and several amino-acids, which had not been detected in the hydrolysate of the soy-bean protein, were isolated.

d) Organic bases in the water extract of the seeds were isolated.

e) Organic bases in the juice of the seedlings germinated in the dark were isolated, and cadaverine which had not been detected in the higher plant was isolated.

A new method for the isolation of cadaverine from a mixture of cadaverine and choline was proposed.

f) Asparagine and several monoamino-acids were isolated from the juice of the seedlings germinated in the dark.

(2) Transformation of carbohydrates during germination in the dark was examined: although the amount of some carbohydrates increased and that of others decreased, the total amount did not show much change during the author's observation.

Starch produced by germination in the dark was isolated, and some of its properties were examined.

(3) Variation of oil during germination in the dark was examined. The oil decreased slowly in the early stage, and rapid afterwards; and there was no evidence of the synthesis of oil during germieation except a chromogenic compound or compounds soluble in ether.

(4) Discussions and presumptions in regard to the results of the experiments are described.

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