SOME BIOCHEMICAL STUDIES ON SEED VIABILITY

Thesis for the Degree of M. S.

Orman E. Street

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Seeds

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II. Reduction of Potassium Permanganate As a Measure of Seed Viability

Thesis presented for the Degree of

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II. Reduction of Potassium Permanganate As a Measure of Seed Viability

Introduction

The possibility of developing a simple method to serve as a measure of seed viability was the basis of this study. In spite of the wide divergence in the chemical and physical composition of different seeds within a group or class, it was hoped that there might be some simple relation, that would operate as a function of the germinating power of the seed, and thus serve to measure that power.

The previous work conducted at this Institution on this problem has been along two lines: the first, the measurement of the electrical conductivity of seed extracts; the second, the determination of the reduction of chemical reagents, notably potassium permanganate. Development of the method of electrical conductivity measurement is described in a paper presented by Fick and Hibbard before the Michigan Academy of Science, Arts, and Letters(12). This work was continued by Miller and is reported in his thesis, presented in 1926. The latter paper contains in addition the study of the chemical methods.

The present study was planned as a continuation of the permanganate reduction method, with a view to refining it sufficiently to establish differences of germination of two or three per cent. Considered from that viewpoint, the results are rather conclusively negative, but with further studies it has been possible to demonstrate some of the factors responsible for the results obtained.

Historical

Of the long list of reagents used in oxidation-reduction reactions, potassium permanganate is undoubtedly the most favored, both for inorganic and organic reductions. Its ability to react in acid, alkaline, or neutral solutions, combined with its intense coloration, which serves as its own indicator, leads to its adoption in a wide range of situations.

Within the more restricted range of plant materials, it is often used. Reichert(35) lists a number of methods for its use in the preparation of soluble starch. Lasser-Cohn(20) in his "Manual of Organic Chemistry", gives the reactions of potassium permanganate with a number of aromatic compounds, including a quantitative estimation of glycerol. Reed(34) reported a reduction of concentrated potassium permanganate by horseradish extract, in which the peroxidases were held to be the reducing agents. Bunzel and Hasselbring(4) refuted the evidence of Reed very soon after his publication, insofar as the peroxidase is concerned as they list ten organic compounds that reduce permanganate. In all the reactions the formation of hydrated peroxides of manganese was noted.

Its direct application to seed extracts, however, is new, hence the feasibility of attempting to establish a quantitative relationship between it and some component of the seed extract.

Methods Other Than Permanganate Reduction

Waller(43) made use of the after-currents aroused by single induction shocks to determine whether seeds of beans were dead or alive. Dead seeds always developed an after-current, or "blaze current", in the opposite direction to the induced current. If the after-current aroused by induced currents of both directions were in the same direction, or if there was no change in current direction between induced and after-currents, the seed was alive.

The electrical conductivity of plant materials has been studied by a number of workers, but not all this work is applicable to this problem. Osterhout(32) studied the resistance of disks of Laminaria, using a modification of the Wheatstone bridge. Brooks(3) made a definite contribution to the field in his studies of conductivity as a measure of vitality and death. He defines "net conductance" as the conductance of the tissue, independent of the conductivity of the bathing fluid. In most cases, conductance of dead tissues was only 35% to 60% of that of live tissues.

Fick and Hibbard(12) applied the method of electrical conductivity to determinations of seed viability. The relative exosmosis of salts was greater in seeds of low germination, and hence the resistance of the extracts to the passage of an electric current was less. A positive correlation was found for timothy and red clover. With a larger number of samples, and improvements in the technique, Miller reports in his thesis that he has not been able to correlate viability and solution resistance,

and that no definite equilibrium is reached, even after twenty four hours.

The decrease in the heat of respiration has been used by Darsie, Elliott, and Pierce(10) as an indication of the laws of germinating power. The temperature which was developed by germinating seeds in silvered Dewar flasks, under conditions suitable for germination, was taken to indicate the viability. A "normal temperature" for each species of plants studied, was used as a basis of comparision. Another application of thermal relations is suggested by Munerati(26) who finds that as seeds of wheat age, they germinate better at temperatures above their normal. This could be taken as an approximation of the age of the seeds, within a relatively narrow range.

Lesage(22) presented a method which had as its basis the ability of seeds to color solutions of KOH. He used solutions ranging from N/1 to N/683 and found that dead seeds colored all, while live seeds colored only the solutions stronger than N/32. He suggested the use of this reagent in a concentration range between N/32 and N/683, as a means of determining viability.

Brocq-Rosseu and Gain(2) were among the earliest workers on the relation of enzymes to viability. They reported peroxidase in wheat estimated as being from two to five thousand years old. All samples of lesser antiquity showed the presence, in an active state, of the enzyme.

McHargue(24) did not verify these findings, as he states that in every case where the seeds showed a weak or zero germination, they showed also a weak or zero peroxidase test. Using tests described by Kastle(18),

he found peroxidases in twenty species, but oxidase and peroxidase in only three species, namely: lettuce, alfalfa, and soy beans. His tests enabled him to classify seeds as high, medium, and low germination.

The relation of enzymes to germination is mentioned by Crocker(7) in his study of the delayed germination of Xanthium. He was unable to detect any differences in digestive activity of extracts from the upper or lower seeds, altho the latter germinated a year sooner. A more extensive study of catalase and oxidase activity appeared in a later work, Crocker and Harrington(8), in which they study Johnson and Sudan Grass seeds. They do not find a very close correlation between either of the enzymes and the vitality of the seed, but stated that the decrease of catalase activity with age was a fair measure of age in continuously dry-stored seeds

Shull and Davis(40), in contrast to Crocker's results, find that the lower seeds show greater catalase activity than the upper seeds, both under laboratory and field conditions. The enzymatic differences were held to be in harmony with other physiological differences, which cooperate to delay germination.

Nemec and Duchon(27) reported investigations of catalase activity which gave much promise. Working on oats and peas, they obtained a remarkable correlation of catalase activity and germinating power. They used 2 grs. of meal, to which was added 15 ccm of neutralized 0.3% hydrogen peroxide. The net volume of oxygen liberated in 5 minutes was about equal in cubic centimeters to the percent germination.

The attempts of other investigators to duplicate these results have not been uniformly successful. In the same year, de Vilmorin and

Cazaubon(42) applied the test to different varieties of peas. They concluded that there was no consistancy between the relation of catalase activity and germinability.

Wilmer Davis(11) found that the meal from dead and live seeds of lettuce often showed a catalase activity nearly parallel. If the meal was soaked in water over night, that from the live seeds showed slight change in catalase activity, while that from dead seeds showed a decrease. This he interpreted as a reduction or chemical decomposition of the catalase. A more reasonable explanation would be to ascribe the difference to greater permeability in the dead cells.

Experimental Work

At the point where work on this problem was terminated by Miller, the procedure in the potassium permanganate method was as follows: The seeds were soaked over night in water and one cubic centimeter aliquots withdrawn for the test. To this was added one drop of N/2 KMnO4 and the time required for reduction noted. In order to obtain a clear end point, a few drops of N/10 oxalic acid was added when the reaction was nearly complete.

It was deemed advisable to germinate the seeds after soaking, as the irregularities within a sample were often greater than the differences between samples. In view of this, a further effort was made to evaluate the vitality of the seedling produced. A system of scoring was arbitrarily set up, based on the vigor of the seedling. As 20 seeds were used, a perfect score for a strong seedling would be 5%. Those of medium vigor were given 4%, the weak sprouting ones, 3%, and those which failed to germinate, 0%. While it is recognized that this is subject to a personal error, it was nevertheless more reliable than a mere plus or minus rating. Further, it is more in harmony with the order of accuracy of a chemical reaction.

After a few preliminary tests, with varying amounts of aqueous extract, and varying amounts and concentrations of potassium permanganate as the subject, the following test was tried. Twenty seeds were soaked in 40 c.cm. of distilled water for a period of 24 hours at room temperature. The extract was decanted, the seeds sterilized with Chlorozene, and placed in sterile dishes to germinate. To 5 c.cm. of the extract, ½ c.cm. of N/61.2 KMnO4 was added, and the time of reduction noted. Oxalic acid as a clarifying agent was also added to the amount of ½c.cm. This in

itself would react with the permanganate in 1 hr. 35 min., hence any solutions requiring nearly that time for reduction would have zero reducing power. Table 1 shows the major aspects of a reaction of this type.

Table 1

Time Rate of Reduction

As a Measure of Viability

Sample	Percentage Germination	Reduction Time in Min.	Sample	Percentage Germination	Reduction Time in Min.
9	100	44.5	14	80	30.2
10	95	45.3	7	72	34.5
13	95	42.2	5	65	26.3
12	95	37.7	4	55	21.2
3	95	31.7	16	45	11.4
8	95	27.6	2	42	10.6
17	90	32.2	1	35	27.8
11	90	28.0	15	0	17.7

There is merely a definite trend in the results, and that is not at all consistent. An end point was found difficult to obtain, as there was a marked tendency for the formation of a colloidal, brown suspension of MnO_2, which was relatively stable. No reason was apparent to explain why a time rate measurement should have any advantage over a conventional titration method. Nevertheless, several more series were run with very mediocre results.

Oxidation-reduction reactions of permanganate are usually conducted at a temperature of 70°C. Since at this temperature, oxalic acid reacts quantitatively with permanganate, it was omitted.

In this and subsequent experiments, several concentrations of $KMnO_4$ were used as work on the method progressed. However, these will all be reduced to a basis of N/10 $KMnO_4$, computed on the pentavalent reactivity of this reagent in an acid medium.

Table 2 shows the results of a run made under the following conditions: 10 c.cm. of extract were heated to 70°C. on a water bath and permanganate added until an end point of a brown suspension was obtained.

Table 2

Titration of Aqueous Extracts of Corn

By Neutral $KMnO_4$

at 70°C.

Sample	Percentage Germination	$KMnO_4$ in c.cm.	Sample	Percentage Germination	$KMnO_4$ in c. cm.
10	95	.130	4	45	.155
11	94	.139	8	45	.130
17	89	.163	1	42	2.371
3	85	.179	6	31	.146
12	82	.179	5	21	.139
13	75	.130	16	12	.285
2	54	.146	14	10	.122
7	51	.114	15	0	.465

A discrepancy in the method was apparent. Sample No. 1 failed to reach any definite end point in its absorption, as there was apparently an accelerative formation of the colloidal hydrated oxides of manganese. The total absorption was low, as may be seen by comparison with Table 3, using the same samples, but with 2% by weight of sulphuric acid added to the extract.

Table 3

Titration of Aqueous Extracts of Corn

By Acid $KMnO_4$ at 70°C.

Sample	Percentage Germination	$KMnO_4$ in c. cm.	Sample	Percentage Germination	$KMnO_4$ in c. cm.
10	95	1.33	4	45	2.24
11	94	1.27	8	45	2.71
17	89	1.74	1	42	2.08
3	85	2.22	6	31	2.39
12	82	1.68	5	21	2.36
13	75	1.75	16	12	2.42
2	54	2.26	14	10	1.36
7	51	1.67	15	0	2.80

The greater amount of reduction was a favorable feature of this series, but the fact that an end point of a clear solution was not attainable would indicate that the reaction was not reaching a point of equilibrium. Inasmuch as the conditions established are exactly comparable with those prescribed for a permanganate oxalate reaction, it was next decided to titrate the excess permanganate with sodium oxalate.

Table 4 shows the results of this procedure, as well as the modification used: Permanganate in excess (1c.cm. N/2 $KMnO_4$) was added to the solution, (acidified to 2% H_2SO_4 and kept at 70°C.) and at the end of exactly ten minutes the unreduced permanganate was titrated back with sodium oxalate.

Table 4

Application of Standard Oxidation-Reduction

Reaction to Aqueous Extracts of Corn

Sample	Percentage Germination	Net c. cm. $KMnO_4$	Sample	Percentage Germination	Net c. cm. $KMnO_4$
12	96	1.25	4	69	2.00
11	95	3.00	6	67	3.90
10	95	3.15	7	60	2.65
8	93	3.15	5	51	1.15
17	88	2.30	1	50	4.30
3	81	2.75	14	42	1.75
13	74	4.67	16	21	4.51
2	73	2.95	15	0	4.25

"It is hardly politic to claim that there is any correlation between the reduction as carried on in this experiment, and the viability of the seed. If there is a relation between reducing power and viability, it does not appear to be a direct function.

Three samples from the same lot of corn were extracted under different conditions. No. 1 was allowed to soak for 24 hours at room temperature, No. 2, placed in a constant temperature oven at 50°C. and No. 3, left in the ice box. The hot-water treatment killed the seeds, but increased slightly their reducing power, as will be shown by table 5.

Table 5

Effect of Temperature Upon

Reducing Power of Extracts

Sample	Percentage Germination	Net c. cm. $KMnO_4$
3	98	3.70
1	90	3.45
2	0	4.05

An attempt was made to apply the test to beans, but no consistent results were obtained altho wide differences in reducing power of the extracts were noted. Steps were also taken to adapt the alkaline permanganate method to these extracts. It took several days to obtain differences in reduction, which fact in itself would disqualify the method.

As permanganate absorption, within definite time limits, closely parallels methods used for iodine absorption, it was thought feasible to try the latter method. In this case, the reagents used

were of the normality recommended in the A.O.A.C. Handbook(1), but the net iodine absorption is computed on the basis of N/10 solution. The solution was left in contact for 15 minutes, and the excess iodine titrated with sodium thiosulphate, using starch as an indicator. The results are shown in Table 6.

Table 6

Absorption of Iodine

by Aqueous Extracts of Corn

Sample	Percentage Germination	Net Iodine	Sample	Percentage Germination	Net Iodine
12	96	.16	4	69	.20
11	95	.24	6	67	.10
10	95	.11	7	60	.11
8	93	.17	5	51	.17
17	88	.13	1	50	.20
3	81	.17	14	42	.19
13	74	.16	16	21	.20
2	73	.14	15	0	.28

There seems to be even less possibility of correlation by this reaction than by the permanganate tests. The low reactivity of the extracts with this reagent, would indicate that there was not a definite reaction, which observation is fortified by the inconsistencies in

amount of net iodine absorbed.

At this point it was decided to substitute an electrometric titration method for the colorimetric titration method, which had failed to give consistent results. The potentiometer set-up which was used is described by Sherrill(39). A mechanical stirrer was connected with a Cenco motor, as a means of insuring a uniform solution. The reference electrode was not the conventional calomel electrode, as it was desired to avoid the presence of the Cl ion. Instead the half-cell, Hg (metal), Hg_2SO_4, H_2SO_4 (1N), technically known as the mercurous sulphate cell, was used.[o] A platinum foil, carefully cleaned and not platinized, was used as the other electrode. This was later changed for a platinum wire, because the latter did not collect bubbles, which usually cause incorrect voltage readings.

Altho diffusion proceeds from the greater to the lesser concentration, and the liquid electrode was 1 N, which was greater at all times than the titrating solution, the electrode yet suffered contamination from the permanganate in the solution. Other salts might also proceed up the arm of the electrode, and being colorless could not be detected. To overcome this error, it was necessary to allow a small amount of the liquid in the electrode to drain into the beaker at intervals during the titration.

A solution temperature of 70°C. was maintained by the use of

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[o] Credit for the suggestion to use this electrode is due Mr. A. M. Malloy, of the Chemistry Department, M.S.C.

a "micro-burner", regulated to give a minimum flame. This feature, together with the use of the special electrode and the mechanical stirrer, were the only deviations from the usual assembly employed in this work.

The method next passed through a period of trial variations. Direct titration of the solution was not possible, because it possessed an apparently endless ability to partly reduce permanganate. The first few runs were made by adding a set amount of $KMnO_4$ and after ten minutes at optimum temperature, titrating the excess with sodium oxalate. The formation of colloidal brown MnO_2 was often a deterring factor, as it was characterized by a sharp drop in voltage at a time when none was justified. It was necessary to add as much as 1 c.cm. of oxalate in excess of the end point, when the colloid would be broken down, and the excess could be run back with permanganate.

The acid concentration was raised to 5% by weight, in order to keep the cell well supplied with active ions, and not have a sluggish change in voltage readings.

The standard solutions used in this phase of the work were more carefully prepared than those formerly used. The potassium permanganate was standardized repeatedly, and the sodium oxalate was electrometrically titrated against the permanganate. The permanganate was .1141 normal, and the oxalate was .0985 normal, but again the results are computed to the basis of .1000 normal permanganate.

These trials led to the development of a technique where-in the permanganate was added at the rate of 1-1.5 c.cm. per minute, so that a

purple tinge was noted in the solution at all times. At the end of ten minutes, the beaker was placed in the apparatus, and oxalate solution added drop by drop until the fall in voltage indicated the end point. By these discrete additions of permanganate, the colloid was eliminated.

While it would be possible to show titration curves for all the reactions studied by this method, there would be no particular justification for the inclusion of such a mass of data. Instead, a few charts, (Fig.1-4) are to be found on the following pages of this paper. It is to be noted that the general form of these curves is nearly identical. Often the identical reading in millivolts was noted for the end-points of different reactions.

Table 7 shows the first series run by this method, with only the net $KMnO_4$ column of the original data sheet included.

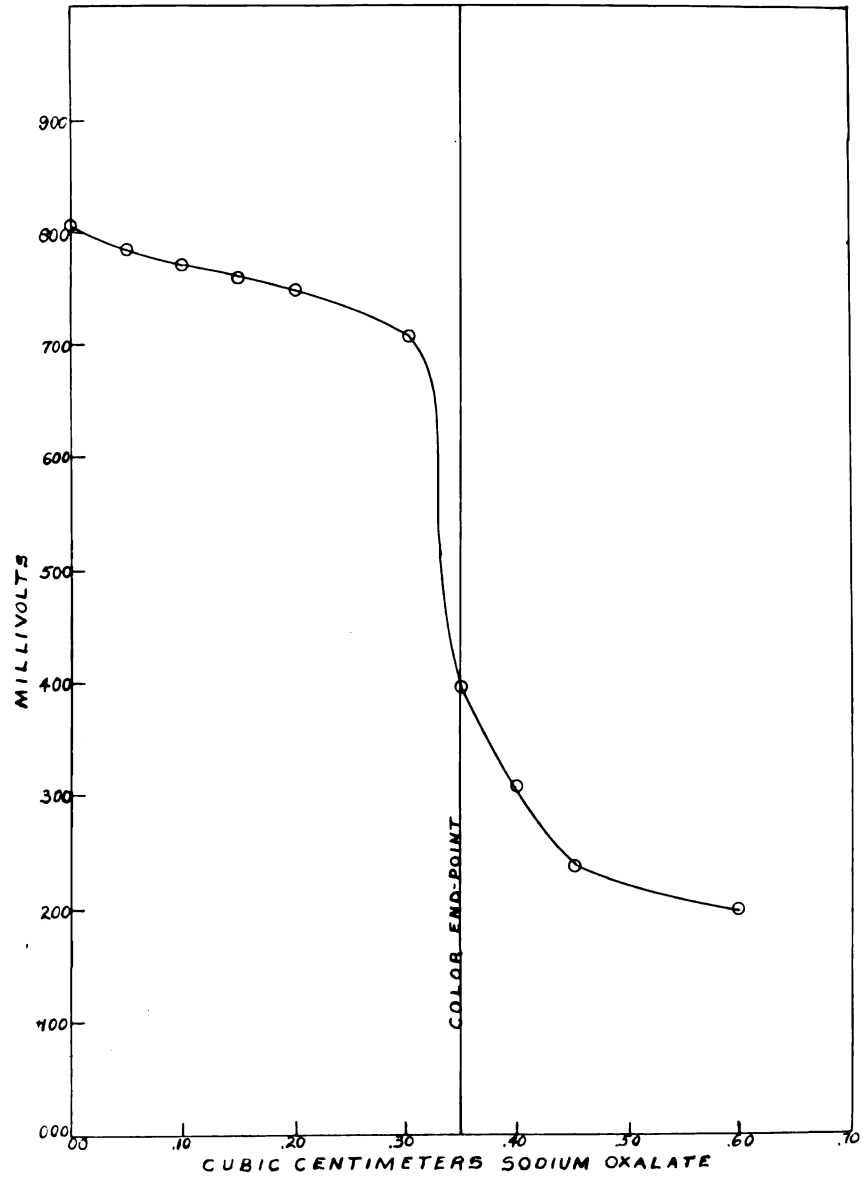
Table 7

Electrometric Titration

As a Measure of Viability

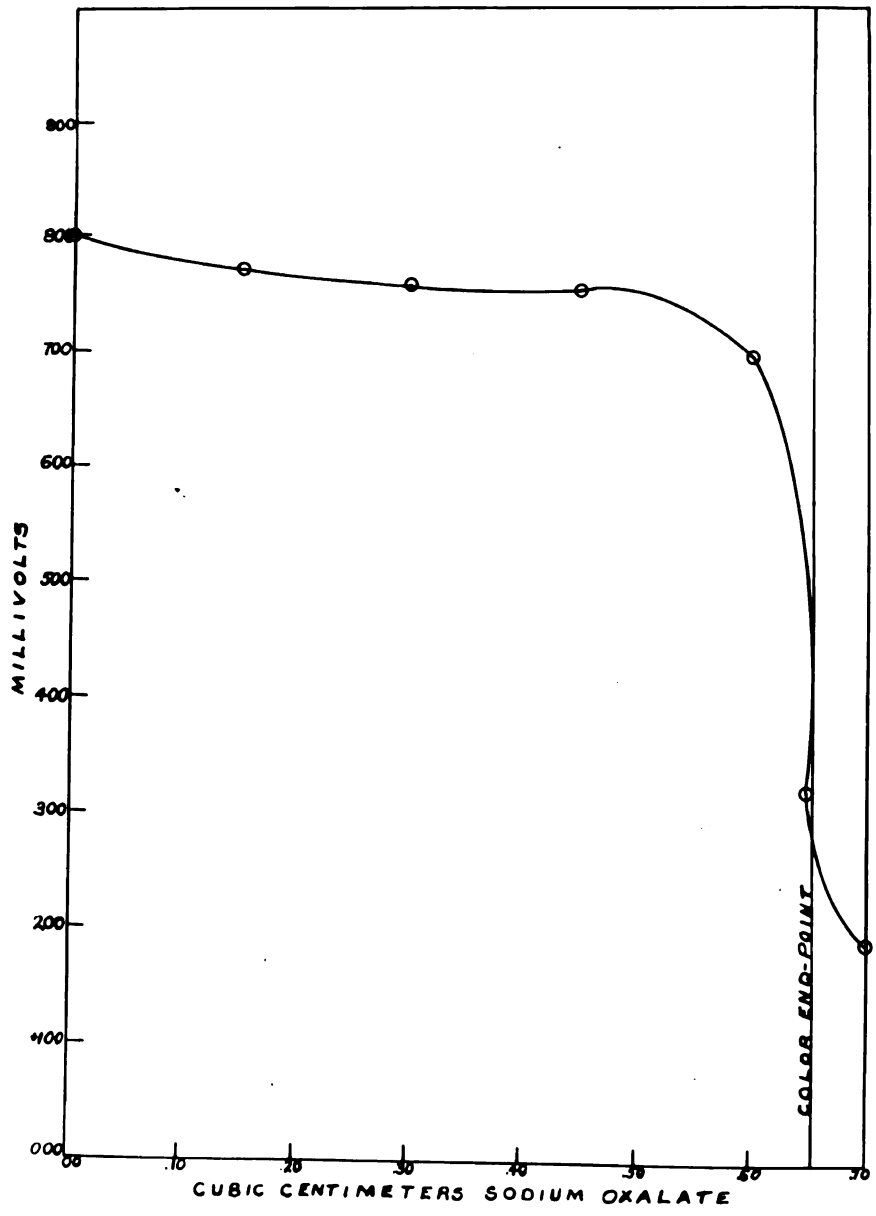
Sample	Percentage Germination	Net c. cm $KMnO_4$	Sample	Percentage Germination	Net c. cm. $KMnO_4$
17	100	16.53	3	64	10.93
11	99	9.36	4	61	7.66
12	95	7.21	5	57	3.50
7	91	13.70	14	33	9.20
10	91	16.90	1	26	1.64
13	90	5.99	6	24	13.99
3	75	7.08	15	0	46.35
16	75	13.48			

Figure 1



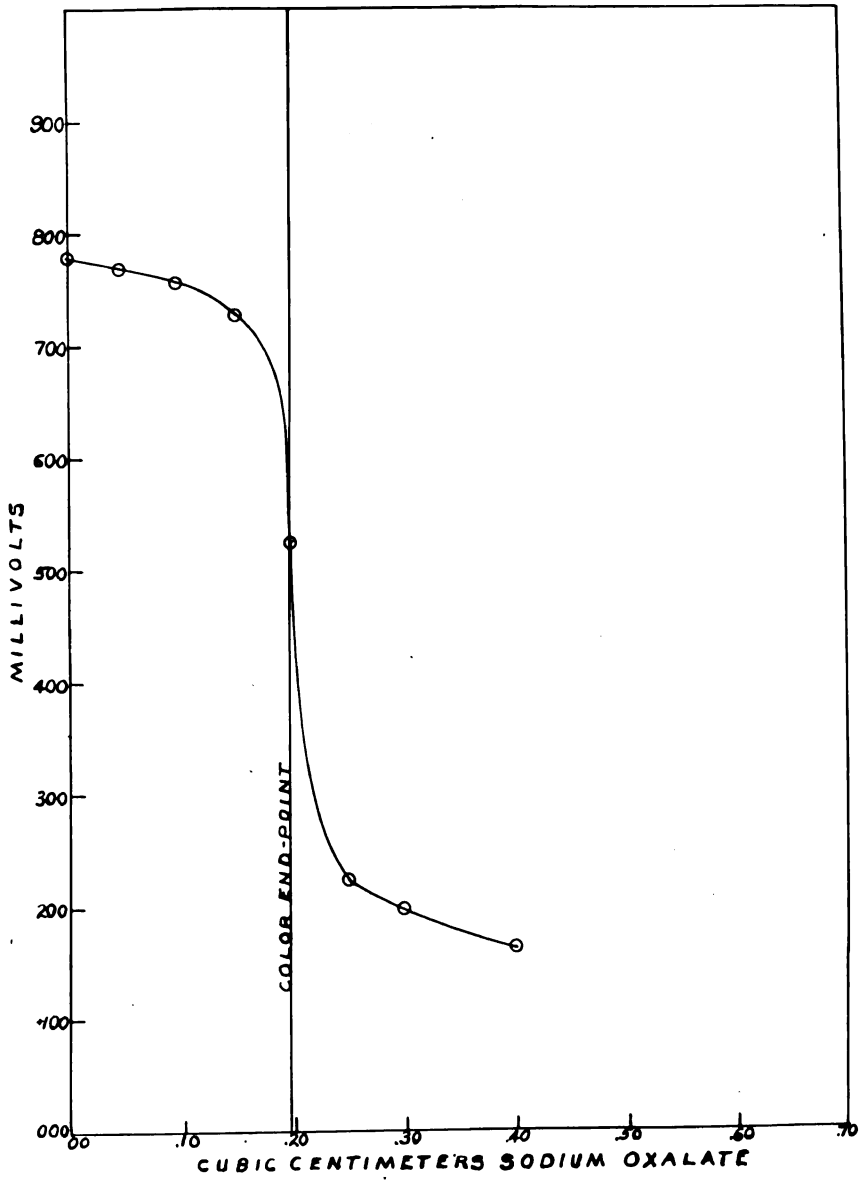
Representative Electrometric Titration Curve of Excess Permanganate Against Sodium Oxalate.

Figure 2



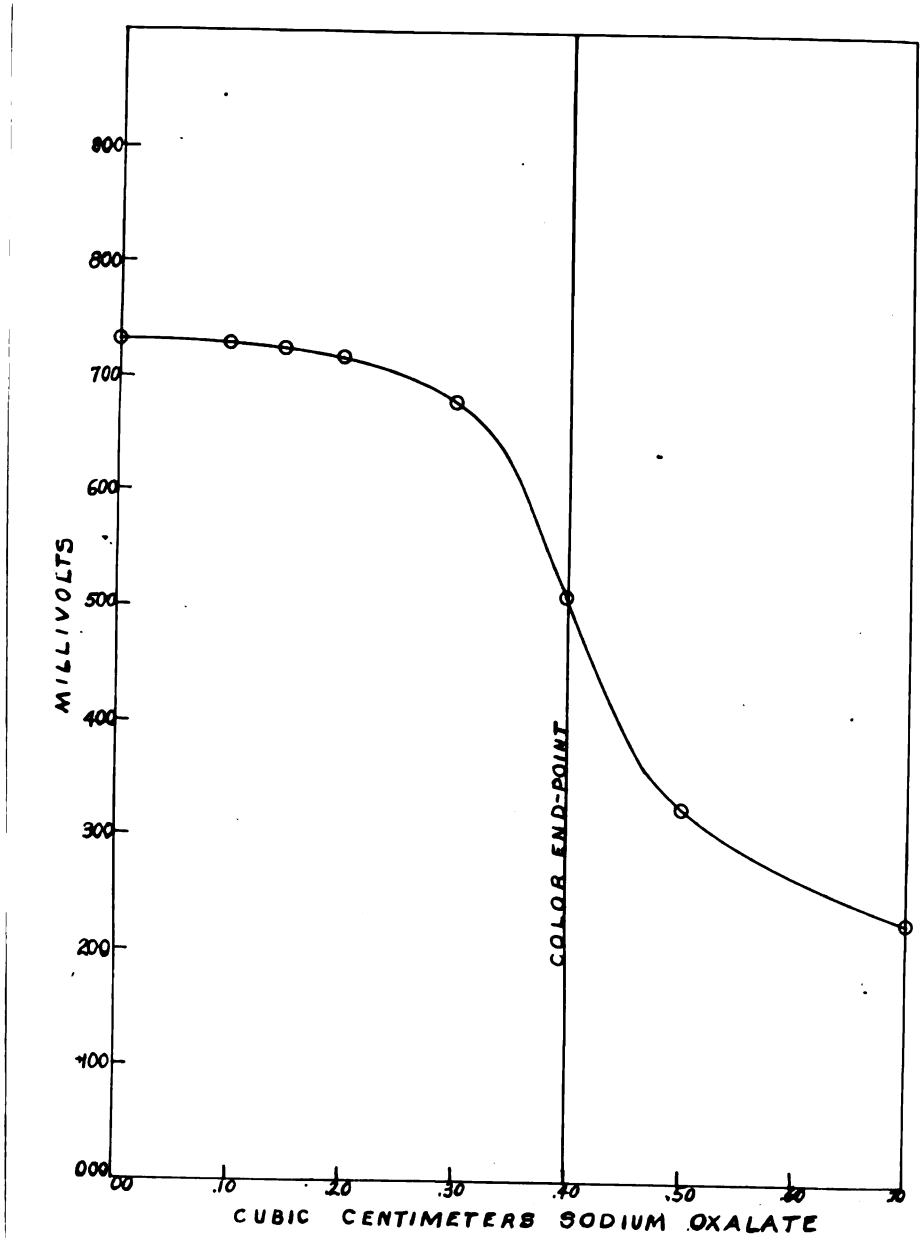
Representative Electrometric Titration Curve of Excess Permanganate Against Sodium Oxalate.

Figure 3



Representative Electrometric Titration Curve of Excess Permanganate Against Sodium Oxalate.

Figure 4



Representative Electrometric Titration Curve of Excess Permanganate Against Sodium Oxalate.

The extremely high reactions shown by most of the samples in this series was hardly explicable. In searching about for a possible flaw in the technique employed, blanks were run on all the reagents employed. As 5% phenol had been used to preserve the solutions of this series, this was included in the test. The results are shown below:

Table 8

Reactivity of phenol with Permanganate

Test Mixture	Net $KMnO_4$
Water, acid, 5 drops phenol	6.27
Extract	0.43
Extract, 0.2 c. cm. phenol	16.57

As the phenol was added to this series with no particular pains to regulate the amount, it is evident that the results are thereby vitiated.

Replicate determinations were run on samples without phenol, and the reduction of permanganate checked exactly in several runs, while in none did it vary more than a few hundredths of a c.cm.

Table 9 shows the results of a series of fresh extracts, run immediately after the completion of the period of soaking. The titrations made here are of a degree of accuracy which would be worthy of better ends. There is every reason to believe that the technique is such that the maximum reduction of permanganate occurs, and the ease with which checks are secured on duplicate determinations leads the writer to believe that the method is chemically sound.

Table 9

Electrometric Titration of Aqueous Extracts of Corn as a Measure of Viability

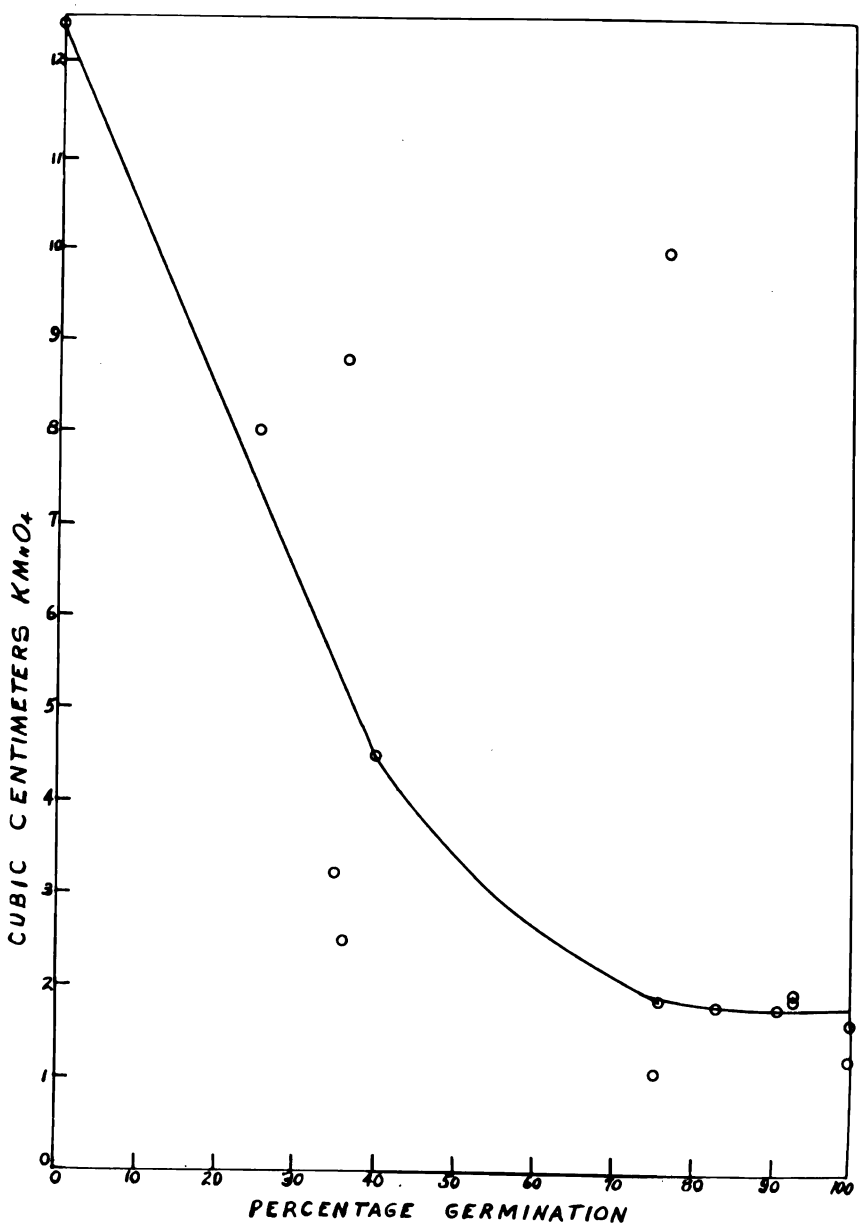
Sample	Percentage Germination	Net c.cm $KMnO_4$	Sample	Percentage Germination	Net c.cm. $KMnO_4$
11	100	1.25	13	74	1.10
7	100	1.61	2	40	4.54
10	93	1.90	5	36	2.48
17	93	1.89	1	36	8.78
12	91	1.80	3	35	3.20
6	83	1.82	16	25	7.96
8	76	9.96	15	0	12.38
14	75	1.88			

The results of table 9 indicate that there is no simple relation between viability and reduction of permanganate. From the graph, (Fig. 5), there is possible evidence that the total reduction is due to a number of components in the extract, and these may be present in such a multiplicity of proportions that it would be impossible to establish a correlation

The determinations reported thus far are not conclusively negative. Yet they are even less conclusively positive. During the time that the work on improvement of the method was in progress, some secondary developments appeared, the pursuit of which justified the continuence of the work.

First among these was the fact that the ability of the solutions to reduce permanganate was diminished quite rapidly by exposure to room conditions. This is apparent in Table 10, the history of which could be

Figure 5



Correlation Curve Between Viability and Reduction Of Permanganate as Measured by Electrometric Titration.

multiplied by that of every other extract used.

Table 10

Effect of Prolonged Standing

on Reducing Power of Extracts

Age of Sample	Net $KMnO_4$
1 day	0.64
2 days	0.39
3 days	0.39
3 weeks	0.00

It is evident that these reactions, involving probably the oxidation of unstable organic compounds, may go on with atmospheric oxygen at room temperatures. Where fungi appeared on the extracts, the loss was hastened. Coons and Klotz(6) report the lowering of the content of certain classes of nitrogenous compounds in the diseased leaves of celery. The loss of reducing power in seed extracts may be due to a progressive break down of protein compounds into a-amino acids.

Inasmuch as the results thus far presented have shown that there are large discrepancies in the correlations attempted, it follows logically that some attempt to discover the bases of these discrepancies should be made. The physical state of the extracted materials might give a clue. It is a primary concept of colloid chemistry that the ability of a material to pass through the pores of a semi-permeable membrane is governed by the state of division in which the material is found. Non-

dialyzable compounds are usually of a high molecular weight, so the possession of the reducing power by that fraction might first point to proteins as the reducing agents. The results of the first experiment in this direction are shown in Table 11. Collodion sacks formed on the inside of a large test tube were used in this experiment.

Table 11

Dialysis of Aqueous Extracts

in Colloidon Sacks

Period of Dialysis	Nature of Membrane	Net $KMnO_4$ Reduction		
		Dialysate	Colloid	Original
16 hrs.	medium	0.17	0.61	0.78
16 "	"	0.17	0.30	0.48
16 "	"	0.17	0.24	0.48
40 "	thick	0.08	0.43	0.48

This would indicate that both fractions have the ability to reduce permanganate, altho the larger part of that ability lies with the material found in a colloidal state. In the above series, there was no attempt to remove the products of dialysis. The sack, with extract, was placed in a beaker containing 75 c.cm. of distilled water and left at room conditions.

The question then arose as to whether the reducing power of the colloidal fraction might not serve as a measure of viability. It would seem from the above that the dialysate did not vary greatly in its reducing power, while the colloidal material showed considerable variation. A complete series was attempted, in which the extracts were placed in

uniform collodion sacks, which were then fitted with tubes and placed in an apparatus for continuous dialysis. Distilled water was siphoned through the beakers from an overhead supply at the rate of 1 liter per hour. At the end of a week, the contents of the bags were tested for reducing power, the results of these tests being shown in Table 12.

Table 12

Reducing Power

of Dialyzed Seed Extracts

Sample	Percentage Germination	Net $KMnO_4$	Sample	Percentage Germination	Net $KMnO_4$
7	100	0.19	3	79	0.32
11	99	0.11	6	68	0.25
10	98	0.17	2	61	0.13
17	90	0.03	14	57	0.00
8	90	0.16	1	47	0.27
5	85	0.00	16	24	0.53
13	83	0.15	15	5	0.37
12	83	0.39			

A check series, consisting of aliquots of the same solutions kept in stoppered flasks, was tested similarly at the end of an equal time period, and the results appear in Table 13.

While theoretically, dialysis might have stabilized the extracts by removing oxidizing agents, the only evidence from these tables is that the loss of reducing power is more rapid when the products of oxidation are removed by dialysis and hydrolysis. The removal of the products enables the reaction to proceed in one direction until nothing remains

to be oxidized or broken down. There is no applicability to measurements of viability.

Table 13

Reducing Power

of Undialyzed Seed Extracts

Sample	Percentage Germination	Net $KMnO_4$	Sample	Percentage Germination	Net $KMnO_4$
7	100	0.53	3	79	1.17
11	99	0.37	6	68	0.49
10	98	0.49	2	61	0.36
17	90	0.35	14	57	0.51
8	90	4.95	1	47	2.77
5	85	0.54	16	24	2.24
13	83	0.66	15	5	10.58
12	83	0.50			

Reducing Power of Extracts of Corn Meal

It is an obvious conclusion that the extract of corn meal would possess greater reducing power than the extract of whole grains. Because it was desired to germinate all the seeds which were tested for reducing power, it had been impossible to work with meal. Comparative results on this were desirable, however, so 500 grains of sample No. 17 were coarsely ground. This sample had an average of 90% germination during the period of experimentation. From Table 9, it will be seen that whole seeds of this sample had a net reduction of 1.89 c.cm. of per-

manganate. When meal was extracted at room temperature for 24 hours, care being taken to use a proportionate amount of water, the net reduction was 22.84 c.cm.; and when the extraction of meal was made at 30°C. for 12 hours, the net reduction was 37.29 c.cm.

Comparision of Electrometric and Colorimetric End-Points

As valuable as the electrometric titrations proved to be in the development of the technique of the method, it is evident from the charts, (Fig. 1-4), that the color end point coincides very closely with the point of maximum change in voltage. The very abrupt drop in voltage was not any more striking than the fading of the purple color, when the appropriate amount of oxalate had been added. In the interest of simplification of methods, there was no reason why a return to colorimetric titrations should not be effected. Thus it is that all succeeding determinations of permanganate reduction are on that basis.

Isolation of Reducing Compounds in Seed Extracts

On the basis of the preliminary experiments so far reported, there seemed ample justification for an attempt to isolate the compound or compounds, which were responsible for the reduction of permanganate. The loss of reducing power on prolonged standing would indicate organic compounds. But such an assumption is not the basis for any conclusions, because of the number and complicity of organic compounds that might diffuse out of the seed. It becomes necessary to examine farther as to the nature of the compounds in question. Empérical considerations would indicate that proteins have no monopoly on the property of reduction, yet they would seem to occupy a commanding position in the study.

Hawk(15) lists lead acetate and ammonium sulphate among the common precipitants for proteins. Saturation with these reagents is supposed to bring down all the proteins. In the case of the former salt, the excess lead in solution is removed by anhydrous sodium carbonate. Table 14 gives the results of tests with these reagents.

Table 14

Reducing Power of Fractions

of Extract of Corn Meal

Description of Fraction	Net $KMnO_4$	Description of Fraction	Net $KMnO_4$
Original Extract	37.29	Original Extract	7.40
Filtrate of Lead acetate	4.53	Filtrate of $(NH_4)2\ SO_4$	2.62
Precipitate of Lead acetate	0.00	Redissolved Precipitate $(NH_4)2\ SO_4$	2.46

In the ammonium sulphate precipitation, the failure of the two portions to equal the original in reducing power may be attributed to the fact that some of the proteins were denaturalized and failed to redissolve in the dilute solution of ammonium sulphate which resulted. The lead precipitate was entirely insoluble in the concentration of acid employed in these tests.

Osborne and his associates(28, 29, 30, 31) devoted a lifetime to the study of the vegetable proteins. Their classifications, nomenclature, and methods of isolations are standard, hence any procedure dealing with proteins well be borrowed 'en tout' from their works. In relation to the proteins of corn, their amounts and properties, the following classification is valuable.

Classification of the Proteins of Corn, "Zea Mays"

1. Protein soluble in pure water---------- Proteose-------0.06%

2. Protein soluble in aqueous extract
(Very dilute salt and acid solution)

A. Re-precipitated by dialysis---------- Maysin---------0.25%

B. Coagulable by heat in presence of NaCl---- Maize globulin-0.04%

3. Protein soluble in 10% NaCl---------- Maize Edestin--0.10%

4. Protein soluble in 80-90% alcohol---------- Zein----------5.00%

5. Protein soluble in dilute alkalines and acids-- Glutelin-------3.15%

In attempting to isolate these compounds, one must follow a laborious scheme, the essential features of which are shown in the description of the actual technique. Forty grams of the ground meal of sample No. 17 were weighed out and extracted in 200 c.cm. of distilled water for a period of twelve hours. The extract was decanted, an equal amount of water added to the meal, and the extraction repeated. The fractions were then combined.

All the proteins and proteoses were precipitated by complete saturation of the solution with $(NH_4)_2SO_4$. The precipitate was redissolved by dilution with water. It was then dialyzed in a collodion sack for a period of ten days, using running distilled water. Maysin separated out within a few days, and filtrate showed only a faint trace of maize globulin by testing with 10% NaCl in HCl solution and heating to 80°C.

Filtrate tested for proteoses and their presence indicated in slight amount upon dialysis of the filtrate into concentrated alcohol, and further concentration of the alcohol to high percentages by addition

of 95% alcohol.

The meal was then extracted for a similar period of time with 10% NaCl, the extraction repeated and the solutions combined and dialyzed. A small amount of maise edestin was obtained, when sufficient salt had been removed to cause precipitation.

The meal was next extracted with 80% alcohol. Zein was obtained in abundance, combined with some alcohol soluble pigments. Petrol ether removed part of the yellow carotinoid pigments, but not enough to justify fractionation. By evaporation of the alcohol and replacement with water, the zein was precipitated in large masses. Redissolution in 85% alcohol and evaporation left the zein as a flaky, horny, hyaline layer on the cover glass.

After these treatments, the meal was devoid of color, and was a powdery, granulated material. No extraction with dilute alkali or acid was attempted. Such treatment would not furnish a protein whose reactions would be characteristic of the compound in its natural state.

It is not claimed that the proteins were obtained in even approximately a pure state, but they were nevertheless recognized as entities, and it was possible to measure their reducing power. Because of the fact that precipitation of the globulins and albumins is apt to cause the formation of irreversible colloids, it was found more valuable in some cases to conduct the tests upon the solutions. In the case of maysin, which is coagulated by removal of the protective ions, it was impossible to entirely redissolve it, only a small fraction being

amenable to boiling with 5% H_2SO_4. Maize globulin was entirely refractory when once precipitated, but it was possible to keep it in solution. The results of the tests are shown in Table 15.

Table 15

Reducing Power

of Proteins of Corn

Material	Amount in gms.	Net $KMnO_4$
Proteose	.001	0.09
Maize Globulin plus Proteose	.004	0.80
Maysin	.020	1.98
Edestin	.006	0.65
Zein	.020	4.24

Thus, instead of finding a single protein capable of reducing permanganate, all were found to possess the ability. Osborne(29) reports the reduction by zein of ferric chloride in an alcoholic solution, but the failure of that protein to reduce potassium ferricyanide. The writer was not able to secure reduction of potassium dichromate, using aqueous extracts which reduced permanganate strongly.

If the proteins reduce permanganate, might it not be that they do so by being themselves broken down by the rigorous conditions of the test, If such an assumption is sound, then amino-acids should be able to reduce permanganate as well. Osborne and Clapp(30) give the products of hydrolysis of the proteins of maize. It was not possiole to obtain

all the amino-acids listed, but several were available. In this connection, it might be noted that non-protein compounds of nitrogeneous nature are found in corn, Schulze and Castoro(38) reporting 0.90 per cent of non-proteins. Schulze(37) found that maize contains 0.25 per cent of lecithin, while Czapek(9,Bd.1, p. 157), reported the same amount in yellow maize and 0.28 per cent in white maize. Jodidi(17), studying the non-proteins of the ungerminated seeds of maize, found polypeptides, free amino acids and acid amides present. An indication of the reducing power of some of these compounds is found in Table 16.

Table 16

Reducing Power of Primary

Nitrogeneous Compounds

Material (20 mg. of each used)	Net c. cm. KMnO4	Material (20 mg. of each used)	Net c. cm. KMnO4
Leucine	2.31	Nucleic Acid (Yeast)	2.49
Asrartic Acid	0.05	Sodium Glycocholate	0.57
Asparagine	0.00	Brucine	13.12
Tyrosine	24.36	Xanthine	0.19
Tryptophane	17.84	Creatine	0.00
Lecithin	1.44		

The first half of Table 16 deals with amino-acids found as products of the hydrolysis of the proteins of corn. Included in this group is the phospho-protein, lecithin. The latter half of the table is not directly applicable to this study, but has general interest in demonstrating the wide range of reactivity of permanganate.

It is interesting to note that all of the high reacting compounds contained a ring structure. According to the classification of Haas and Hill(14, p. 324) these are as follows: tyrosine, an aromatic compound, B-parahydroxyphenyl, a-amino proprionic acid; tryptophane, a heterocyclic compound, B-indole a-amino proprionic acid; brucine, a complex alkaloid of the quinoline group, characterized by two six-membered rings condensed together. Lasser-Cohn(20) mentions also that permanganate reactivity is a means of distinguishing between unsaturated acids and saturated acids containing open or closed chains, and carboxylic acids of benzene or similar bodies.

The role of this group of compounds was thus sufficiently established, but there had also been indications that the simple sugars were not lacking in reducing ability. Qualitative tests on glucose confirmed the suspicion, so the tests tabulated below were performed.

Table 17

Permanganate Reduction

by Common Sugars

Sugar (20 mg. of each used)	Net c. cm. $KMnO_4$	Sugar (20 mg. of each used)	Net c. cm. $KMnO_4$
Arabinose	9.13	Galactose	9.34
Xylose	8.80	Sucrose	9.96
Dextrose	6.38	Maltose	5.13
Mannose	6.01	Lactose	3.24
Levulose	8.36	Raffinose	4.43
Sorbose	8.68		

While the data on reducing power of sugars in no way constitutes a scientific novelty, the direct application of permanganate is not mentioned in the literature. The nearest approach is the indirect method wherein the reduced copper is measured by permanganate titration. In this connection, mention might again be made of the action of permanganate on starch, noted by Reichert(35). The significance of sugar in the aqueous extract is differently interpreted by Miller and Hibbard(25). They considered it as a stabilizing agent in the formation of silver sols by reduction of silver nitrate, while proteins were given the power of reduction.

The actual presence of sugar in the aqueous extracts was positively determined by a standard method. Clarification of the extract was by use of Horne's anhydrous lead sub-acetate, which would remove the proteins, but not the nitrogenous compounds of non-protein character, or the sugars. The excess lead was then removed with Na_2HPO_4. The test for sugar was based on the Munson and Walker method of precipitation(1,p.190), and the Schaffer-Hartmann iodometric titration of copper(34). The only modification was that 9.2 N H_2SO_4 to the amount of 17 c.c. was used, instead of 5N to the same amount.[o] Fading of the end-point was overcome by this change.

With proteins and sugars aligned in respect to their property of reducing permanganate, the only remaining group of water-soluble compounds of any importance, are the non-protein nitrogenous derivatives. A rather involved method of proof was employed in their case. The

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[o] Credit is due Mr. H. F. Clements, of the Botany Dept. U.S.D., for this change.

clarified extract prepared as described above was submitted to both the standard sugar test and the permanganate reduction test. By means of the data of table 17, the permanganate reduction for that quantity of glucose was computed. The difference in permanganate reduction might then be attributed to the non-proteins. Extract of corn meal was used for this test, in order that larger differences might be obtained and thus give a firmer basis for comparision. The scheme is shown in the following table.

Table 18

Non-Proteins as Agents in

the Reduction of Permanganate

Description of Test or Procedure	Net $KMnO_4$
Permanganate on clarified meal extract	34.46 c.cm
Standard sugar on same (24.4 mg. glucose)	
Computed $KMnO_4$ for 24.4 mg. glucose	11.66 c.cm
Difference attributable to non-proteins	22.80 c.cm

It is not possible, in the light of later findings, to give much weight to the results of table 18, except in conceding it would certainly show that the non-proteins are not immune to the action of permanganate.

With only a few tests, it is not possible to attempt the establishment of a correlation between the amounts of these components and the total reduction of permanganate. The various constituents are all

influential in the reduction, yet not in equal measure, or even intensity of reaction. The fact that the reducing power is quite rapidly diminished upon standing may be in part a matter of actual decrease of the materials, or it may be an oxidation without any other quantitative differences.

A test of the correlation of the content of protein, non-protein, and sugar, with the reduction of permanganate, was next attempted. It was hoped that some clue to the unusual reactivity of several samples of good germination, might be obtained.

The methods were conventional. The sugar test was as given in the preceding pages of this paper. Total nitrogen was run by the Kjeldahl method, using $CuSO_4$ as the catalyst. The acid used to absorb the NH_3 given off was found to be exactly N/10 by gravimetric determination.

On the first few samples, it was attempted to run the non-protein nitrogen from the clarified sugar-test extract. This proved impossible because the lead contained considerable nitrogen as a contamination. Precipitation by phospho-tungstic acid proved more consistent. The test is as follows: 5% phospho-tungstic acid in 5% H_2SO_4 is added to a seed extract made acid to 5% with H_2SO_4 and heated to boiling. Fortunately, a sufficient amount of the original extract from the samples remained to repeat the test with the latter reagent. The protein nitrogen was obtained by difference. Direct determination was unaccountably inconsistent, and it was not considered germane to this study to spend time on that problem.

Permanganate reduction was run on the original extract and the sugar-test extract. The reacting power of the latter to permanganate

exactly equaled that property in the extract from the phospho-tungstate precipitation, so that the results shown in table 19 are as applicable as if run on the identical solutions.

Table 19

Test of Correlation of Content of Proteins, Non-Proteins, and Sugars, with Reducing Power in Terms of Permanganate

Sample	Percentage Germination	Total $KMnO_4$	Total N-in mg.	Non-Pro. N-in mg.	Glucose in mg.	Second $KMnO_4$
10	94	0.53	.195	.130	0.29	0.42
3	85	1.16	.146	.122	3.80	0.91
8	83	6.32	.326	.204	8.83	2.73
7	73	0.71	.130	.082	2.28	0.49
2	71	1.08	.163	.130	0.80	0.46
6	64	0.52	.114	.082	2.24	0.42
5	60	0.77	.212	.203	1.52	0.42
1	29	5.05	.489	.185	2.72	2.95
16	21	5.49	.619	.521	6.53	3.82
15	0	9.15	1.695	1.385	12.77	1.82

In this series, 100 seeds of each sample were placed in a flask with 200 c.cm. of distilled water and allowed to soak for 24 hours at room temperature. The extract was decanted, filtered through a coarse filter paper, and made up to 200 c.cm. 50 c.cm. of this was precipitated

with lead for sugar and permanganate tests, 50 c.cm. with phospho-tungstic acid for non-protein nitrogen, 50 c.cm. used for duplicate determinations of total nitrogen, and the remainder devoted to total permanganate reduction tests.

As the data in previous tables is all on the basis of 10 c.cm. of extract, the amount obtained from five seeds, the results of these determinations will be similarily reduced. In that proportion the amounts of some of the constituents would be too small for detection, but as the results were obtained on samples averaging five or ten times the minimum amount, checks were very consistent.

From table 19 it may be computed that the amount of sugar in the extract varied from .02% to .80% of the average weight of the corn, while the amounts of nitrogen were of a lower order. But within the range presented, wide differences are evident.

The small amounts of soluble material in the unsprouted seed was the subject of early investigations. In 1885, Portele(33) made a study of the chemical nature of yellow corn at various stages in its growth. Starting from the time of flowering and running to the time when the kernels were hard, there was a steady decrease in sugars and soluble nitrogenous compounds, and a steady rise in starch.

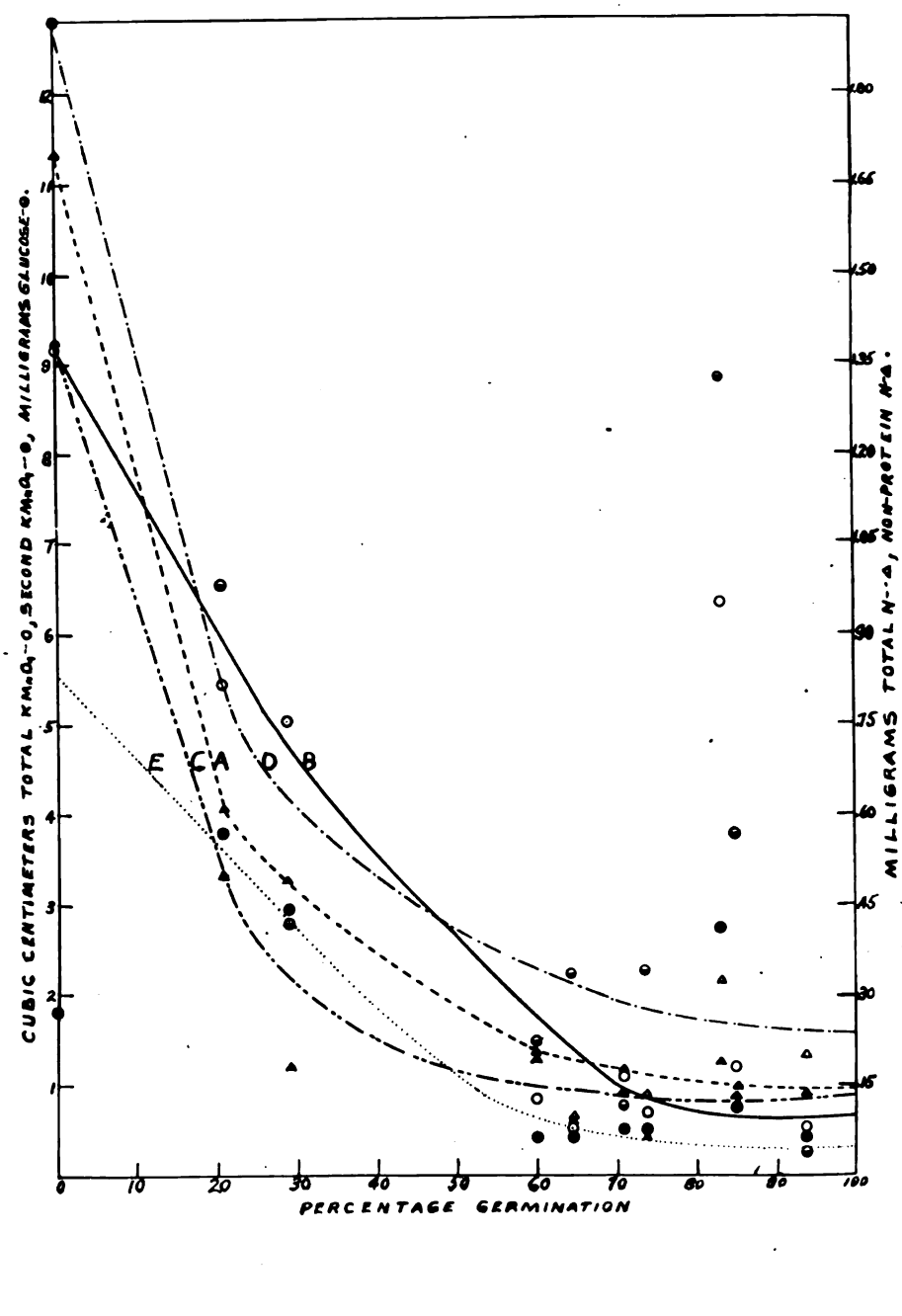
But it is recognized that the act of soaking seeds would encourage enzymatic activity and initiate chemical changes tending toward the formation of simpler compounds. The diffusion of these products into the solution might be at different rates, however. As early as 1879, Green(12) reported on the changes accompanying germination in the castor

been. He recognized the presence of "ferments", which, to use his own words, 'were existing in consequence of the available activity stirred up in the cells by the conditions favorable to germination, especially moisture and warmth'.'

As far as the possibility of establishing a close correlation between the components and their total reduction is concerned, a study of the graph, (Fig.6) will show that it is not to be expected. It is clear that proteins, non-proteins, and sugars are all workers in the enterprise of producing permanganate. But just how active each one may be is difficult to determine by this data. For instance, if only samples No. 5 and No. 6 were considered, it would seem that the protein-free extracts of most reduced permanganate equally. But No. 6 had a lesser amount of non-protein nitrogen, and No. 5 a lesser amount of sugars, and the differences were compensating. Beyond these two samples, the correlation in this part of the table was very inharmonious.

A more important correlation, and one that seems to have a sound basis, is the relation of the total permanganate to the total nitrogen plus the glucose. If it is remembered that the amounts of albuminoid nitrogen are to be multiplied by 6.25 and the proteins thus estimated, the comparative amounts of nitrogenous and carbohydrate material would seem more reasonable. Rearrangement of the samples with regard primarily to their total permanganate reduction, and next to digestibility, and a comparison of this reduction with the combined amounts of nitrogen and sugars, gives interesting results. Only one sample, No. 2 is radical in its departure from the type, as shown in Table 20.

Figure 6



Correlation Curves Between Viability and Various Chemical Components as follows: A. Total Nitrogen, B. Total $KMnO_4$, C. Non-Protein Nitrogen, D. Glucose, E. 2nd $KMnO_4$(Reduction of Protein- Free Extract).

Table 20

Comparative Reducing Power

of Sugars and Nitrogeneous Compounds

Sample	Total $KMnO_4$	Total N-in mg.	Glucose in mg.
6	0.52	.114	2.24
10	0.53	.195	0.29
7	0.71	.130	2.28
5	0.77	.212	1.52
2	1.08	.163	0.80
3	1.16	.146	3.80
1	5.05	.489	2.72
16	5.49	.619	6.53
8	6.32	.326	8.83
15	9.15	1.695	12.77

Even granting the fact that the nitrogeneous compounds are present as proteins or similar compounds, they are nevertheless more active, gram for gram, than the sugars. A difference of .08 mg. of nitrogen or .50 mg. of protein, between samples No. 6 and No. 10 is compensated by 1.95 mg. of sugar to give an equal permanganate reduction. But between samples No. 7 and No. 5, an equally great difference of nitrogen is compensated by .76 mg. of sugar, so it is hardly wise to draw any hard and fast conclusions.

With only general relations under consideration, it is convenient to consider the samples in pairs of nearly equal reducing power. In all cases of this sort, an excess of sugars in one is compensated by an excess of nitrogen-bearing compounds in the other. In the latter part of the table, samples of markedly greater reducing power are found to surpass those of lesser activity in content of both sugars and nitrogeneous compounds. That the reducing power of aqueous extracts rests on the sugars and nitrogeneous compounds is clearly demonstrated.

The variation in germinability of a sample of seed upon the conventional method of growing the seeds may amount to as much as five or ten per cent. If there was any correlation between viability and reducing power, it might be expected to stay within the same limits. But when the variation in reducing power is very much greater, as was found in table 19, it is sound to conclude that a positive correlation is lacking.

Discussion on Experimental Data

On the basis of results presented in this paper, it cannot be assumed that there is any correlation between the viability of seeds and the reducing power of their aqueous extracts. As far as the relative position of samples in a series was concerned, there was a fair consistency in the reaction when tested from time to time. But it was only infrequently possible to find a group of samples which would give gradations in reactivity at all comparable to the viability.

Why are the results inconsistent? A number of reasons might be advanced, and no one alone suffice to interpret the situation. For the first line of approach let us consider the chemical phases of the question. A great amount of work on the chemical composition of corn has been published. With special reference to the variations encountered, mention might be made of the analyses reported by Bushey(5), Ladd(19), Leach(21), Lindstrom and Gerhardt(23), and Portele(33). This group might be greatly augmented, but the type conclusions found are much the same.

The content of all the important constituents may be varied by a host of circumstances. The state of maturity enters very strongly in influencing the composition. Bushey(5) found that corn killed by frost had a high percent of non-proteins in the form of polypeptides and amino-acids. Immature corn is also known to have more sugars and less starch than riper samples. Genetic differences are the bases of great differences in composition, as has been shown, among others, by Lindstrom

and Gerhardt(23). Lack of chemical uniformity is so conclusive that no further mention need be made of it.

Added to these differences, the fact that in permanganate reduction, several groups of compounds were active, makes the task of establishing a correlation on chemical grounds well nigh impossible. The various possible combinations in amount of these components, combined with the differential reactivity of the groups, makes the relation still more complex.

The physical state of plant membranes is not the least important factor in establishing differences. Shull(40),in his study of the semipermeability of seed coats, found that even dead plant membranes might be semipermeable. The entirely impermeable nature of the coats of many seeds, and the deterrant effect of this on germination, has been the subject of investigation.

The effect of the colloidal state on the permeability is none too clearly defined. Whether changes in permeability are due to the coagulation of the proteins, a view advanced by Crocker(7), is hardly definitely proven.

The recent findings of Hottes and Huelson(16) on sweet corn constitute an interesting study of physico-chemical state. Although their studies were on the relation of the seedling vigor to the colloidal properties of the aqueous extract, the relation of the latter to viability was also indicated. Between samples of zero germination and those of 95% to 100% germination, the colloidal index, as measured with a Leitz nephelometer, varied considerably. The

results show that denser suspensions were found in extracts from seeds of low viability, but as far as the applicability of these findings to measurements of lesser differences in germination power is concerned, the method shows little promise.

In consideration of the data presented in table 19, it would seem that the most important change accompanying loss of germinating power is an increase in permeability. Yet even this rule is violated in at least two cases out of ten, and it is necessary to assume some uncommon occurrences in the history of samples like No.8 and No.3 in order to explain their high rate of exosmosis. Subject to these deviations, the difference in permeability seems to bear a fundamental relation to the phenomonon of death.

Summary

1. The literature on methods for determining viability was reviewed.

2. The permanganate reduction method as a measure of viability was made the subject of a process of refinement.

3. Electrometric titrations were substituted for colorimetric titrations with a view toward development of a more discriminating technique.

4. The method was perfected sufficiently to insure consistent results.

5. No correlation between differences of viability of as much as ten per cent, and permanganate reducing power of extract, was established in any case.

6. Colorimetric titrations under the conditions established were proven as accurate as electrometric.

7. In a supplementary experiment, iodine absorption of aqueous extracts was measured, but even less promise was shown by this method.

8. The reducing power of dialyzed extracts was found to have no correlation with viability.

9. Isolation of compounds causing reduction of permanganate was attempted.

10. The following classes of compounds were found to have reducing power: 1, proteins found in corn; 2, some amino-acids found in corn, and other primary nitrogeneous compounds; 3, common sugars; 4, nitrogeneous compounds of non-protein character found in corn.

11. A correlation between content of proteins, non-proteins, and sugars of extract, and reducing power of extract, was attempted on basis of standard analyses.

12. Positive correlation between permanganate reducing power of solution and total nitrogen plus sugar content was indicated.

13. No correlation between viability and amounts of any of the constituents was found.

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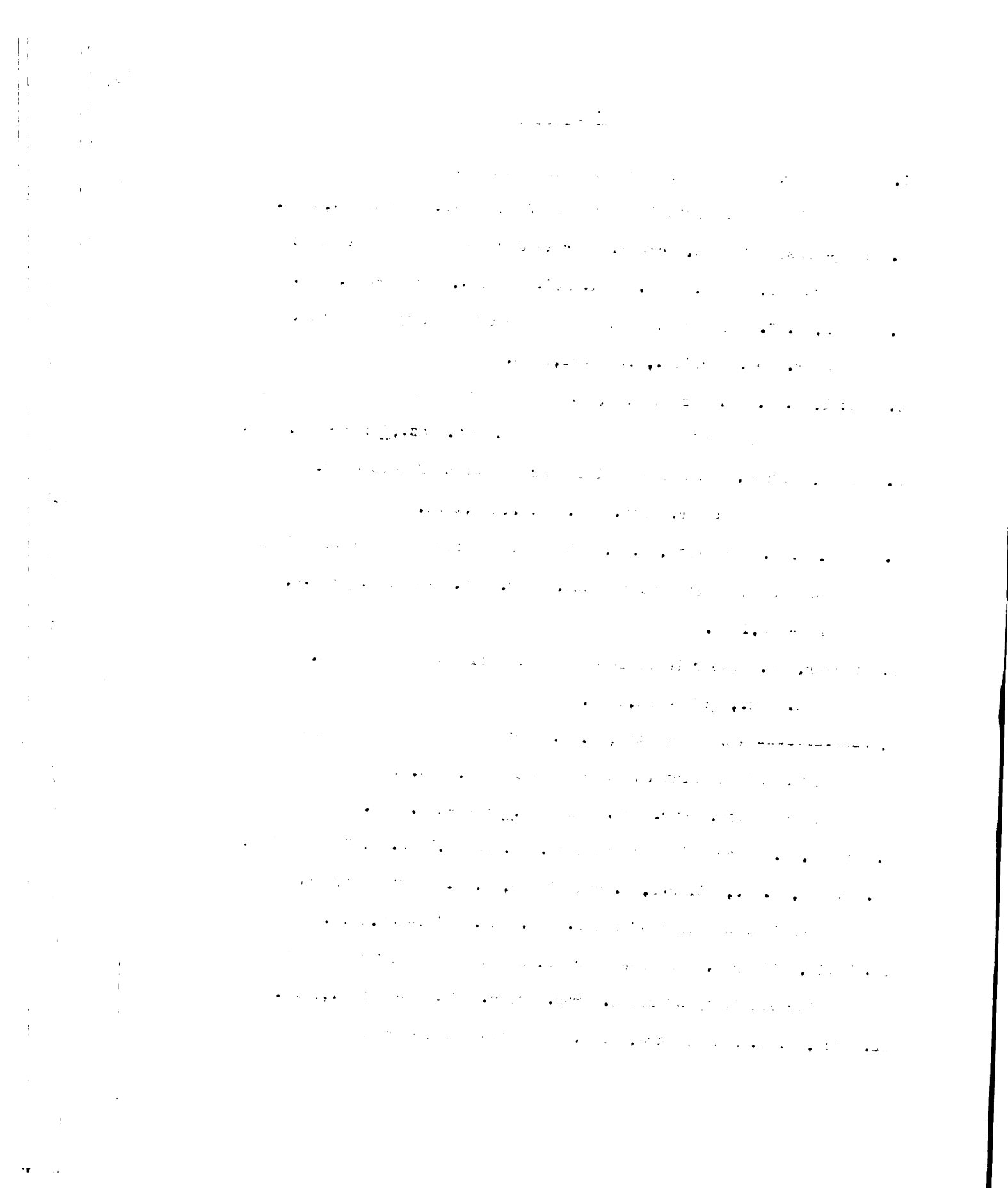

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