Changes in Chemical Composition of Kafir Corn during the first fifteen days of growth.

by

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PREFACE

This work was suggested by Dr. V. G. Heller, under whose direction the work was done, in connection with some vitamin work that he was doing with germinated kafir corn. This investigation was carried out in connection with a series of vitamin studies and conducted by V. G. Heller concerning the changes of potency of seeds during the germination period. It was thought that some correlation could be shown between vitamin potency and chemical change. This thesis deals only with some of the changes in chemical composition.

Some studies have previously been made of the chemical composition of seed at various stages of germination by other workers but the results vary for different seeds so that they are difficult to compare. Dr. N. B. Guerrant, of this institution, did similar work on kafir corn with special reference to the Phosphores and Sulphur changes. His total protein and fat change in the light and dark compared nicely with the results reported in this paper.

HISTORICAL

In all probability there has been considerable work done in this field but only a few studies are listed as such in the literature possibly due to the fact that germination work has been done in connection with other problems.

The general procedure has been to take a certain variety of seed and germinate, taking out samples at intervals and analyzing each sample for the different substances that are to be studied. For example, DeLeano(3) made daily determinations of fat, water, soluble and insoluble material, total nitrogen and nitrogen precipitated with phosphotungstic acid. Zlataroff(8) took samples every six days of germinating Cicer Arietinum and determined the ash, water, nitrogen and phosphorus. Other research problems of this nature are similar; the time between the sampling ranging from one to nine days.

Little is said about inorganic nitrogen. Zlataroff (8) finds traces of ammonia formed in germination and Ravenna(5) says that hydrocyanic acid is synthesized from the ammonia present.

The nitrogen content is, of course, mostly protein and a study of the nitrogen distribution along this line has been worked out by Zlataroff (8) on the Cicer Arietinum. He found that at twelve day periods of germination all the combined nitrogen had increased except the protein nitrogen which had decreased. At the end of twenty-five days the total nitrogen had dropped to one-third its original value.

Suzuki(6) in his work with the lima bean, separated the seed from the stem and cotyledon and determined the nitrogen distribution of each. He found that all protein except peptones showed a decrease in the cotyledon at six and twelve day periods of germination. This was most conspicuous in the case of the coagulable proteins. Peptones, diamino acids, monoamino acids and ammonia showed increase at six day periods and afterwards decrease due to decomposition of higher proteins.

He found also that the water soluble proteins go into the stem, while the stems contain only a trace of peptones. The insoluble proteids increase greatly in the stem showing that fixed proteins play an important part in the formation of stem. This formation of insoluble and coagulable proteids in the stem is more active in the sunlight and causes a decrease of albumineses, peptones and diamino compounds.

Choate(2) found in working with wheat that amino acids were absent until the fourth day and then increased gradually.

CHANGES IN CARBOHYDRATES

Very little literature can be found on carbohydrate changes, presumably, because the methods of analysis are not as good as they should be. Gatin(4) in working with juices of Borassus found that mannose gradually disappeared while the reducing power of the seed remained constant. Van Laer(7) determined the amount of pentosans in dry barley and after nine days germination. De Leano(3) found sugars to increase slightly.

The only material found in reference to changes in fat content was the work of De Leano(3) who found that fat remained constant until the eighth day of germination and then rapidly disappeared.

CHANGES IN OTHER ELEMENTS

De Leano(3) found that inorganic phosphorus increases with germination at the expense of organic phosphorus. According to Bernardine(1) lecithin remained constant until the formation of chlorophyl and hence there was no change in germination.

EXPERIMENTAL

For germinating the seed an ordinary seed corn tester with five trays one above the other was used. These trays had a groove about one-half inch deep around the edge. Water trickled in from the top and when this groove became full it overflowed into the tray below. Towelling was spread over these trays and this made an automatic watering system. However, in order to avoid leaching the automatic drip was discontinued and distilled water was added to each tray separately.

The germinator was heated by placing an electric bulb inside and a temperature of approximately 33° was maintained. Yellow milo maise, a member of the grain sorghum family was germinated first but it was impossible to germinate this particular sample without molds outgrowing the seed. Treating with HCHO, $HgCl_2$ and $CuCO_3$ singly and sterilizing the germinator and towels did not stop the molds which developed and it became necessary to change to a sample of the darso variety, combating the molds by soaking in 1% $HgCl_2$ for five minutes before placing in the germinator.

Five lots of 250 grams each were taken, soaked in 1% $HgCl_2$ five minutes then washed to remove the $HgCl_2$. These lots were spread evenly over the towels in the germinator trays, moistened and kept at 35° C. At the end of two days the seeds had a half-inch sprout and one sample was taken out dried for 24 hours at 105° and weighed. At two day intervals the others were taken out, dried and weighed and placed in sample jars. These germinated sample weights are given in the following table:

Sample #	1	2	3	4	5	6
Days Ger.	0	2	4	6	8	10
Dry Wt.	240 g.	227	215	205	195	186

Five other lots of 100 g. each were grown for the same lengths of time as the preceeding lots but instead of being dried were placed under enough boiling 95% alcohol to make a total of 80% alcohol for carbohydrate analysis. It has been shown that carbohydrates undergo change on drying due to emzymic action and this method of storing is generally used.

Since it is hard to get two germinations alike, 300 g. lots were germinated and samples taken at three day intervals. They were placed in the sun as the weather was warmer and they could be placed in the

open in the day time. Aliquots were taken of each sample and put under 95% alcohol for carbohydrate analysis and the rest dried. These weights are given and the methods of aliquoting can be readily seen:

Wt.off Germ.	Days Germ.	Wt. for Carbo.	Carbo. dry wt.	Total dry wt.	% loss during ex.
520	3	30	14.56	252.6	15.8
1150	6	57.3	16.43	230.	23.3
1117	9	50	14.12	217.	27.7
793	12	50	13.05	207.	31.
790.5	15	50	12.85	203.3	32.2

Since it was possible that some of the loss in weight during germination could be due to leaching the ash content of the samples was determined as a criterion. These values varied within narrow limits, probably due to a nonrepresentative sample and to the fact that the error of a two gram sample is multiplied by 250 and 300 to give the results shown in the table.

LIGHT SERIES

Sample #	1	2	3	4	5	6
Ash in 250 g. of original	3.07	3.00	3.07	3.43	2.95	3.26

Sample #	1	2	3	4	5	6
Ash in 300 g. of original	3.84	3.74	3.56	4.12	3.99	4.17

As a check on leaching petri dishes were taken with five layers of filter paper in the bottom and 10 grams of darse placed in each. After germinating for 3, 6, and 9 days, the whole petri dish and contents was dried and weighed. The percent loss compares favorably with the first samples and the curves are similar.

Sample #	2	3	4	5	6
Percent loss light series	15.8	23.3	27.7	31.	32.2
Percent loss petri dishes	17.5	29.3	39.7		

CARBOHYDRATE ANALYSIS

In analyzing the green samples for carbohydrates which had been stored in alcohol the alcohol is filtered and evaporated. The residue of green material on the filter was extracted with warm water (30-35°) and this extract was combined with the alcohol resi-

due and made up to volume. After precipitation of proteins the reducing power of this solution was determined with quantitative Fehlings solution made up according to A. O. A. C. methods. This determination was called the free reducing power or glucose. Another portion of this solution was inverted with HCl at 69° for ten minutes and the reducing power determined in the same way. 95% of the difference between these two determinations should give the sucrose content. However, in every case the solution had less reducing power after it was inverted than before. This fact was borne out in about 50 determinations. This suggests several explanations.

(1) The hydrochloric acid reacts with the carbonyl group of compounds present making the group inactive.

(2) Volatile aldehydes are driven off by the heat.

(3) Certain aldehydes are destroyed by heat.

The last two are not probable since the solutions were heated up in the reducing reaction. But one thing is certain; that the reducing power of the solution was not all due to glucose since it is not destroyed by such mild methods.

The residue of green material after it had been

extracted with water was placed in a 250 cc. erlenmeyer flask with water and hydrochloric acid and autoclaved for two hours at twenty-five pounds pressure. The water extract of this material was allowed to react with quantitative Fehlings solution after precipitation of proteins and the determination was called "starch" or acid hydrolyzable material. Care was taken in all these Fehlings reactions to carry out the analysis in the same way. The reduction was done in porcelain casserols and heated on a boiling water bath for exactly 15 minutes and filtered immediately through a gooch crucible, dried, ignited at a dull red heat in a muffle and weighed.

DARK SERIES

No.	2	3	4	5	6
Glucose in 20 g. original	1.59	3.22	3.26	1.15	2.25
Starch in 20 g.	6.38	5.96	5.91	2.08	1.53

LIGHT SERIES

No.	1	2	3	4	5	6
Glucose in 300 g.	3.35	6.31	11.7	22.1	15.9	15.9
Starch in 300 g.	92.5	69.5	80.5	69.9	54.2	51.4

Protein nitrogen was determined by the Kjeldahl method (A. O. A. C) and gave the following results:

No. Dark	1	2	3	4	5	6
% protein	12.9	13.9	13.9	13.9	15.6	15.7
Protein in 250 g. original	30.9	31.5	30.0	28.6	30.4	29.3
No. Light	1	2	3	4	5	6
Nitrogen in 300 g. original	5.1	5.03	5.23	5.16	5.2	4.89

The percent protein in the samples is seen to increase but when this is calculated on the basis of the total protein present in the entire sample a slight decrease is found. Several workers have noted that ammonia and hydrocyanic acid are formed during germination and that protein is lost. The odor of hydrocyanic acid was easily detected while grinding the green samples but no qualitative test was made. Ammonia would be expected to be a decomposition product of proteins since they would be first hydrolyzed and then deaminized. This is borne out by determining the free amino acids on the hot water extract

of the samples. The samples(4g.) were digested with hot water for four hours on a boiling water bath covered with watch glasses and care taken to keep the solution sterile. They were filtered hot and made up to volume. Hot water was used since the amino acids are more soluble in this medium. Amino nitrogen was determined on these solutions at once by Van Slykes method (as given by Plimmer) before bacterial action set in. Results follow: (Table, page 13)

The proteins evidently break down rapidly as shown by the steepness of the curve(P.) by the eighth day deaminization is equal to hydrolysis.

To determine just what amino acids were deaminized, Van Slyke's analysis for protein distribution was run on the three day samples.

VAN SLYKE ANALYSIS(with modifications)

Fifty grams of dry sample was extracted with .2% sodium hydroxide for twenty four hours. The first eight hours the bottles were shaken in a mechanical shaker. This method was used to extract the proteins of the Mung bean by Heller and Guerrant(10) and found

DARK SERIES

No.	cc.gas	cc. in Blank	cc. gas from sample	Amino nitrogen	N per gram	total wt. sample	Mg.free amino N total
1	.54	.37	.17	.0942	.471	240	113.
2	.61	.37	.24	.133	.666	227	151.3
3	.70	.37	.33	.183	.916	215	197.
4	.80	.37	.43	.238	1.19	205	244.
5	.81	.37	.44	.244	1.22	195	238.
6	.82	.37	.45	.249	1.245	186	232

to be very satisfactory. After they had stood the rest of the twenty-four hours the mixture was centrifuged and the supernatent liquid siphoned off. Portions of this extraction were analyzed by Kjeldahl method to determine the percent protein extracted.

LIGHT SERIES

No.	1	2	3	4	5	6
%	35.25	24.6	35.66	39.97	42.9	47.45

Five hundred cc. of the extract was neutralized with concentrated hydrochloric acid and then 550cc. concentrated HCl added. The mixture was then heated in the autoclave in pressure bottles for twelve hours at 25 lbs. pressure. The Biuret test for protein was negative on all samples after hydrolysis. This mixture was partially evaporated to remove the excess hydrochloric acid. Kjeldahl determinations for protein were run again to determine the amount of nitrogen lost during hydrolysis.

The apparatus used for vacum distillation was practically the same as that used by Van Slyke as given in Klimmer(9). Three checks were run on all samples and the best two taken.

Two hundred fifty cc. of the sample was placed

in the distillation flask and the water bath around the distillation flask heated to 42°. The distillation was carried on until most of the acid was distilled off. The apparatus was then washed carefully with air and water and N/14 acid placed in the receivers. 250 cc. 10% lime suspension was placed in the flask with the sample and distilled until about one-half the water was carried over. This took about four hours. From the acid neutralized the amide nitrogen can be easily calculated.

The lime reaction mixture was filtered and the well washed residue subjected to Kjaldahl analysis for humin nitrogen.

The filtrate from the humin nitrogen is* neutralized with hydrochloric acid, returned to the vacum distillation apparatus and concentrated to about 100 cc.

It is then washed into a 250 cc. flask and 18 cc. of concentrated HCl and 15 g. of phosphotungstic acid in water are added.

The entire solution is diluted to 200 cc. with water and heated in a water bath, until the precipitate has nearly, or quite, redissolved. On cooling,

the precipitate separates in crystalline form.

After standing for at least 48 hours the crystals are filtered off and washed in the following manner:

A three inch Buchner funnel is covered with a hardened filter paper of such a size that it fits against the bottom and side walls. The portion of the paper against the side walls is folded into about 20 plaits so that it fits snugly all round.

The precipitate is poured into this pocket and the mother liquor removed by suction and by pressing down the precipitate with a flattened rod.

The filtrate is returned to a beaker.

Washing is effected with 10 cc. of solution containing 2.5 g. of phosphotungstic acid and 3.5 g. of HCl per 100 cc. This is first used to dislodge the particles remaining in the flask. It is then poured upon the precipitate which is stirred up until all lumps are broken and until there is only a granular suspension. It is then sucked dry as before. The washing is repeated three to four times in this manner. Then the precipitate on the filter is washed five to ten times with the same solution from a wash

bottle commencing around the edges and sucking dry each time.*

The whole phosphotungstic acid precipitate was subjected to Kjeldahl analysis and the hexone bases and cystine nitrogen determined as a unit. The results are given in the next table.

The filtrate from the phosphotungstic acid precipitate which contains the mono amino acids, was concentrated to 250 cc. and the amino nitrogen determined by Van Slykes method(9).

Nitrogen Distribution (expressed in grams of nitrogen on the basis of 300 grams of original sample)

No.	1	2	3	4	5	6
Amide	.5135	.328	.872	.964	.672	.945
Humin	.55	1.03	1.39	1.57	1.77	1.52
Hexon Cystin	.56	.30	.27	.41	.21	.58
Mono amino	2.44	2.15	2.03	1.37	1.22	1.27

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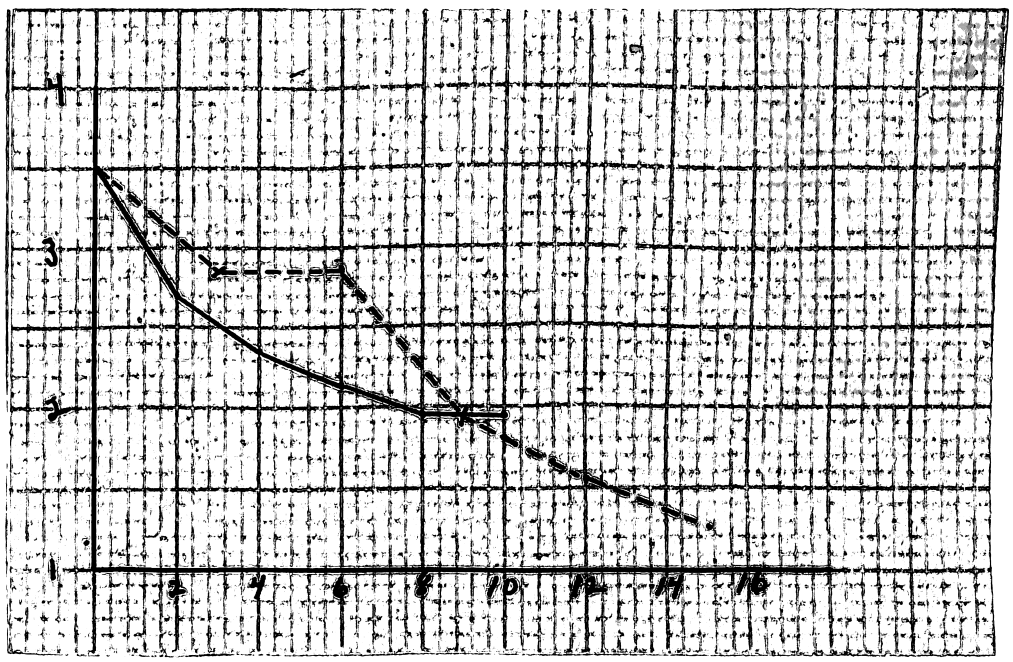
* The material between these two * is taken verbatim from Plimmer's Monograph on Proteins(9).

DeLeano (3) states in his work with Ricenus Communcis that the fat content is constant until the eighth day and then rapidly disappears. It was found that the fat content of kafir disappeared in a regular manner from the first, forming the curves given in plate I. It can be seen that the fat content decreases more rapidly in the seeds germinated in the dark up to the eighth or ninth day when the curves cross. At this point the fat in the dark series approaches a constant value while the light series only slows up slightly. The dark germination was not followed farther than the tenth day.

Fats are oxidized in plants similar to carbohydrates and there is no reason to believe that the process would be periodic unless we hold to the optimum P_h idea. That it takes place faster in the dark is surprising but it reaches a stage about the eighth day where the plant no longer has the power to oxidize.

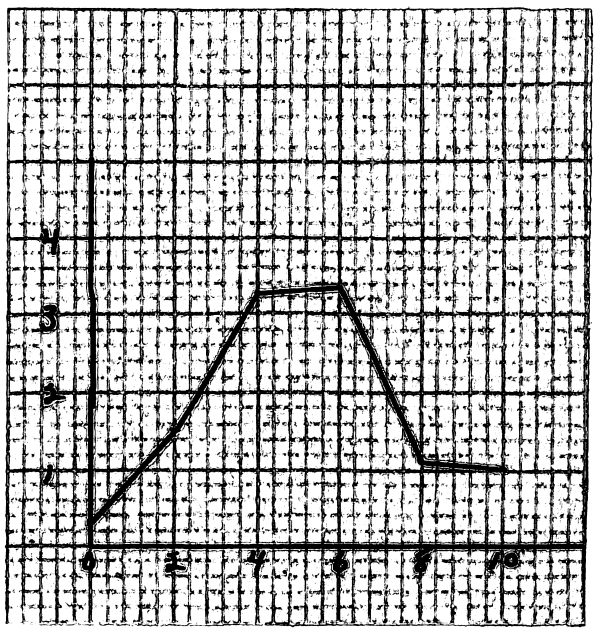


Abscissa - Days germination.

Ordinate - Grams of fat per 250 g. original sample.

The oxidization rate becomes equal about the 8th day and continues to oxidize at a decreased rate.

Plate II, Dark Series; Plate III, Light Series



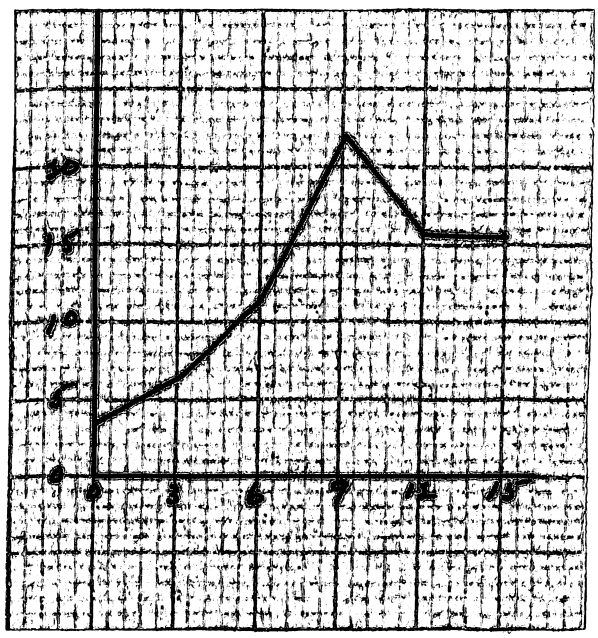
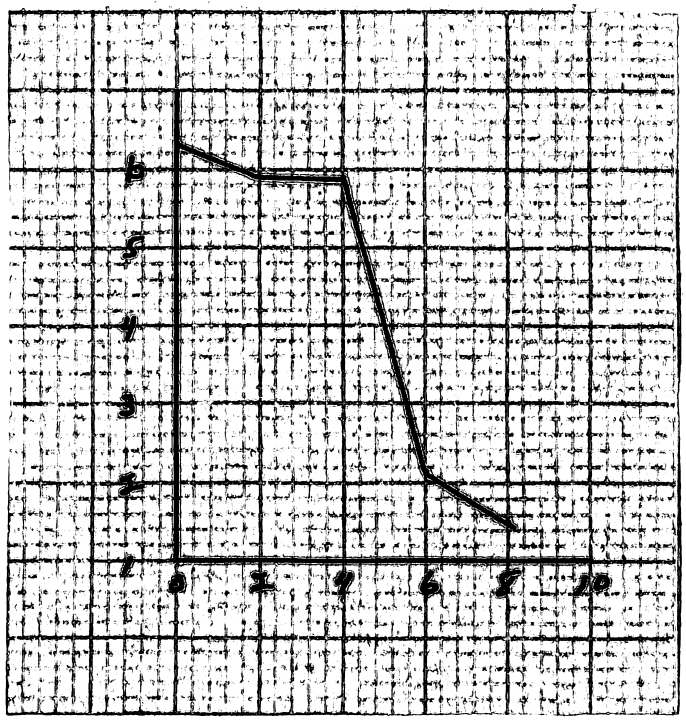
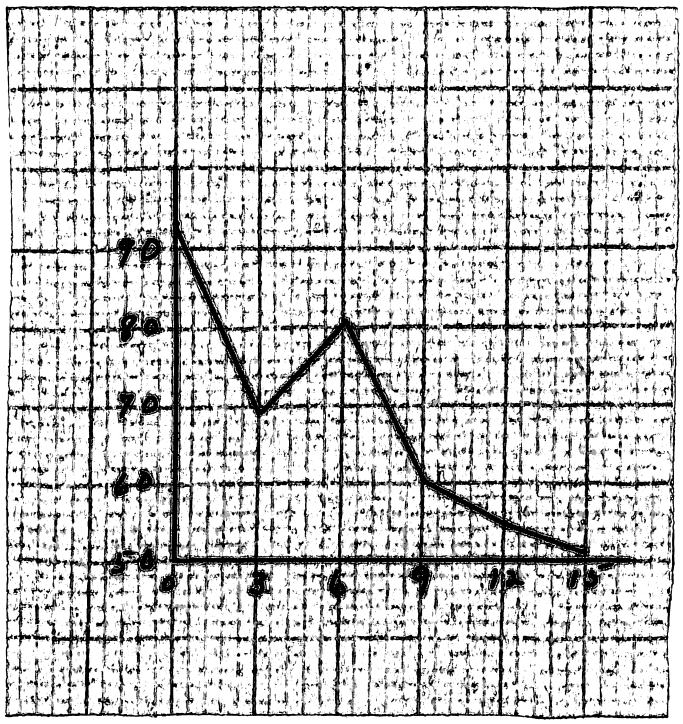


Plate IV, Dark Series; Plate V, Light Series



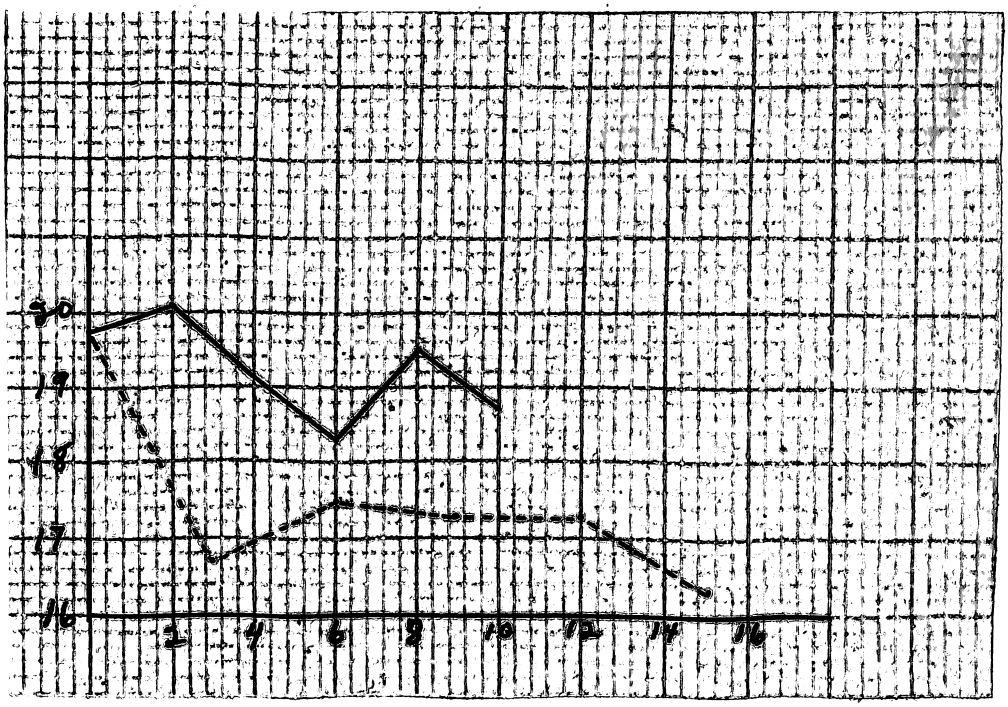


The Fehlings method for the determination of glucose is not so satisfactory as it might be when working with plant tissue. However, conditions were kept the same throughout as heretofore stated and several determinations made so that the results can be taken as relative but not absolute. Plates II and III show the relative amounts of glucose present. In the sample grown in the dark glucose increases rapidly until the sixth day, whereas the light sample reaches a maximum at the ninth day but only about one-half the value of the dark maximum. Possibly there is better chance for enzyme action in breaking down the polysacchrides and less chance for respiration since there is no chlorophyl. This looks more probable on looking at plates IV and V. The starch lost over an equal period of time is greater in the dark and perhaps accounts in part for the great increase of glucose. It would seem that the plant grown in the dark did not have the power of oxidizing glucose that the light grown plant had.

It would be very interesting to get a complete record of the hydrogen ion concentration through the

germinating period as probably a change in hydrogen ion accompanies all these changes and perhaps this power to oxidize glucose.

PLATE VI, TOTAL PROTEIN

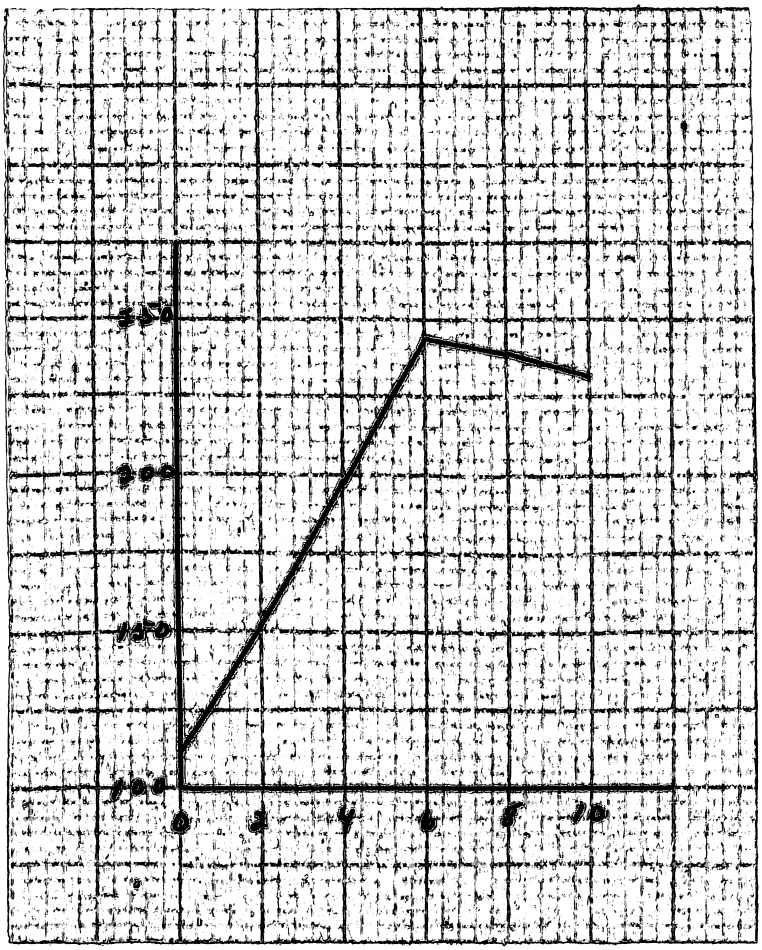


The irregularity of the protein curves could not be accounted for except by non-representative samples but it is apparent that the more rapid decomposition takes place in the light. The dark curves show a downward trend but not much outside the experimental error. That there is a decompo-

sition of protein is seen from the next curve of free amino acids. Since the changes are more marked in the light series we took those for protein distribution analysis.

PLATE VII

Free Amino Acids, Dark Series

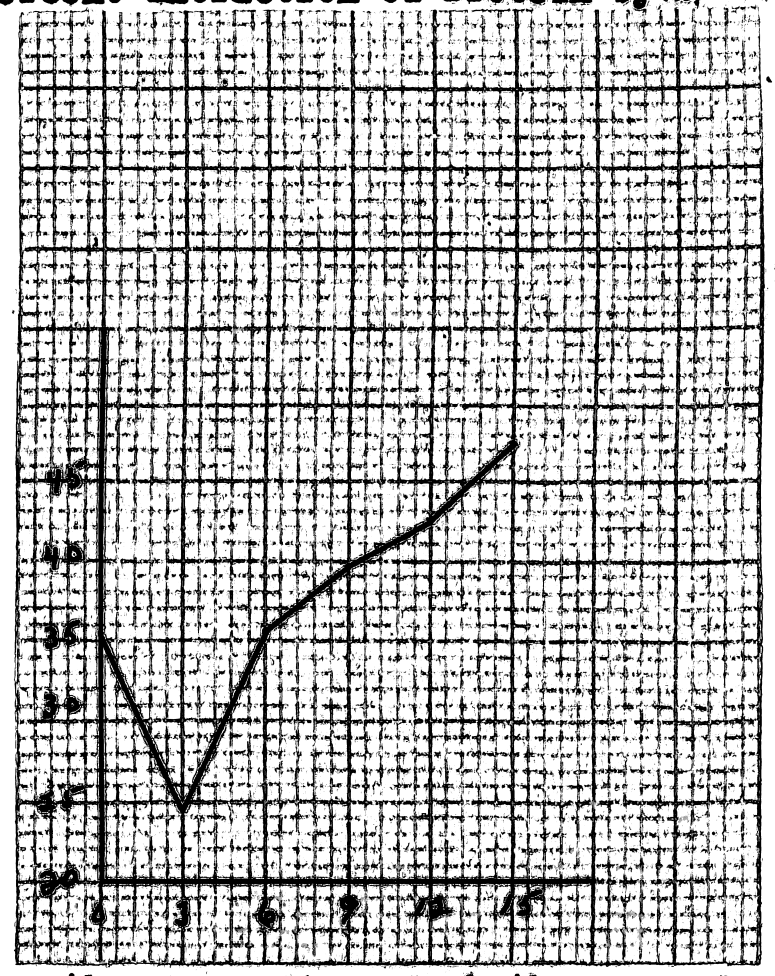


The free amino acids increase about two and one-half times during a period of six days. It is probable that some deaminization is taking place before the

sixth day but evidently the correct P_h for this reaction is not reached until this time for then it becomes a little greater than hydrolysis.

PLATE VIII

Percent Extraction of Protein by .2% NaOH



From the preceeding graph the increase in extracting power of .2% NaOH is partly understood. Possibly the amino acids react with the NaOH to give

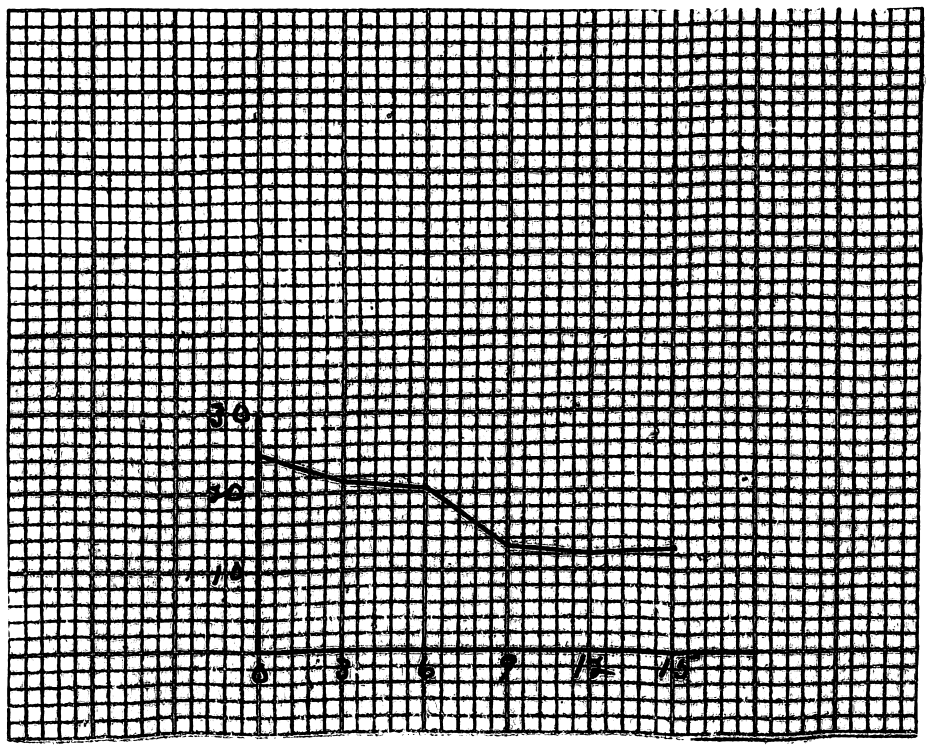
soluble salts thus increasing the amount of protein extracted.

It was thought that perhaps the fatty material in the seeds was interfering with the extraction so the work was repeated but the fat was first removed with ether and absolute alcohol. No improvement in extraction of protein was effected.

Why the solubility of the protein should be reduced 30% after three days germination, has not been explained.

PLATE IX

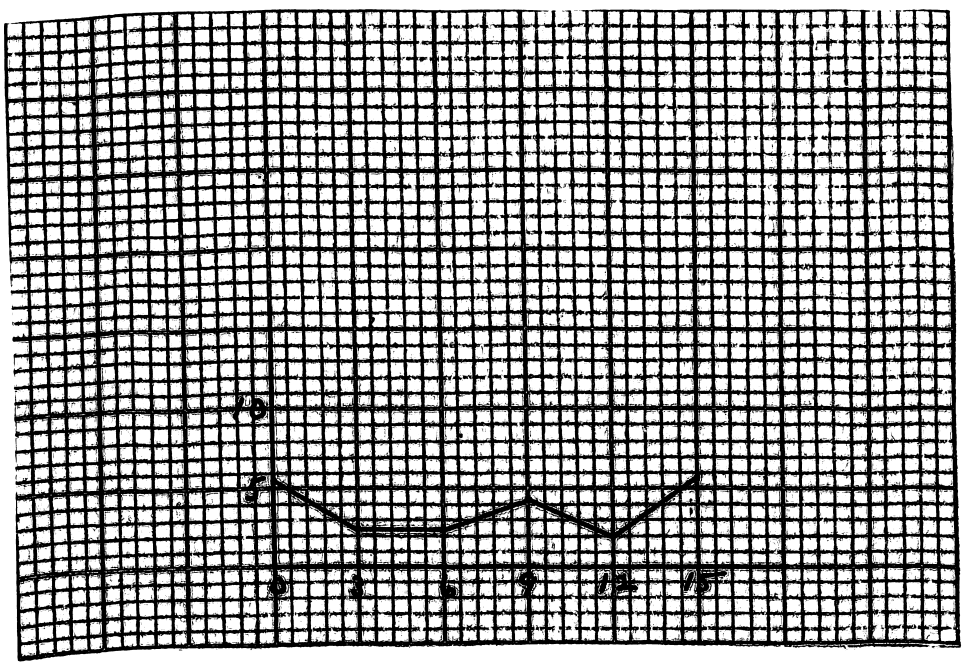
Monoamino Acids



Since the monoamino acids are the most reactive of the amino acids, it is to be expected that they would be the first to be deaminized. They decrease about forty percent over a period of nine days and remain almost constant for the next six. Again the correct P_h for the deaminizing bacteria or enzymes is possibly not reached before the ninth day. Then too, the free amino acids in large quantities are absent until the sixth or ninth day.

Plate X

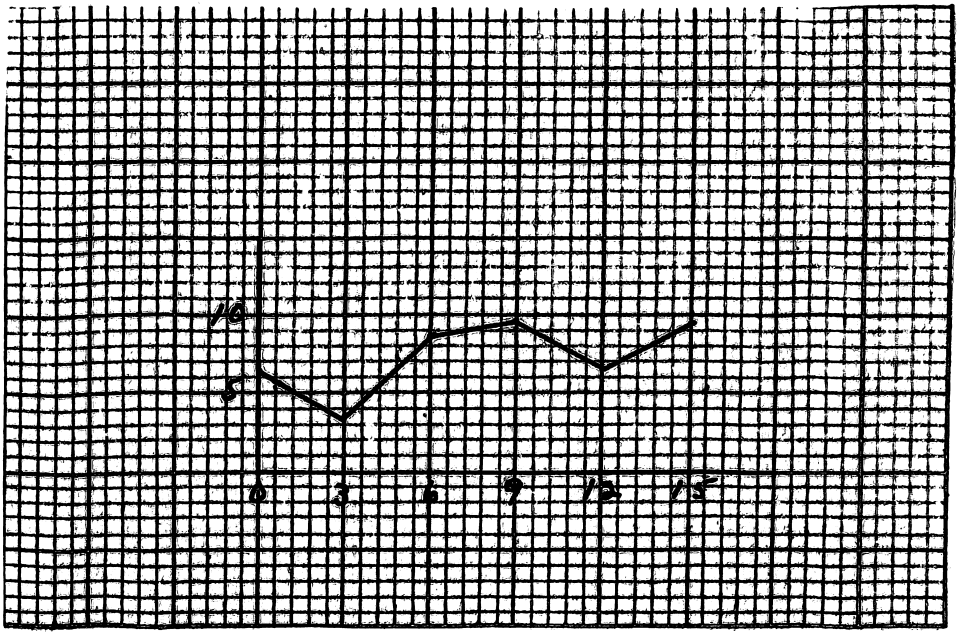
Hexone Bases and Cystine



The changes of the hexone bases and cystine seem to be very slight. If anything, there is a gradual decrease in the amount of these amino acids as germination progresses.

PLATE XI

Amide Nitrogen



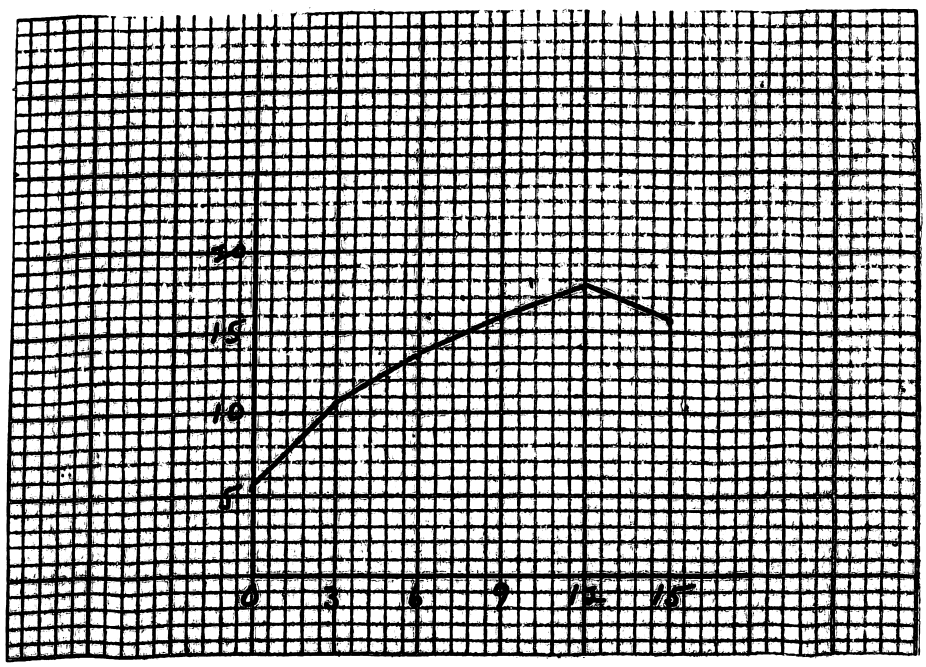
The decomposition of amide nitrogen to ammonia and deaminization seem to work opposite each other.

During the first three days of germination before the deaminization rate has become fast, the amides decrease rapidly and about the sixth day when deaminization has reached its peak, the amides increase.

It might be that the amide is an intermediate step between the amino acid and ammonia.

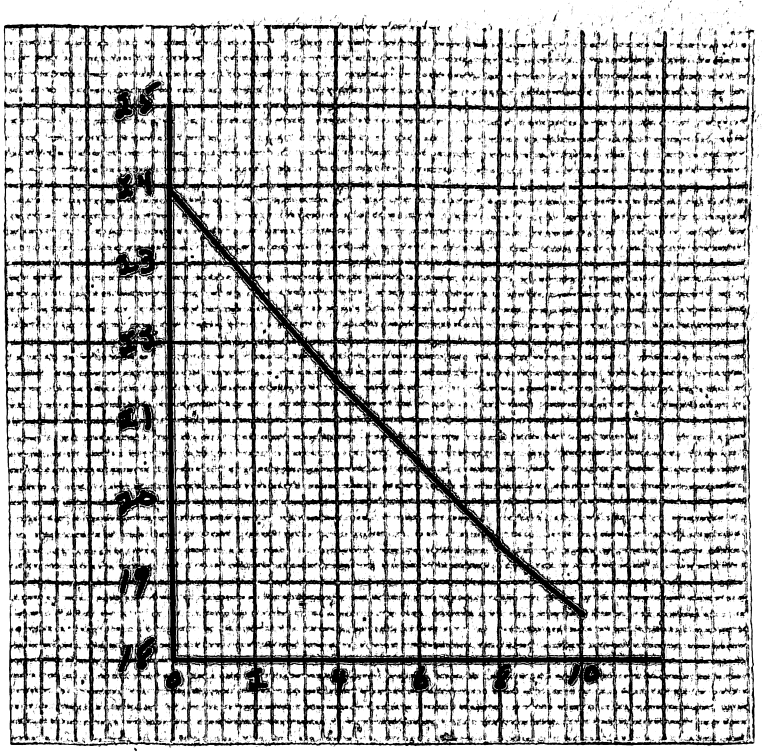
PLATE XII

HUMIN NITROGEN

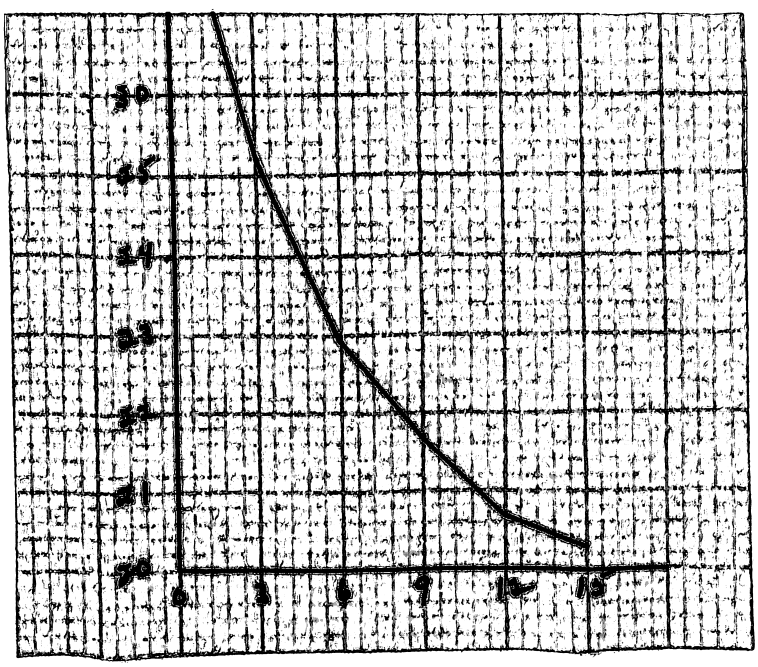


Humin nitrogen is thought to be a decomposition product of tryptophane and if this ratio is constant it seems that tryptophane reacts similar to the total monoamino acids.

Loss in Weight, Dark Series



Light Series



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