EGG PROTEIN DETERMINATION

Separation of Egg Yolk Proteins by Paper Electrophoresis

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Isolated egg yolk proteins differ in properties from preparation to preparation. Proteins of whole unextracted yolk were separated from each other by the ridgepole paper electrophoresis technique of Durrum using a pH 8.6 diethylbarbiturate buffer of 0.05 ionic strength. The proteins were dyed with bromophenol blue and the lipides with Oil Red O. The protein color was eluted with dilute sodium hydroxide solution for quantitative studies and absorbance was determined at 590 mμ in the spectrophotometer. Fresh egg yolk contained 8.6% livetin, 41.7% lipovitellenin, 46.4% lipovitellin, and 3.3% of other proteins. Extraction with ether changed the electrophoretic behavior of the proteins so that lipovitellenin was not separated from lipovitellin, probably because part of the lipide is linked to lipovitellenin by a bond that is easily broken by contact with ether even in the cold. Paper electrophoresis offers a method for the study and segregation of egg yolk proteins without prior ether extraction.

EGG YOLK PROTEINS have not been studied as extensively as the proteins of egg white, because until recently there have been no indications of any such important biochemically active materials in egg yolk, and because these proteins are difficult to isolate. Some studies of egg yolk proteins have been made using the conventional moving-boundary electrophoresis technique (*3*, *14*, *20*), but the data obtained were fragmentary and not very clear-cut. Evans and Bandemer (*5*) used paper electrophoresis for the separation of egg white proteins and their quantitative determination. Nothing has been published on the use of paper electrophoresis for the separation of egg yolk proteins from each other. The purposes of the experiments reported herein were to study the behavior of egg yolk proteins when subjected to electrophoresis on paper and to determine the effects of different methods of preparing the yolk on the electrophoretic behavior of the protein.

Method of Paper Electrophoresis

The filter paper electrophoresis cell used was of the ridgepole type (Spinco Model R, Specialized Instruments Corp.) (*2*, *19*). The procedure is the same as that used in a previous study (*5*) and the diethylbarbiturate (barbital) buffer (also described previously) of pH 8.6 and 0.05 ionic strength was used.

Some of the paper strips were stained for lipide, instead of protein, by placing them, for 16 hours, in a saturated solution of Oil Red O in 50% ethyl alcohol (*4*). They were rinsed twice for 10 minutes each with distilled water and air dried. The Oil Red O stain was prepared by allowing an excess of dye to stand in 60% ethyl alcohol for 16 hours with occasional shaking to mix the dye with the solvent.

The filter paper strips were "scanned" in a Welch Densichron densitometer using the white light source. Absorbance was then plotted against distance traveled by the protein or lipide.

Procedure

Preparation of Egg Yolk Samples Most of the work was done either on whole egg yolk, diluted with an equal weight of 10% sodium chloride solution, or water, or on similar yolk solutions extracted one to three times with ether. However, in some early work the yolk solutions were exhaustively extracted with ether. The yolks of several eggs were separated from the whites, and any adhering white was removed from the yolk by rolling

it on cheesecloth. The yolks were then well mixed with an equal weight of 10% sodium chloride solution or water. Several methods were used to extract the "free" lipides with ether. Extraction of the saline-diluted yolk in the cold (0° C.) caused part of the protein to precipitate after the second or third ether extraction. A clear solution remained after the first extraction, which removed most of the yolk pigments. Peroxide-free, reagent grade, absolute ether was used in part of the experiments and untreated U.S.P. ether was used in the rest.

Cold egg yolk diluted with an equal weight of 10% sodium chloride solution was mixed with cold, peroxide-free, reagent grade ether and allowed to stand in the cold. An emulsion formed which took 3 days to break. The yolk-saline-ether emulsion and the yolk-saline solution were used for paper electrophoresis within 4 hours after the emulsion was made and after 1, 2, and 3 days' standing in the cold.

Preparation of Egg Yolk Proteins

Lipovitellin. Lipovitellin (*12*) was prepared by a modification of the method of Alderton and Fevold (*1*). The yolks of 12 eggs were separated from the whites and rolled on cheesecloth to remove all adhering white. The yolks were well mixed with twice their weight of distilled water, and the diluted yolk emulsion was centrifuged in a Servall angle centrifuge at full speed. Most of the lipovitellin was deposited as a waxy translucent solid, which was dissolved in 5% sodium chloride solution to give the material used for electrophoresis.

Lipovitellenin. Lipovitellenin (*6*) was prepared from the supernatant of the lipovitellin precipitation by dialysis with distilled water in the cold until no more chloride was detected in the dialysis water. The solution was adjusted to pH 6.2 with sodium hydroxide solution and centrifuged at full speed in the Servall angle centrifuge. This broke the emulsion and part of the lipovitellenin rose to the top as a butterlike solid. The mixture was poured into a separating funnel and allowed to stand in the refrigerator (0° C.) overnight. Because there was not a clear-cut separation of solid and liquid into two layers, the mixture was filtered onto a wet paper. Lipovitellenin remained on the paper.

Livetin. No special separation of livetin (*8*) was made, but the protein remaining in the filtrate from the lipovitellenin preparation was considered to be essentially livetin. No attempt was made to obtain pure and uncontaminated proteins in this study but only to get preparations in which most of the protein was the one desired, so that it could be used to identify the electrophoresis bands obtained with whole egg yolk. Livetin, especially, was contaminated with unprecipitated lipovitellin and lipovitellenin.

Quantitative Determination of Protein Distribution in Egg Yolks

Evans and Bandemer (*5*) separated egg white proteins from each other by paper electrophoresis and determined the amounts of each by extracting the blue color formed in the reaction of protein with bromophenol blue and by reading its absorbance at a wave length of 590 mμ in the Beckman Model B spectrophotometer. A similar method was used to fractionate and determine the amounts of the different proteins in unextracted egg yolks.

Results and Discussion

Typical paper electrophoresis curves obtained with unextracted egg yolk diluted with salt solution are presented in Figures 1, *A* and 2, *A*. Three distinct protein peaks are evident, and a fourth, corresponding to the position of ovalbumin in the egg white curve, was often present. Seven bands were present on the bromophenol blue-stained paper strips, but some were not far enough apart from each other to be picked up as separate peaks with the densitometer used, although the bands were visually distinctive. The protein fractions were designated A to G. Protein A band corresponded in position to egg white ovalbumin. Protein B band was not associated with lipide and corresponded in position with the principal peak from the livetin preparation (Figure 1, *C*). Protein C band corresponded in position to the ovomucoid plus ovoglobulin band of egg white. Protein C may also have been livetin, because Shepard and Hottle (*13*) obtained three principal livetin bands with conventional electrophoretic separation of livetin preparations. The lipovitellenin plus livetin solution had as its principal peak one corresponding in position to protein D (Figure 1, *C*). Protein E was a lipoprotein and was nonmobile under the conditions of electrophoresis used. Lipovitellin was nonmobile under these conditions (Figure 1, *B*). Protein F was a lipoprotein, but protein G was not. The lipovitellin preparation had a protein F band as well as the protein E band (Figure 1, *B*), and crude lipovitellin is probably made up of two components, one nonmobile and the other charged so that it migrated slightly towards the cathode. Mecham and Olcott (*11*) isolated phosvitin, a protein with high phosphorus content, from crude lipovitellin preparations. No attempts were made in the present study to isolate phosvitin from egg yolk for study nor to determine the phosphorus distribution in the proteins fractionated by paper electrophoresis, but lipoprotein F could possibly be phosvitin.

The distances that each protein migrated on the electrophoresis papers are given in Table I for a number of isolated egg yolk proteins and egg yolk preparations. Data are presented only for proteins A, B, D, and E, which have distinct peaks on the plotted curves. An average value for fresh egg white is given for comparative purposes. Egg white conalbumin migrated to approximately the same point that protein D of unextracted egg yolk did.

Egg yolk proteins soluble in distilled water after exhaustive ether extraction and dialysis had no protein E band, and the protein D band (if it can be so designated) migrated almost three times as fast as the protein D from fresh egg

Table I. Distance That Proteins Migrated under Conditions of Experiment

(Millimeters from origin to peak of curve)

Sample	Protein Fraction A	B	D	E
Egg white	−84.4	...	− 7.8	...
Fresh egg yolk[a]	−80.4	−44.1	− 7.5	0.6
Fresh yolk + ether	−76.5	−45.3	−11.4	0
1x ether-extracted yolk	−73.2	−41.1	−13.5	1.3
2x ether-extracted yolk	−79.5	−36.0	−12.0	3.0
3x ether-extracted yolk	−64.5	−42.0	−15.0	0
*n*x ether-extracted yolk	−81.6	−47.3	−12.0	0
Yolk protein-sol. glycine-NaCl soln.	−85.5	−45.0	−12.0	0
Yolk protein–sol. H_2O	−78.0	−45.0	−18.0	...
Old normal egg yolk	−88.9	−51.0	−19.5	0
Salmon-colored yolks	−76.5	...	−24.0	0
Butterscotch yolks	−78.0	−48.0	−27.0	0
Water-diluted yolk	...	−48.0	− 8.0	0
Lipovitellin	...	...	...	1.0
Ether-extracted lipovitellin	...	...	...	1.0
Lipovitellenin + livetin	...	−46.5	−13.5	−1.5
Ether-ext. lipovitellenin + livetin (ppt.)	...	−45.0	−24.0	0
Ether-ext. lipovitellenin + livetin (sup.)	...	−45.0	−21.0	0
Lipovitellenin	...	...	−30.0	0
Livetin	...	−47.5	−12.0	0

[a] Values are averages of 23 different electrophoresis runs from yolks of fresh eggs obtained from M.S.U. laying flock as required.

yolk. The protein that was insoluble in distilled water but soluble in 15% glycine and 10% sodium chloride solution had a main band that did not migrate.

Included in Table I are data for yolks of eggs—stored at 0° C. for over 6 months—from hens that had received crude cottonseed oil in their ration. These are the yolks designated as salmon-colored and butterscotch. The lipoprotein band, D, moved much faster towards the anode in old eggs than it did in fresh eggs. Band D from "cottonseed" eggs migrated over three times as fast as band D from fresh eggs.

Band D of lipovitellenin increased in speed of movement as the lipovitellenin was freed of impurities. Removal of lipovitellin increased distance migrated from −8.0 to −13.5 cm., and removal of other soluble materials further increased it to −30.0 cm.

The averages of eight determinations of protein distribution on a sample of fresh egg yolk are presented in Table II. The lipoproteins (D, E, and F) made up 88.1% of the total yolk proteins in the sample of egg yolk studied.

Effect of Ether on Egg Yolk Proteins

Young and Phinney (*20*) used moving - boundary electrophoresis techniques for the separation of the proteins in ether-extracted egg yolks, and obtained three peaks using a pH 8.5 barbiturate buffer of 0.32 ionic strength. The values given in Table II for lipoproteins add up to 88.1% compared to the 85.1% lipovitellin found by the same workers. Proteins B and C accounted for 8.6% of the yolk protein (Table II) compared to the 10.2% livetin also obtained by them.

The paper electrophoretic curves, shown in Figures 1, *A* and 2, *A*, differ in one important respect from the curves of Young and Phinney and that is in the presence of two major lipoprotein peaks rather than one. However, the paper electrophoresis curve obtained with ether-extracted yolk (Figure 2, *D*) is very similar to their curves.

During attempts to isolate and study the individual yolk proteins, each step was followed by electrophoretic protein separation on paper. Extraction of the yolk with ether did something besides extracting the noncombined lipides, as is shown by a comparison of Figure 2, *A* (nonextracted yolk) and Figure 2, *D* (ether-extracted yolk). Along with the loss of lipide, there was a change in protein D, so that instead of migrating in a negative direction, most of it, along with the previously nonmobile protein E, migrated about 3 mm. in a positive direction. The remaining lipoprotein D (weak band) migrated twice as far toward the anode as did lipoprotein D in untreated egg yolk (Table I). Thus, ether extraction not only removed part of the lipide associated with the lipoproteins but also changed the electrical behavior of the lipoproteins.

Previous methods for the study of egg yolk proteins have consisted essentially of dilution of the yolk with saline solution, extraction of the "free" lipides with ether, and making further studies on the ether-extracted material. According to Warner (*17*), ether solubility does not make a very clear distinction between free and bound lipide and methods for fractionating lipoproteins in general are poorly developed. Results of the present investigation support Warner's viewpoint.

Extraction in the cold with U.S.P. diethyl ether was used in the first studies. After the second or third ether extraction, part of the protein precipitated.

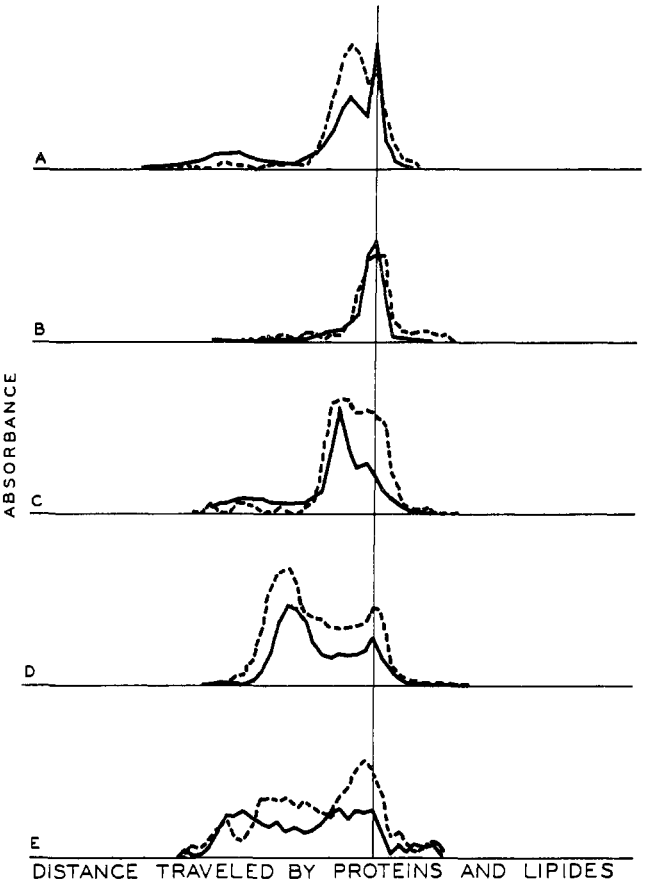

Figure 1. Paper electrophoretic patterns of egg yolk and isolated egg yolk proteins

A. Egg yolk
B. Lipovitellin
C. Lipovitellenin + livetin
D. Lipovitellenin
E. Livetin
——Protein stained with bromophenol blue
– – –Lipide stained with Oil Red O

Table II. Protein Composition of Egg Yolk Determined by Paper Electrophoresis

Protein Fraction		*Total Protein, %*
A	Ovalbumin	1.7
B	Livetin	5.5
C	Livetin	3.1
D	Lipovitellenin	41.7
E (Nonmobile)	Lipovitellin	34.5
F	Lipoprotein	11.9
G		1.6

The precipitated protein was at least partially lipoprotein, although the lipide content was relatively low (Figure 2, *E*). Two lipoprotein bands were obtained. One remained at the origin, but the other migrated 6.0 mm. towards the cathode. No protein precipitated when ether extraction was carried out at room temperature, but the electrophoretic curve was little different from that of the soluble portion of the yolk proteins extracted with ether in the cold.

Egg yolks mixed with salt solution were also extracted in the cold with peroxide-free, reagent grade, absolute diethyl ether. Part of the protein precipitated and a similar paper electrophoresis curve was obtained with the soluble protein as with the soluble protein from the egg yolks extracted with cold U.S.P. ether—showing that the change in lipovitellenin when extracted with ether was not caused by the presence of peroxide or alcohol in the U.S.P. ether.

The next problem was to determine if the presence of free lipide caused the lipoproteins to migrate differently than they did in its absence or whether there was actually some lipide-protein bond broken by the ether. Cold, peroxide-free ether was mixed with cold egg yolk–saline solution. The electrophoretic peak for lipoprotein D rapidly decreased in size during contact of the yolk with ether, and one large nonmobile peak replaced peaks *D* and *E*, indicating that some type of linkage of lipide to protein was broken by contact with ether (Figures 2, *B*, and 2, *C*). The remaining band D migrated further toward the anode as time of contact of yolk with ether increased.

Egg yolk was mixed with an equal weight of water and the mixture extracted with cold peroxide-free ether, as used by Lea and Hawke (*9*) for the preparation of lipovitellin, to see if the salt present caused the rupture of the protein-lipide linkage (*10*). Undiluted egg yolk was also extracted with ether. In both cases, ether extraction resulted in changes in the electrophoretic curves similar to those that occurred when the egg yolk was diluted with sodium chloride solution and extracted with ether.

Ether extraction of isolated lipovitellin did not change the mobility of the protein peak but it did remove much of the lipide associated with the protein (Figure 3, *A* and *B*). Lipovitellin was nonmobile under the conditions of the experiment. Because paper electrophoresis separations were not made at other pH values or with other buffers, it is not known whether lipovitellin is nonmobile because it is bound to the filter paper, or denatured, or because pH 8.6 is very near its isoelectric point. It is also impossible to know whether or not ether extraction would change the electrophoretic behavior under other conditions.

The solution remaining after the precipitation of lipovitellin contained both lipovitellenin and livetin (Figure 3, *C*). This solution was extracted in the cold with ether according to the procedure of Fevold and Lausten (*6*) and three layers (lipide in ether, precipitate, and water-soluble material) were obtained. The ether layer was discarded. The precipitate and water-soluble materials were separated in a separatory funnel, and the precipitate was dissolved in 5%

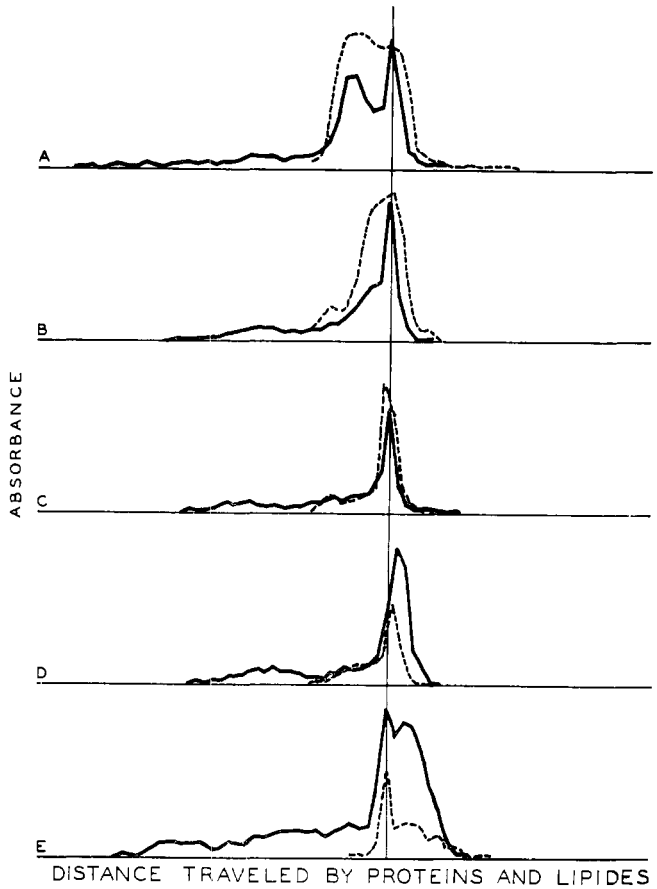

Figure 2. Paper electrophoretic patterns of egg yolk and egg yolk preparations

A. Egg yolk
B. Egg yolk + ether
C. Egg yolk + ether after standing 3 days
D. Ether-extracted egg yolk
E. Precipitated protein from three times ether-extracted yolk
——Protein stained with bromophenol blue
– – –Lipide stained with Oil Red O

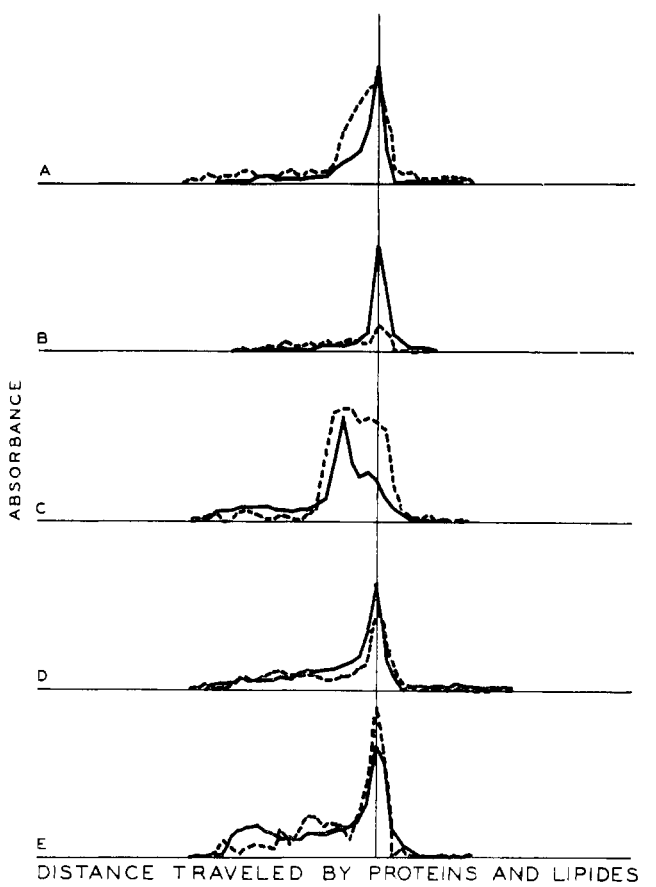

Figure 3. Paper electrophoretic patterns of isolated egg yolk proteins and ether-extracted proteins

A. Lipovitellin
B. Ether-extracted lipovitellin
C. Lipovitellenin + livetin
D. Precipitate from ether-extracted lipovitellenin + livetin
E. Supernatant from ether-extracted lipovitellenin + livetin
——Protein stained with bromophenol blue
– – –Lipide stained with Oil Red O

sodium chloride solution. Most of the livetin remained in solution and so did some of the lipovitellenin (Figure 3, *D* and *E*). Ether extraction changed lipovitellenin from a protein that migrated toward the anode to a nonmobile one (Figure 3, *C*, *D*, and *E*). Vandegaer, Reichmann, and Cook (*16*) observed that the solubility and stability of both lipovitellin and lipovitellenin are impaired by ether extraction.

Prior extraction of egg yolk with ether makes impossible the separation of lipovitellin and lipovitellenin. Only where separations have been made prior to ether extraction have the two been separated. The use of ether by Young and Phinney (*20*) in the preparation of their yolk sample for electrophoresis accounts for their inability to confirm the presence of lipovitellenin in egg yolk.

The data presented indicate that perhaps most of the egg yolk lipides are combined with the proteins and that at least two types of combination exist. The first is a relatively stable combination which is not broken by ether. Lipovitellin and lipovitellenin as commonly obtained are compounds of protein and lipide combined in this way. A weaker lipoprotein-lipide complex also appears to occur, but this complex is broken by contact with ether even in the cold. Recently, Weinman (*18*) as the result of ultracentrifugation studies with unfractionated egg yolk concluded that nearly all egg yolk lipides are bound to protein.

Hawke and Lea (*7*) observed that triglycerides and cholesterol were more readily removed from their lipovitellin preparation by different solvents than were the phospholipides. It has been postulated (*15*) that in lipoproteins the neutral lipide is mainly bound to the phospholipides, and that phospholipides form the link between neutral lipide and protein.

Conclusions

Extraction of egg yolk with ether, either in the cold or at room temperature, changed the electrophoretic behavior of the yolk proteins on paper. Two lipoprotein peaks, lipovitellenin and lipovitellin, were obtained with unextracted yolks, but only one was obtained with ether-extracted yolks. The difference in electrophoretic behavior of ether-extracted yolk proteins and nonextracted proteins was apparently caused by the breaking of an actual lipide-protein bond by the ether. Extraction of lipovitellenin with ether changed its properties so that it gave a peak at the same point as lipovitellin.

Paper electrophoresis methods offer a new procedure for the study of egg yolk proteins. The results of experiments reported herein emphasize the necessity for a new look at the proteins of egg yolk and for the development of isolation techniques that do not involve treatment with ether.

Acknowledgment

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CORRESPONDENCE

Silicon Determination in Ashed Plant Material

SIR: In the application of the silicon method of Straub and Grabowski [*Ind. Eng. Chem., Anal. Ed.* **16,** 574–5 (1944)] to the analysis of plant material, many ashed samples were found to contain significant amounts of ferrous iron. The presence of this ferrous iron, in conjunction with the phosphorus normally contained in plants, caused falsely high silicon readings. Such results have been reported by Bunting [*Ind. Eng. Chem., Anal. Ed.* **16,** 612–15 (1944)] for samples containing both phosphate and ferrous iron. This difficulty was overcome by the addition of hydrogen peroxide to ensure the oxidation of the iron.

In practice, 50 to 100 mg. of dried and ground tomato plant material were placed in a platinum crucible in a cool furnace and were heated until the temperature just reached 650° C. This heating took about 1 hour. The crucible was immediately removed from the furnace and allowed to cool, after which the ash was washed from the crucible into a polyethylene container. The crucible was then washed with 1 ml. of 2.4*M* hydrochloric acid, and this acid, together with subsequent distilled water rinsings, was added to the ash suspension. One milliliter of 0.15% hydrogen peroxide (30% hydrogen peroxide diluted 1 to 200) solution was added, and the volume was made to 25 ml. From this point, the analysis was carried out as outlined by Straub and Grabowski (starting with the addition of ammonium molybdate), except that half of the recommended volume of each reagent was used, to conform to the 25-ml. sample volume. The strength of the hydrogen peroxide solution was not critical—solutions of 0.03% and of 1% being satisfactory. If solutions much stronger than 1% were used, however, the final reduction of the silicomolybdate was prevented.

The amount of plant material that could be analyzed depended largely upon its phosphorus content, as more than about 50 micromoles of phosphate drastically reduced the final silicon reading. This factor limited the size of sample to 100 mg. in most cases. The lowest reliably detectable amount of silicon by this method was about 5×10^{-9} gram atom in 100 mg. of dried plant material, or about 1.5 p.p.m.

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