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Fractionation of the egg white proteins in media of low dielectric constant and low ionic strength

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FRACTIONATION OF THE EGG WHITE PROTEINS IN MEDIA OF LOW DIELECTRIC CONSTANT AND LOW IONIC STRENGTH

by

Richard Hamilton Forsythe

A Dissertation Submitted to the
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INTRODUCTION

The importance of egg white as a biological system is well recognized. With the possible exception of the plasma proteins, egg white has been studied more than any other natural system. Much of the interest in egg white has stemmed from the curiosity of physiologists intent on discovering the mode of formation of egg white and its possible significance in embryonic development.

That eggs and egg products are also items of commercial importance is demonstrated by the fact that in 1948 89,564,000 lbs. of frozen and 2,678,000 lbs. of dried egg white were produced in this country (133). The current value of these products probably exceeds $35,000,000. In 1948, more than fifty-five billion eggs were produced which contributed $2,154,911,000 to the gross income of the nation (134).

Many investigations have been completed in attempts to improve the technology of eggs and egg products. The deterioration of the thick white gel in shell eggs has been studied and some procedures developed which can minimize this loss. Frozen egg white is in general satisfactory for the preparation of most food products in which it is an ingredient. Dried egg white, on the other hand, loses some

of its functional properties, such as ability to produce stable foams or satisfactory angel cakes, during the dehydration process. Off odors and discoloration are often found in stored powders. These defects have seriously limited the use of dried egg white in such products as prepared cake mixes.

It has been pointed out by many interested in this field that a study of the individual protein components of egg white might throw additional light on some of the technological problems involved. The egg white system is so complex, and some of the factors involved so subtle, that an alteration in one constituent may be masked by the large quantities of "impurities". The role of the proteins of egg white in embryonic development has remained unsolved because of the difficulty in studying individual proteins. If a satisfactory procedure were developed for the preparation of each protein in its native form, such problems might be reinvestigated with more promise of satisfactory results. The physical chemistry of the proteins of egg white, other than ovalbumin, has not been studied extensively because of the difficulty of preparing them in pure natural form. The availability of such protein preparations would certainly give impetus to such investigations.

This study was designed in an attempt to develop better methods of separation and purification of the egg white proteins so that problems such as those mentioned above might be investigated. The present procedures for fractionation of egg white have many disadvantages. In most cases, the insolubility of the proteins in high concentrations of ammonium sulfate has been used to effect separations. The effect of high concentrations of salts on protein denaturation has not been definitely established, but it seems quite reasonable that under such conditions proteins might be unstable. Preparation of salt free proteins, when ammonium sulfate procedures are used, is a tedious and time-consuming process.

The insolubility of the salts has often prevented operating at lower temperatures where proteins are more stable. The lack of specificity in the "salting-out" techniques often necessitates long purification procedures. "Salting-out" procedures do not lend themselves to commercial practice both because of the difficulties already mentioned and the high costs involved.

The successes of Cohn and co-workers at the Harvard Plasma Fractionation Laboratories prompted an investigation into the possibility of applying their aqueous ethanol procedures to the egg white system. Their schemes offer many advantages over the classic "salting-out" techniques.

The five variable system employed in the plasma studies presents an extremely wide choice of conditions for removal of each fraction. A high degree of specificity can be obtained by proper control of the variables. Low temperatures are employed to improve protein stability. Since low salt concentrations are employed, prolonged dialyses are avoided. Ethanol can be removed easily and rapidly by lyophilization. The ethanol fractionation procedures are adaptable to commercial operations so that valuable by-product production is a possibility.

This study may be divided into two parts: Part I, a study of the protein composition of egg white and Part II, the fractionation of the proteins of egg white. The protein composition of egg white had previously been investigated electrophoretically, but it was considered necessary to study several factors which might affect the electrophoretic composition of egg white. The relative concentrations of the protein constituents of egg white were determined in a large number of experiments so that the magnitudes of the errors involved in these analyses could be measured. Accurate data were needed for the mobilities of the individual proteins so that fractions could be properly identified. Experiments were carried out to determine the influence of ovomucin removal, pH and ionic strength, genetic characteristics, and the age of the eggs on the normal composition of egg white.

In fractionation studies there seem to be at least two approaches. The first, and what appears to be the more logical, is to assemble all the solubility data available for the components of the system, and from these data design a fractionation scheme. This might conceivably be possible in the case of egg white by preparing the fractions by ammonium sulfate procedures and determining their solubility characteristics in media of low dielectric constant and low ionic strength. This procedure would involve a tremendous amount of time and the data might be quite useless when applied to whole egg white. The second approach to a fractionation is much less systematic than that just described. This procedure involves setting up arbitrary conditions for the separation of the proteins, measuring the degree of separation obtained, and then improving the procedure by repeated trial and error. The second plan has been followed in developing this new fractionation procedure for the egg white proteins.

NATURE AND COMPOSITION OF EGG WHITE

During the development of a fractionation scheme it is essential that all existing information regarding methods of preparation, chemical composition, and physical properties be readily available. Available solubility data are of utmost importance in planning separations.

Grossfeld (50), in 1938, completed a very extensive summary of the literature of the avian egg in his book "Handbuch der Eierkunde". A second comprehensive review of the egg white literature became available early in 1949 when "The Avian Egg" appeared (108).

No attempt has been made in this review to discuss all of the literature of egg white and its constituents, but only those investigations which seemed pertinent to this problem. A considerably larger number of references have been consulted than have been listed.

Structure of Egg White

During the development of the egg of the domestic fowl, Gallus domesticus, several processes take place which result in a highly differentiated structure in the egg white. As the yolk passes through the magnum, or "albumen secreting portion" of the oviduct, an extremely viscous mass of

protein material is deposited around the yolk (113). Two types of cells are believed to be responsible for the secretion of this dense white, one secreting ovomucin and the other a less viscous protein solution. In the extreme anterior portion of the magnum a dense sheet of mucin fibers, the chalaziferous layer, envelops the yolk. As the yolk passes to the posterior portion of the magnum, a mixture of ovomucin and liquid egg white is laid down in a more or less concentric manner (12, 30). The chalaziferous layer continues to increase in thickness as the twisting motion of the yolk pulls the fibrous material from the dense protein solution (8, 32). In this manner, two compact ropy bundles of ovomucin fibers, the chalazae, are formed at either end of the yolk (8, 33). The formation of the chalazae from the dense white results in a thin layer essentially free of any apparent structure. As the egg leaves the posterior portion of the magnum, all of the protein has been secreted while the volume is only about 50% of that of the final egg white (23).

In the isthmus are secreted shell membranes, composed of keratin or mucin like protein, which enclose the thick white (94, 105). In the uterus, a fluid containing potassium bicarbonate, potassium chloride, sodium bicarbonate, and sodium chloride is secreted. This solution passes through

the membranes and extracts some of the soluble proteins from the dense protein solution, forming an outer layer of thin white (23, 24). This outer layer has practically no mucin fibers and is typical of a concentrated protein solution.

There are many reports in the literature concerning the proportion, composition, and changes in the four layers of egg white. Much of this work has stemmed from the interest of physiologists in the manner of formation and the purposes of these layers in the egg during incubation. Perhaps an even larger amount of work has resulted from the observed changes in the structure of egg white during commercial handling and storage of shell eggs.

Romanoff and Sullivan (109) reported the relative proportion of these layers on a volume basis in the fresh egg as follows:

chalaziferous	2.7%
inner thin	16.8
thick	57.3
outer thin	23.2.

These are to be taken only as average values as Almquist and Lorenz (9) report variations of from 30-80% for the thick white, from 10-60% for the outer thin layer and from 1-40% for the inner thin. These differences result from individual hen variation and can be controlled somewhat by breeding. Certain environmental conditions are also

believed to effect the relative proportion of parts (58, 62, 78, 115).

Romanoff (107) reported that the solids content of the four layers increased from the outer thin to the chalaziferous layer. The percentage of minerals increases in the same manner. Frampton and Romanoff (47) concluded, on the basis of electrophoretic evidence, that no major protein component in any one layer could be identified in any of the others, but Forsythe and Foster (45) found no justification for this conclusion.

The portion of the egg white which is normally called the thick white is believed to consist primarily of an ovomucin gel in which protein solution is occluded. The thin portions are composed of all of the component proteins but contain considerably less mucin than the thick. McNally (82, 83) found that the thick white of a fresh egg contained 94.5 mg. of mucin nitrogen as compared with 10.4 mg. in the thin.

The breakdown of the mucin gel results in an egg white which is very fluid and exhibits no tendency to stand up when broken out on a flat plate. Much work has been done in an attempt to determine the mechanism and means of prevention of this breakdown. As was described in the section on egg formation, the gel is partially broken down

by the formation of the chalaziferous layer and the chalazae, as well as by the extraction of the soluble proteins by the uterine fluids.

One school of thought with regard to the probable mechanism of the dispersion of the ovomucin gel, and the one which appears more nearly correct in the light of present day evidence, attributes the change to the effect of pH on the properties of ovomucin. So much information (9, 10, 17, 51, 114, 137) has been accumulated as to the effect of CO_2 and high temperature on the thick white of the egg that there can now be little doubt that the dispersion of the mucin gel results from the loss of carbon dioxide, and subsequent rise in pH. Almquist and Lorenz (9) believed that the liquefaction might be due to two processes that take place under different conditions. In the presence of excess carbon dioxide they believed the fibers became shorter and squeezed from their gel the solution of the other proteins just as had taken place in the development of the egg; at lower concentrations of carbon dioxide, the liquefaction might be caused by breaking up of long fibers into short fibers. They proposed that the difference between firm and fluid white was only in the physical form, not in chemical make-up.

Balls and Hoover (15) demonstrated that while the

total mucin in the egg white did not change during the breakdown of the gel, the relative amounts in the thick and thin did change. They attributed the breakdown to the dispersion of the mucin fibers and their distribution into the various layers.

Hoover (55) studied the physical chemical properties of mucin with particular emphasis on its unique gel structure. He found mucin could be broken down by homogenization and certain reducing agents, such as hydrogen sulfide, cysteine and neutralized thioglycollic acid; the breakdown product closely resembled that of naturally thinned whites. Although not in the gel form, mucin was present in this reduced white and could still be precipitated by five fold dilution with water, but in case of either natural or artificial thinning, acetic acid had to be added to effect precipitation. He studied the efficiency of various dispersing agents on the mucin gel and found that urea completely dispersed the water precipitated mucin.

The author feels that many of the discrepancies noted in these researches may be due to differences in the treatment of the thick white prior to the actual experiments. In the experimental portion it will be shown that treatments which might be assumed to cut the fibers into smaller fragments have a marked influence on the behavior of the ovomucin.

Another school of thought postulates a proteolytic breakdown of the mucin of the thick white by a tryptic type enzyme occurring naturally in egg white. Balls and Swenson (16) suggested that an anti-tryptic factor, known to be present in egg white, normally inhibited this reaction. Hughes, Scott and Antelyes (57) concluded the reported anti-tryptic factor to be concentrated in the inner layer of thin white. Van Manen and Rimington (135) disagreed with these conclusions on the grounds that the methods employed to determine if proteolysis had taken place were not sensitive enough. By using more refined techniques they were unable to demonstrate any such breakdown in the thick white. Balls and Hoover (15) later confirmed the work of Van Manen and Rimington and concluded that proteolytic activity was not responsible for the breakdown of the mucin gel.

Recent studies of Lineweaver, et. al. (71) demonstrated that the usual agents added to egg white to prevent microbial growth were not effective. They pointed out the possibility of bacterial growth in the studies of the above workers and furnished additional evidence that the primary cause of the liquefaction of egg white is not due to enzymes occurring naturally within the egg.

Non-protein Composition of Egg White

Inorganic constituents.

The inorganic composition of egg white has been reported by several investigators. Their findings are summarized in the following table:

TABLE I.

Inorganic composition of egg white.

Substance	Percent	Reference
Calcium	0.01	Almquist & Lorenz (9)
Chlorine	0.15	Rose and Vahlteich (110)
Copper	0.85 ppm	"
Iodine	Trace	"
Iron	0.0001	"
Magnesium	0.01	"
Phosphorus	0.10	"
Potassium	0.16	"
Sodium	0.16	"
Sulfur	0.19	"
Total Ash	0.80	"
Water	87.77	"

A list of the trace minerals found in the chicken egg has been compiled by Romanoff and Romanoff (108, p. 358). Calculated to a molar basis, the figures for potassium, sodium and chlorine become:

K = 0.041
Na = 0.069
Cl = 0.042

If it is assumed that these elements are all present in the ionic form, and that electrical neutrality is maintained by the presence of bicarbonate and protein anions, the ionic strength of the fresh egg white may be approximated. This calculation yields the figure 0.11-0.13 for the ionic strength of fresh egg white.

Organic constituents.

Lipids. Egg white contains a very small amount of lipid material. Grossfeld (50) reported that egg white contained 0.0013-0.0019% cholesterol. Cruickshank (35) reported 0.05% fat.

Carbohydrate. Carbohydrate is found in the egg white both as free sugar (0.45%) believed to be glucose, and as protein-bound carbohydrate (119). Sørensen (119) reported the carbohydrate (as mannose or galactose) content of the egg white proteins to be as follows:

Globulin	4.0%
Mucin	14.9
Albumin	1.7
Conalbumin	2.8
Mucoid	9.2

The carbohydrate moieties of the several proteins will be discussed in more detail below.

Vitamins. The only vitamin which has been found in any appreciable amount in the egg white is riboflavin. The amount of this vitamin present is dependent on the diet of

the hen, but the average figure of 6 micrograms per gram of egg white has been reported by Bird (19).

Protein Composition of Egg White

Number of egg white proteins.

Bechamp (18), in 1873, was among the first to observe that egg white contains more than a single protein. He separated egg white into three fractions, one of which was soluble in alcohol. A year later, Gautier (48) reported that the egg white system consisted of two heat coagulable and two non-coagulable proteins. By fractional heat coagulation Corin and Berard (34) found that egg white could be separated into five components, two albumins and three globulins.

In 1898, Eichholz (39) observed that egg white could be separated into three constituents, each of which contained a substance yielding an osazone similar to phenylglucosazone. He obtained a fraction which he called ovomucin by diluting egg white with four volumes of water. This fraction had many of the characteristics of other natural mucins.

Osborne and Campbell (99) investigated the proteins of egg white in 1900. After a series of fractionations based on ammonium sulfate precipitation, dilution with water,

and a heat fractionation scheme, they concluded that egg white consisted of four fractions which they described as ovomucin, ovalbumin, conalbumin, and ovomucoid. They proposed that the glycoprotein, ovomucin, found and named by Eichholz was identical with the globulin of earlier workers.

Obermayer and Pick (97) found four proteins in egg white; globulin, ovalbumin, conalbumin, and ovomucoid. The globulin fraction was further separated into four constituents; ovomucin and euglobulin which were insoluble in water; dysglobulin and pseudoglobulin which were soluble in water.

Piettre (104) described a method for the separation of the proteins of egg white employing acetone as precipitant. Four fractions were obtained: ovalbumin, two globulins and a glycoprotein. He claimed this technique resulted in a sharper separation of albumin and globulin, with the inclusion of fewer impurities. Denatured proteins probably were obtained and from the results given it is difficult to determine the purity of the fraction.

Hektoen and Cole (52) reviewed the literature in 1928 and concluded that with the information available at that time there were two albumins in egg white, one of which was readily obtained in crystalline form. Extensive ammonium sulfate fractionations were carried out. Precipitin reactions

were used to check the purity of their fractions and to determine the immunological similarity of various proteins. They concluded that there were five distinct antigens in the system which they identified as ovoglobulin, ovomucin, crystalline ovalbumin, non-crystallizable conalbumin, and ovomucoid.

Needham (95, p. 1301-1380) still believed in 1931 that there were but four proteins in the egg white system. He reported two albumins, in agreement with the early investigators, and two glycoproteins, ovomucin and ovomucoid. He obtained no globulin and agreed with Osborne and Campbell that this protein was probably a mixture of denatured proteins. This was in disagreement with Hektoen and Cole who had reported the presence of both a globulin and ovomucin fraction.

M. Sørensen (119) prepared the various fractions of egg white by fractional precipitation with ammonium sulfate. She obtained five fractions: ovalbumin, conalbumin, globulin, ovomucin and ovomucoid but pointed out that the hypothesis of "reversibly dissociable protein systems", first proposed by S. P. L. Sørensen (121) might allow other fractions by modifications in the fractionation procedures. S. P. L. Sørensen regarded the natural proteins in biological fluids as systems held together by secondary valencies which could be broken by the treatment given to the system.

He concluded this dissociation to be reversible in most instances.

Young (140) investigated the egg white proteins in a further attempt to separate the different fractions. He found no characteristic globulin but obtained an ovomucin fraction. He admitted the possibility that the ovomucin might in reality be denatured globulin with unusual nitrogen and carbohydrate content.

Young stated, "The natural protein of egg white is suggested to be a mucoprotein-albumin complex separated into fragments dependent on the reagent employed and having no separate identity in egg white" (140, p. 9). Such a system would be very similar to that in the cereal proteins as proposed by McCalla and Rose (81) and would also be in agreement with the previously mentioned hypothesis of S. P. L. Sørensen. It is possible that some natural protein systems do contain no separate isolable molecular species. In such a case, it is clear that the number and characteristics of the fractions might be dependent on the procedures employed.

In a later paper, Young (141) studied egg white electrophoretically, observed five or six boundaries and stated that possibly his earlier postulate of a natural complex might be erroneous. He was unable to prepare an electrophoretically homogenous fraction with his ammonium sulfate

procedures. This inability to prepare homogeneous fractions forced him to the conclusion that at that time chemical methods were insufficient to separate the egg white proteins into distinct fractions without modification of the natural state.

In 1940, Longsworth, Cannan and MacInnes (77) undertook a more extensive and critical electrophoretic investigation of the egg white proteins. They studied egg white diluted eight times at several pH's (3.92, 4.45, 6.12, 8.0) and at ionic strength of 0.1. The following components were found: ovomucoid (which did not appear homogeneous), two albumins, conalbumin and three globulins (G_1, G_2, G_3). The presence of ovomucin was acknowledged, but due to its insolubility, was not included in their analyses. They observed that pH had a marked effect on the number of peaks in the electrophoretic patterns. All of the above components were observed at pH 4.45, but not at higher pH's. On the basis of limited fractionation studies, it was concluded that the value of the electrophoretic techniques was analytical rather than preparatory in the case of these proteins.

Conant (31) applied various modifications of the ammonium sulfate procedures to egg white. On the basis of cystine and methionine content he concluded there were five distinct fractions in egg white: Globulin, ovomucin,

ovomucoid, conalbumin, and ovalbumin.

Bain and Deutsch (13) completed an electrophoretic study of the egg white proteins of various birds. Electrophoretic analyses of chicken egg white at pH 8.6, ionic strength 0.1 gave patterns indicating the presence of at least six proteins. Two albumins, globulin, two conalbumins, and a small component with a mobility higher than ovalbumin were observed. In addition to these listed, ovomucoid was believed to be present in the globulin gradient.

In addition to what might be designated the major protein components, several other proteins possessing unique biological activity have been isolated from egg white. In 1922, Fleming and Allison (43, 44) observed that egg white contained a powerful bacteriolytic agent. The term lysozyme was applied to this and similar substances found in tissues of a number of animals. Lysozyme has been isolated from egg white by Roberts (106), Meyer, et. al. (93), and Alderton, et. al. (6). The latter group suggested that lysozyme is probably identical with Longsworth's G_1 globulin.

Eakin, Snell and Williams (36) isolated highly active concentrates from egg white which combined with biotin, making it unavailable for various organisms. They called this substance avidin because of its high affinity for biotin. Avidin has been prepared in crystalline form by

Pennington, Snell and Eakin (103).

Several other enzymes have been found in egg white in low concentrations. Lineweaver, Morris, Kline and Bean (71) reinvestigated earlier reports and found the following enzymes to be present in small amounts in fresh eggs: tributryinase, peptidase, and catalase. Phosphatase, oxidase, cytochrome oxidase, and peroxidase were absent within the limits of experimental error.

The following compilation is given to summarize the best existing information as to the number and quantities of the proteins in egg white:

TABLE II.

The proteins of egg white.

Constituent	Amount	Method	Investigation
Ovalbumin (A_1 & A_2)	60.5%	E	Bain and Deutsch (13)
	60.0	E	Longsworth, et. al. (77)
	70	I	Sørensen (119)
Ovomucoid	14.0	E	Longsworth, et.al. (77)
	11.7	I	Sørensen (119)
	11.4	I,A	Lineweaver and Murray (72)
Globulin (G_2 & G_3)	8.9	E	Longsworth, et. al. (77)
	6.7	I	Sørensen (119)
	15.0	E	Bain and Deutsch (13)
Conalbumin (C_1 & C_2)	13.8	E	Longsworth, et. al. (77)
	9.0	I	Sørensen (119)
	22.5	E	Bain and Deutsch (13)
Ovomucin	1.9	I	Sørensen (119)
Lysozyme (G_1)	2.8	E	Longsworth, et. al. (77)
	2.5	I,A	Alderton,Ward and Fevold (6)
Avidin	0.06	A	Alderton,Lewis and Fevold (5)

E = Electrophoretic, I = Isolation, A = Assay.

Table II (Continued)

Constituent	Amount	Method	Investigation
Tributyrinase	Present	A	Lineweaver, Morris, Kline and Bean (71)
Peptidase	Present	A	Lineweaver, Morris, Kline and Bean (71)
Catalase	Present	A	Lineweaver, Morris, Kline and Bean (71)

Preparation and properties of ovalbumin.

The primary protein constituent of egg white, ovalbumin, has been available in crystalline form for over fifty years. Its physical and chemical properties have been investigated as much as those of any other single protein. Many of the physical methods now available for protein studies have evolved from investigations on crystalline egg albumin.

Preparation. The preparation of crystalline ovalbumin, first devised by Hofmeister (54), has undergone only slight modification since its inception. Hofmeister prepared ovalbumin by half saturating whipped egg white with ammonium sulfate and removing the precipitated globulins. The solution was then allowed to evaporate, increasing the ammonium sulfate concentration. After considerable evaporation, a second precipitate formed, which after repeated

treatment was obtained in crystalline form.

Hopkins and Pinkus (56) modified this procedure by the addition of acetic acid after the removal of the globulin precipitate. Crystal formation resulted in much less time than that required in the original procedure.

In 1917, S. P. L. Sørensen and co-workers (120, 121, 122, 123, 124, 125) described the first of their classic experiments on egg white. After removal of the globulins with half saturated ammonium sulfate, the pH of the solution was adjusted to 4.8 with dilute H_2SO_4. Upon further addition of ammonium sulfate, crystallization took place quite rapidly and after three crystallizations essentially pure ovalbumin was obtained.

LaRosa (67), and Kekwick and Cannan (61) described procedures which resulted in improved yields of the crystalline protein. Their procedures embodied the improvements of the earlier investigations. At the time the present study was begun, the ammonium sulfate procedure was the only one which had given crystalline ovalbumin.

Chemical composition. Because of the relative ease of preparation, the chemical composition of crystalline ovalbumin has been investigated extensively. The total nitrogen content has been determined by many investigators. Chibnall, Rees and Williams (27), in an extensive investigation of the Kjeldahl procedure for nitrogen, reported that

ovalbumin contained 15.76% nitrogen.

Tables of amino acid composition of ovalbumin have been compiled by Cohn (28), Block and Bolling (20), and Vickery (136). It is not desirable to discuss here the details of the many analytical studies that have been reported. No marked differences are observed when the amino acid composition of ovalbumin is compared with other water soluble proteins except for the slightly higher content of the sulfur containing acids, methionine and cystine. When compared to water insoluble proteins, e. g. zein, the greater number of free basic and acidic groups easily explains the difference in solubility.

On the basis of molecular weight and data from the above sources, the following estimate of the ionizable groups of ovalbumin has been made (26).

TABLE III.

Ionizable groups of ovalbumin.

Amino Acid	Ionizable Group	Equiv./mole
Arginine	Guanidine	14
Histidine	Imidazole	4
Lysine	Amino	15
Tyrosine	Phenolic hydroxyl	10
	Total basic	43
Glutamic Acid	Carboxyl	40
Aspartic Acid	Carboxyl	24
	Total carboxyl	64
Amide N		31
	Free Carboxyl	33

The phosphorus content of ovalbumin is reported to be roughly one mole of phosphoric acid per mole of ovalbumin. This phosphoric acid would contribute two equivalents of hydrogen ion per mole to the albumin, one titrating at pH 2, the other in the imidazole region, pH 7.

There has been some question as to whether ovalbumin contains any bound carbohydrate, or whether the carbohydrates found arise only from impurities. Osborne, Jones and Leavenworth (100) analyzed acid-hydrolyzed ovalbumin and reported 3-5% of carbohydrate (reported as glucose). They contended that this amount could not have arisen from impurities.

M. Sørensen (119) determined the carbohydrate of ovalbumin by use of the colorimetric orcinol method and found 1.7% carbohydrate as mannose. Neuberger (96) reinvestigated Srensen's analyses and reported that seven times recrystallized ovalbumin contained 1.8% of a hexose. He found that the carbohydrate remained attached to the protein even after denaturation, and suggested that this carbohydrate might be present as a polymer consisting of two moles of hexosamine, four moles of mannose, and another unidentified constituent. He proposed that the molecular weight was about 1200, one unit of the polysaccharide being bound to one molecule of ovalbumin. On the basis of existing

information, ovalbumin should be classed as glycoprotein, yielding on hydrolysis, about 1.7-1.8% of a reducing sugar.

Amphoteric properties. As was indicated above, the amphoteric properties of ovalbumin have received considerable attention. Acid-base titrations have been carried out by Cannan, Kibrick, and Palmer (26) on recrystallized ovalbumin. On the basis of their dissociation curves they concluded that egg albumin behaved as a polyvalent ampholyte containing 51 carboxyl, 5 imidazole (possibly including 1 phosphate), 23 amino, and 14 quanidine groups.

The discrepancy between the twenty-three amino groups reported from dissociation data and the fifteen from analytical figures may be partially explained by the presence of some α-amino groups and the possibility that some other diamino acid, as yet unreported, may be present. The difference between the thirty-three free carboxyl groups reported in the analytical and the fifty-one from the titration data may be due to an incomplete recovery of aspartic and glutamic acid, as well as to the presence of some unidentified dicarboxylic amino acid.

Sørensen, Linderstrøm-Lang, and Lund (127) prepared ovalbumin by dialysis of the recrystallized protein against dilute ammonia followed by quantitative neutralization of the ammonia with HCl. The pH of the resulting solutions

was 4.88 at 18° C. They identified this as the isoionic point and noted that it did not shift appreciably when the ammonium chloride content was changed. Cannan, Kibrick, and Palmer used 4.8 as the isoionic pH but demonstrated that it was not independent of the KCl concentration.

Smith (117, 118) reported that the apparent isoelectric point of ovalbumin is influenced in a definite manner by the nature and composition of the buffer ions. Protein concentration also influenced the isoelectric pH.

Longsworth (74) measured the electrophoretic mobility of 0.5% ovalbumin solutions at ionic strength 0.1. He arrived at the value of 4.58 for the average isoelectric pH under the conditions of the experiment. He observed "that substitution, at constant pH and ionic strength, of one monovalent buffer salt for another, or of the neutral salt, sodium chloride, does not result in appreciable changes of mobility" (74, p. 273). He found, however, that when divalent buffer ions were employed instead of monovalent ions, the mobility was changed appreciably; at pH 6.8, ionic strength 0.1, the mobility of ovalbumin was increased 14.5% by the substitution of phosphate for monovalent buffer ions.

Electrophoretic behavior of crystalline ovalbumin. Longsworth (73) was the first to recognize that several times recrystallized ovalbumin contained two components,

A_1 and A_2, with different mobilities at most pH values. This phenomenon was independently observed by Tiselius and Eriksson-Quensel (132). In a further study Longsworth, et. al. (77) observed that at both high and low pH values only one component was observed. Mobilities in various buffers, at ionic strength of 0.1, were reported for these albumins. Their figures are summarized in Table IV.

TABLE IV.

Effect of pH on the electrophoretic mobility of ovalbumin.

Buffer	pH	Mobilities x 10^5	
		A_1	A_2
Acetate	3.93	+2.63	--
Acetate	4.64	-0.18	--
Acetate	5.33	-2.88	-2.59
Phosphate	6.80	-6.07	-5.57
Veronal-chloride	7.82	-5.99	-5.50
Phosphate chloride	6.71	-5.50	-5.04

Alberty, Anderson and Williams (3) observed three separate electrophoretic components in crystalline ovalbumin at pH 4.72, $\Gamma/2 = 0.1$. The amounts and mobilities of these components were determined: A = 75%, $\nu = -0.2$; B = 15%, $\nu = +0.5$; C = 10%, $\nu = +1.7$. This report was substantiated by Cann (25) in 1949, who reported that the

apparent number of components increased with decreasing ionic strength.

MacPherson, Moore, and Longsworth (84) studied the effect of age on the electrophoretic composition of ovalbumin. When salt free isoelectric solutions were stored, the amount of A_2 increased until after one year it was the only component present. This material was crystallizable and not insoluble at the isoelectric point. The same changes took place in stored powders, but at a much slower rate.

Molecular weight. The molecular weight of ovalbumin has been determined in several ways. Sørensen, et. al. (122), on the basis of osmotic pressure measurements, estimated a molecular weight of 34,000. By early ultra-centrifuge studies, Svedberg and Nichols (130) arrived at a similar value of 35,000. Later, Marrack and Hewitt (85) reported a value of 43,000 from their osmotic pressure studies. Sørensen's data were reconsidered by Adair (2) who arrived at a value of 43,000. Bull (22) reported that same value by osmotic pressure measurements in 1941. Re-examination of ovalbumin by later ultracentrifugal techniques has given the value of 44,000 from the sedimentation velocity and diffusion measurements, and 40,500 from sedimentation equilibrium. Edsall and Cohn (38) arrived at a molecular

weight of 43,000 ± 3000 after reviewing the various physical chemical studies.

Molecular shape. Oncley (98) discussed the measurements involved in describing the size and shape of protein molecules. For ovalbumin, a frictional ratio of 1.27 has been calculated from sedimentation and diffusion measurements. This frictional ratio (f/f_o) is a measure of the departure of the molecule in solution from a compact, spherical, unsolvated particle. The frictional ratio may be considered as consisting of two parts, one of which describes the amount of hydration, and the other the degree of asymmetry of the unsolvated molecule. Oncley calculated that the hydration of ovalbumin was about 0.2 grams of water per gram of protein from dielectric dispersion and crystal density data. The axial ratio of the ovalbumin molecule, i. e., the ratio of the major to minor axis of an ellipsoid of revolution, has been calculated to be 4.0 from the f/f_o given above.

By consideration of amino acid analyses, Lundgren (79) has tabulated some dimensional data for ovalbumin. If considered as one single chain, the length would need to be about 1400 Å. When calculated as four chains, as he suggests, this length would be 350 Å. The cross section of a single chain measured about 9.5 Å to give an axial ratio of 36. The wide discrepancies from this value and the one

previously mentioned may be considered as evidence for the natural coiling of the ovalbumin polypeptide chains into more symmetrical units.

Solubility. Of utmost importance in any fractionation plan is the availability of solubility data for each component of the mixture. The solubility of most proteins is so dependent on temperature, ionic strength, dielectric constant (as in the case of aqueous alcohol systems), pH, and other factors that it is extremely difficult to predict solubility characteristics from existing information. Sørensen, et. al. (126) observed that ovalbumin prepared by repeated crystallization conformed to the rigid requirements of the phase rule. They concluded that previous investigators had failed to observe agreement with the phase rule because they had not considered pH to be a component of the system.

Sørensen and co-workers' (120, 126) studies on ovalbumin included investigations of the solubility characteristics. The solubility of ovalbumin in water at pH 4.8 was reported to be 400 grams/liter. Extensive solubility studies were carried out in ammonium sulfate solutions because of the widespread use of this salt for "salting out" techniques. The following solubilities in 2.14 M ammonium sulfate were reported:

TABLE V.

Solubility of ovalbumin in 2.14 M ammonium sulfate.

T°C.	Ovalbumin, grams/liter
0	3.18
12	2.09
20	1.81
29	2.24

Ferry, Cohn and Newman (42) studied the solubility of crystalline ovalbumin in various sodium chloride-water-ethyl alcohol solutions. They reported that the solubility of ovalbumin in 25% ethanol at -5° in the absence of salt was 0.13 grams/liter. The addition of neutral salts greatly increased the solubility in this system.

The following table has been compiled from their data to show the magnitude of this effect:

TABLE VI.

Effect of NaCl on the solubility of ovalbumin in 25% ethanol.

Conc. NaCl Mole/liter	Solubility of ovalbumin grams/liter
0.0	0.13
0.02	0.26
0.05	0.52
0.10	1.2
0.20	4.1
0.49	41.4

Preparation and properties of ovomucoid.

Preparation. Ovomucoid has been prepared in a number of ways, most of which have involved preliminary separation of the other egg white proteins by "salting out", acetone and alcohol precipitations, or heat coagulation.

Hektoen and Cole (52) prepared ovomucoid by boiling diluted egg white which had been made acid to litmus. After concentration, the ovomucoid was precipitated by the addition of four volumes of ethyl alcohol. The preparation was purified by repeated treatment to give a product which was still soluble in water at its isoelectric point.

Sørensen (119) isolated ovomucoid from the filtrates after the other egg white proteins had been removed with varying amounts of ammonium sulfate. The ovomucoid was precipitated by three volumes of 96% alcohol; after washing with 96% alcohol the preparation was still soluble in water at all pH's studied.

Balls and Swenson (16) described a procedure for isolating a substance from egg white which acted as a trypsin inhibitor. They started with egg white which had been dried by repeated treatment with acetone and ether. The dry egg white was extracted with dilute ammonium hydroxide, the extracting liquid heated at 75-80° C. at pH 5, and the soluble solids precipitated with alcohol. As will be described below, this preparation had the

characteristics of ovomucoid isolated in other ways.

In Young's (140) fractionation of egg white, ovomucoid was obtained by ammonium sulfate saturation of the filtrate from which the other proteins had been removed by boiling at pH 5.

Lineweaver and Murray (72) described several modifications of a trichloroacetic acid-acetone procedure for the preparation of ovomucoid. Their method consisted of removing the other egg white proteins by adjustment of the pH to 3.5 with a trichloroacetic acid-acetone mixture. After the precipitate was removed acetone was added to remove the ovomucoid. The preparation was dried by washing with acetone and ether.

It should be noted that all of the methods described depend upon rather drastic procedures for removal of the other proteins. Yields in most cases are rather low because of the occlusion of ovomucoid upon the voluminous protein precipitates.

Chemical composition. Lineweaver and Murray (72) have summarized the existing information as to the chemical composition of ovomucoid. From their summary, the average nitrogen content can be calculated as 12.4%. The best existing information yields a value of 2.2-2.5% sulfur. Romanoff and Romanoff (108, p. 333) have also compiled data giving the partial amino acid composition.

A considerable amount of work on the carbohydrate radical of ovomucoid has been reported. The presence of mannose and glucosamine in ovomucoid has been demonstrated by Fraenkel and Jellinek (46) and later by Levene and Mori (69). The results of these investigations were in fairly close agreement, both indicating the presence of a polymeric structure containing one glocosamine to two mannose residues. Karlberg (60) reported that ovomucoid contained 13.5% glucosamine and 10.2% mannose. Masamune and Hoshino (86) found an equimolecular mixture of mannose and glucosamine. Meyer (90) reviewed the earlier work on the carbohydrate moiety, and on the basis of his own and other investigations, concluded that the ratio of one mannose to one glucosamine residue was probably correct.

Stacey and Woolley (128, 129) reported that the polysaccharide in ovomucoid consisted of glucosamine, mannose and galactose in the proportions of 7:4:1. Quantitatively, the methylation procedures they employed are less satisfactory than the admittedly poor colorimetric analyses used by previous investigators. It is doubtful if there is any justification for assuming other than a one to one ratio for mannose and glucosamine.

Little is known as to the molecular weight of the polysaccharide in ovomucoid because of the procedures used for isolation. From a consideration of other mucoids and

mucopolysaccharides, however, it may be quite high. The total carbohydrate content (as glucose) in ovomucoid has been reported as 20 to 25% (60, 72, 90).

Electrophoretic behavior. Hesselvik (53) showed, by electrophoresis experiments, that ovomucoid was isoelectric at pH 4.5. As his method for the preparation of the ovomucoid is not clearly stated, quantitative comparisons with more recent studies are difficult.

Longsworth, et. al. (77) studied the electrophoretic mobility of ovomucoid, in acetate and phosphate buffer at an ionic strength of 0.1. Under these conditions, they reported an isoelectric pH of 4.3. Ovomucoid mobilities in purified preparations were the same as in whole egg white. It was reported that this protein exhibited "reversible boundary spreading" in the electrophoresis experiments. This phenomenon has been described as due to the electrophoretic inhomogeniety of the preparation.

Molecular weight. Mazza (87) reported a molecular weight of 49,300 which is considerably higher than that measured by Lineweaver and Murray (72), who reported a molecular weight of 29,000 by osmotic pressure measurements. The latter workers observed no difference in the molecular weight of active and inactive preparations.

Biological activity. The fact that egg white has

anti-trypsin activity has been known for forty years. Balls and Swenson (16) isolated the active principle from egg white and observed that it had many of the characteristics of ovomucoid. Lineweaver and Murray (72) established the identity of this trypsin inhibitor and ovomucoid isolated by a variety of procedures. Repeated attempts to fractionate the active inhibitor from ovomucoid preparations were unsuccessful. They observed that less than one molecule of ovomucoid is required to effect 50% inhibition of one molecule of trypsin.

Ovomucoid may be denatured by heating in alkaline solution. It remains soluble at its isoelectric point when denatured, but the inhibitory activity is decreased, accompanied by an increase in detectable -S-S- groups. From these data it appears that many of the earlier methods for preparing ovomucoid resulted in a denatured product.

Preparation and properties of globulin and ovomucin.

The globulin fraction of egg white has been the subject of much controversy since this system was first investigated. This is due primarily to the fact that most workers have been unable to separate, satisfactorily, ovomucin from the globulins. Ovomucin and globulin are discussed together here, in spite of the fact that their properties are somewhat different, because of the many investigations in which

both were considered.

Preparation. Eichholz (39) prepared ovomucin by diluting egg white with four volumes of water. Osborne and Campbell (99) prepared, in the same manner, a glycoprotein which comprised 7% of the total protein in egg white. They found no characteristic globulin fraction. Obermayer and Pick (97) obtained a crude globulin fraction by dilution with water and half-saturation with ammonium sulfate. They further separated the fraction into a euglobulin, which was insoluble in water, but soluble in dilute sodium chloride solutions, a pseudoglobulin, which was soluble in water, ovomucin, which was insoluble in water and dilute salt, and a dysglobulin which was soluble in water but not further described.

Hektoen and Cole (52) removed a fraction from egg white by half-saturation with ammonium sulfate. The original precipitate was washed with water, leaving the ovomucin fraction. The water soluble portion of the original precipitate was again brought to half-saturation with ammonium sulfate and euglobulin obtained. This appears to be a rather unsatisfactory separation because of the probability of some water insoluble globulin remaining in the ovomucin precipitate. The presence of only one globulin was reported.

Sørensen (119) using essentially the same technique,

found about 2% of the total protein to be ovomucin, and about 7% globulin. No attempt was made to separate the globulin fraction further.

Kuchel and Bate-Smith (65) studied the globulins of egg white after first removing the ovomucin by dilution or filtration. Globulin was prepared from the "mucin-free" egg white by half-saturation with ammonium sulfate. Two globulins were obtained, a pseudoglobulin, soluble in water, and euglobulin insoluble in water but soluble in dilute sodium chloride. The yields obtained were probably low as their values of 0.2-0.4% of euglobulin and 0.15% pseudoglobulin are much lower than in other reports.

Young (140) prepared ovomucin by half-saturation with ammonium sulfate and by dilution of the egg white with water. In each case an ovomucin precipitate was obtained, but he concluded that no globulin was present in the egg white.

Longsworth, et. al. (77) precipitated ovomucin from egg white by dilution with an equal volume of 0.25% sodium chloride and acidification to pH 6.0. After ovomucin was removed, the globulins were precipitated with half-saturated ammonium sulfate. Dialysis to remove the ammonium sulfate reprecipitated the globulins which were then extracted with various buffers. Three globulins were obtained by this procedure which they called G_1, G_2, and G_3. They

observed all three of these components in electrophoretic patterns of "mucin-free" egg white. G_1 has since been identified with egg white lysozyme.

Hoover (55) claimed that ovomucin could be separated from the globulins by removing the fraction formed upon one-third saturation with ammonium sulfate at pH 7.9. The globulins were then precipitated by increasing the ammonium sulfate to 0.4 saturation. It is difficult, because of his analytical methods, to believe that a satisfactory separation was obtained.

Chemical composition. Any data regarding the chemical composition of the ovomucin and globulin fractions must be considered as doubtful until the purity of the preparation can be evaluated. Nitrogen determinations on ovomucin range from 12.5-14.5% (99, 140). Langstein (66) reported that the egg globulin fraction contained 15.1% nitrogen. Sørensen (119) observed that ovomucin contained 14.9% carbohydrate and globulin 4.0%. Young (140) found 11-12% hexosamine in ovomucin. Pseudoglobulin was reported to contain 2.3% hexose and euglobulin 3.7% by Kuchel and Bate-Smith (65). The only reported amino acid analysis for ovomucin is for cystine. Young (140) found 4.6% of this amino acid in his preparations.

Electrophoretic behavior. Little is known of the electrophoretic behavior of ovoglubulin, and ovomucin has

not been studied because of its insolubility under the conditions of previous electrophoresis experiments. Longsworth, et. al. (77) measured the electrophoretic mobilities over the pH range 3.9-8.0, ionic strength 0.1. From their data, the isoelectric point of G_2 was in the vicinity of pH 5.8-5.9, while G_3 had a somewhat lower range, 5.5-5.6. G_1 was not isoelectric below pH 3.0 in these experiments. Mobility measurements on the globulins were complicated by the poor degree of resolution from the impurities, and different mobilities were observed in the purified globulins and whole egg white. The isoelectric points of the globulins cannot be considered well established.

From the many observations that mucin can be precipitated from egg white by dilution and acidification to pH 6.0, it has been generally assumed that this protein is isoelectric in the range, pH 5.8-6.0. Kuchel and Bate-Smith (65) report that the insoluble portion of the thick white (ovomucin) is isoelectric at pH 2.5 but no details as to the method of measurement were given.

Data relating to the molecular size and shape of ovomucin and ovoglobulin have not been found in the literature.

Preparation and properties of conalbumin.

Preparation. Osborne and Campbell (99) reported the presence of an albumin which remained after the ovalbumin

had been crystallized from ammonium sulfate solutions. They called this protein conalbumin since, in spite of its apparent similarity to ovalbumin, crystallization attempts were unsuccessful.

Hektoen and Cole (52) prepared conalbumin from the ovalbumin mother liquors. A heat treatment, which resulted in a denatured preparation, was used to purify the conalbumin. Sørensen (119) also used the ovalbumin mother liquor for preparation of conalbumin. Conalbumin was prepared by adjusting the pH to about 4.5, and adding sodium sulfate to "salt-out" the protein. Complete removal was effected by heat coagulation. The preparation was dried by washing with alcohol and ether. It is doubtful if native conalbumin was obtained after such harsh treatment.

Longsworth, et. al. (77) removed the ovalbumin from mixtures of ovomucoid, conalbumin and ovalbumin by shaking. They stated that neither conalbumin nor ovomucoid were denatured by this treatment; the conalbumin was then removed by saturation with ammonium sulfate. They observed that when the ovalbumin fraction obtained with saturated ammonium sulfate was allowed to stand at a pH below 4, a precipitate formed. This was shown to have the same properties as the conalbumin prepared in the "shaking" procedure.

Alderton, Ward, and Fevold (7) precipitated a fraction

from egg white with 2.3-2.6 M ammonium sulfate at pH 5.8. This was then dialyzed free of salt, the pH lowered to 3.0, and ammonium sulfate added to give a concentration of 1.5 M. They demonstrated that the fraction obtained was identical to conalbumin.

Bain and Deutsch (14) separated conalbumin from the rest of the egg white protein by application of aqueous ethanol fractionation procedures. Their fractionation resulted in a product which was electrophoretically pure conalbumin, but in a yield of only 12%. The techniques employed offer many obvious advantages over those previously described.

Chemical composition. Nitrogen analyses on conalbumin preparations range from 14.8-16.4% (14, 77, 99, 119). The best values probably lie in the range 16.0-16.4%. Limited amino acid analyses are summarized by Romanoff and Romanoff (108, p. 333). Sørensen (119) reported, on the basis of the orcinol reaction, that conalbumin contained 2.8% hexose. Employing spectrophotometric measurements, Bain and Deutsch concluded that the riboflavin of egg white is associated with conalbumin. The flavin could be dialyzed from the protein only at pH's acid to the isoelectric point. Their results indicated somewhat less than one mole of flavin was bound to one mole of protein.

Electrophoretic behavior. Longsworth, et. al. (77) carried out electrophoretic analyses on conalbumin preparations. They reported that conalbumin consisted of two components which they labelled C_1 and C_2. At low pH's C_2 was the main component, but as the pH was increased more C_1 was observed. They concluded that during dialysis at pH 3.6, the equilibrium was shifted and C_2 was formed. Similar results were obtained in whole egg white experiments. In egg white, at pH's above 4, C_1 was the normal component, but dialysis at lower pH values increased the amount of C_2. This transformation is reversible and hence is not a typical denaturation reaction.

Isoelectric points, from pH - mobility data, of 5.8 and 6.0 were reported for C_1 and C_2 respectively (77). These data were obtained in acetate and phosphate buffers of ionic strength 0.1. Anderson and Alberty (11) reported the isoelectric point of conalbumin, with flavin removed, as 7.1 at an ionic strength of 0.01. The difference from the value reported at ionic strength of 0.1, may be partially due to the flavin removal, but points out the necessity for close control of isoelectric precipitations in protein fractionation studies.

Bain and Deutsch (14) found only one component in the pH range 3.0-8.6, except when the conalbumin had been dialyzed below pH 3.0. The difference between this and the

Longsworth, et. al. (77) report may be partially explained by the different method of preparation. When conalbumin was subjected to electrophoresis at low (0.01) ionic strengths, two components were observed.

Ultra-centrifugal behavior. Longsworth, et. al. (77) observed two peaks in the sedimentation patterns of conalbumin. The main constituent, C_1, had a molecular weight of around 70,000 while C_2 had a somewhat higher sedimentation value. Bain and Deutsch (14) observed only one peak in the ultra-centrifuge and reported a molecular weight of 87,000.

Biological activity. Schade and Caroline (111) observed that egg white was capable of binding large amounts of iron, making it unavailable for certain microorganisms and inhibiting their growth. Alderton, Ward, and Fevold (7) identified the iron binding ability with conalbumin.

Preparation and properties of lysozyme.

Preparation. Lysozyme is probably the easiest of the egg white proteins to prepare in crystalline form. Abraham (1) isolated lysozyme by extracting acetone dried egg white with 50% alcohol containing 10% acetic acid. The product was purified by fractional precipitation with acetone. Alderton, Ward and Fevold (6) prepared lysozyme by absorbing the active portion of egg white on bentonite

and eluting with pyridine. The pH of the eluting solvent is very important as the lysozyme is eluted only between pH 3.0-6.2, with a sharp maximum at 5.0. Alderton and Fevold (4) reported that lysozyme could be crystallized directly from egg white by adjusting to a pH of 9.0-9.5, and a sodium chloride concentration of 5%. Several lysozyme salts were crystallized under suitable conditions.

Chemical composition. The analytical data available for lysozyme indicate a nitrogen content of 15.0% (40). Abraham (1) reported that lysozyme contained 11.6% arginine, 7.0% lysine, 4.4% tyrosine, and 2.6% histidine. On the basis of a molecular weight of 18,000, he calculated that there were twenty-two basic groups per molecule. Data for the acidic groups are apparently not available in the literature.

Electrophoretic behavior. Longsworth, et. al. (77) reported mobilities of their G_1 globulin from pH 3.6-8.0. Extrapolation of the pH mobility curve indicates an isoelectric point at pH 10-11. Alderton, Ward and Fevold (6) demonstrated the close similarity between G_1 and lysozyme and reported an isoelectric point of 10.5-11.0. Anderson and Alberty (11) determined the electrophoretic mobility of crystalline lysozyme at ionic strengths of 0.01, 0.02, and 0.05. The isoelectric pH was found to be 11.0-11.2 and not strongly dependent upon ionic strength.

Molecular weight and crystallographic studies. The molecular weight of lysozyme has been reported as 17-18,000 from ultra-centrifuge, diffusion, and osmotic pressure measurements (1, 6). Palmer, Ballantyne, and Galvin (101) determined the molecular weight of lysozyme by X-ray diffraction techniques. They found the molecular weight of dry, chloride free lysozyme to be 13,000 ± 600. The later value is probably much more reliable than those previously reported.

Jones (59) has discussed the optical and crystallographic properties of a number of lysozyme salts.

Biological activity. The ability of lysozyme to effect a break down of the cell structure of several microorganisms has been previously mentioned (p. 20). Organisms which are particularly susceptible to the action of lysozyme are Micrococcus lysodeikticus and Sarcina lutea (43, 44). Airborne varieties of bacteria usually are less resistant to lysozyme than are others, but resistance can be developed within a species.

Meyer and co-workers (91, 92) have studied the mode of action of lysozyme. A highly polymerized mucopolysaccharide has been isolated from a number of organisms which undergoes depolymerization upon addition of lysozyme. The degree of degradation was determined by viscosity measurements.

A relationship between the biological activity of lysozyme and avidin has been proposed (68, 89) but strongly contested (5). There seems to be no conclusive evidence for any real relationship between these two proteins.

Preparation and properties of avidin.

Preparation. Eakin, Snell, and Williams (36) have described three methods for the preparation of avidin. In one method, all of the egg white proteins were precipitated with acetone and the curds extracted with salt solutions. The active principle was extracted. Avidin has been concentrated with ammonium sulfate or heat coagulation. Crystalline avidin was obtained by the use of ammonium sulfate procedures (103).

Properties. Pennington, et. al. (103) reported that crystalline avidin contained 12.1-12.8% nitrogen and gave a positive Molisch test for carbohydrate.

On the basis of its biotin binding capacity, Eakin, Snell and Williams (36) calculated a maximum molecular weight of 87,000. Woolley and Longsworth (139) studied avidin electrophoretically and reported it was isoelectric at pH 10. On the basis of sedimentation and diffusion measurements, they arrived at a molecular weight of 70,000.

It has been proposed (36, 139) that one molecule of

avidin is capable of binding one molecule of biotin. The biotin is then rendered unavailable for utilization by certain bacteria and yeasts. Biotin can not be removed from the avidin by dialysis.

FRACTIONATION OF PROTEINS UNDER CONDITIONS OF LOW DIELECTRIC CONSTANT AND LOW IONIC STRENGTH.

Edsall (37) has adequately discussed the principles of protein fractionation and their application to the plasma proteins. The following remarks are intended to summarize the information pertinent to this study.

Principles of Protein Solubility

Thermodynamically, considerations of the solubility of proteins do not differ qualitatively from those involved in the solubility of simple ions. In a saturated solution at equilibrium, the chemical potential of the solid phase must be equal to that of the dissolved solute. The chemical potential (μ) and the activity a are related by the expression $\mu - \mu_o = RT \ln a$ where μ_o is the chemical potential of the substance in a defined standard state, μ is the chemical potential under any other specified conditions, and a is the activity under these same conditions. If the same standard state is chosen, the activity of the solute in equilibrium with the same solid phase in any solvent must be identical.

The activity of the solute is the product of two factors: the concentration (usually expressed as mole

fraction) and the activity coefficient f. If the same solid phase is in equilibrium in two or more saturated solutions, the activity of the solute is the same in all of the solutions as previously pointed out. Now if it is assumed that the activity coefficient in a dilute aqueous solution is unity, the activity then equals the concentration or the solubility. In any other solvent, f will probably be different than unity, but since a must remain constant, the solubility is changed. Thus any change which affects the activity coefficient of the solute will change the solubility. From a consideration of the Debye-Hückel theory, the factors involved in the evaluation of the electrical free energy (and hence activity coefficient) may be determined. The dielectric constant, the temperature, and the ionic strength influence the activity coefficient of the electrically charged solute. The effects on the activity coefficient of a singly charged inorganic ion are greatly magnified when a protein is considered. Edsall points out that a relatively small change in the composition of the solvent may produce a great change in the solubility of the protein, and, moreover, these effects may be highly specific.

Another factor affecting the solubility of a protein is the crystal lattice energy involved. Edsall has described the process of a molecule dissolving as follows:

The molecule must first be removed from the crystal lattice; the energy required to accomplish this increases with the strength of the crystal lattice bonds. The second step is the creation of a hole in the solvent; the free energy change necessary to accomplish this depends upon the energy involved in the vaporization of the solvent. The third step is the insertion of the molecule into the hole in the solvent. While the first two steps require the expenditure of energy, the third step is an energy recovery step. The amount of energy recovered depends upon the interaction of the solute with the solvent molecules. Obviously then, whether a protein molecule dissolves or not depends partially upon whether the energy recovered in the last step is sufficient to supply that needed for the first two. Edsall points out that these same considerations apply to proteins in an "amorphous solid phase" because there must be intermolecular forces involved even though no crystal lattice actually exists.

The many polar groups present in protein molecules exert a strong influence on the solubility. If positive and negative charges are in close proximity in the crystal lattice the binding strength is increased, while if two positive charges are adjacent the crystal lattice energy is decreased. When in aqueous solution, all of the polar

groups react with the water dipoles increasing the solubility of most proteins in aqueous media. Non-polar groups tend to reduce the solubility in water but increase the solubility in non-polar solvents. The position of the polar groups in the molecule also has a marked effect on the solubility. In general, the greater the dipole moment, the greater the solubility in polar solvents such as water.

The effect of added salts is that, in general, the solubilities of proteins are increased at low ionic strengths. This observation is in agreement, at least qualitatively, with predictions made from the Debye-Hückel theory. Edsall (37) shows that the Kirkwood relationship is a good representation of the experimental data. Kirkwood's equation gives the relative solubility N/N_o as a function of the ionic strength ($\Gamma/2$) at low ionic strengths for a spherical dipolar ion of radius (b) and a point dipole (μ) located at the center of the sphere:

$$\ln \frac{N}{N_o} = \frac{2\pi N \epsilon^2}{DKT}\left[\frac{3\mu^2}{2DaKT} - \frac{b^3}{a}\alpha(\rho)\right]\frac{\Gamma}{2} \qquad \text{I}$$

where N, ϵ, k, D, and T have their usual significance, a, the collison diameter, is equal to b plus the mean radius of the salt ions in solution; and $\alpha(\rho)$ is a function of ρ which is the ratio b/a. Thus it is seen that in a medium of a given dielectric constant, the logarithm of the solubility

is proportional to the first power of the ionic strength. In this equation the first expression in the brackets, usually referred to as the "salting-in" term, represents a solvent effect due to electrostatic forces. The second, a "salting-out" term, increases in proportion to the volume of the molecule. In low dielectric media the second term, because it varies only with the reciprocal of the dielectric constant, is relatively unimportant compared to the first, and the primary effect of adding salt is to increase the solubility. The effect of low concentrations of salts on the solubility of the globulins is well recognized but in the case of the albumins a similar effect, due to their high water solubility, has but recently been observed. The addition of such reagents as alcohol or acetone reduces the dielectric constant. The decrease in dielectric constant results in increased electrostatic forces between the charged groups of the protein. Increased interactions of this type lower the solubility in the absence of salts. Under such conditions of low dielectric constant, the effect of added salts is very similar to that observed for the globulins. In addition, Edsall comments (37, p. 419),

> "the decrease in dielectric constant of the solvent, which is responsible for reducing the solubility in the absence of salts, also enhances the electrostatic interactions between

> the ions of the salt and the charged groups of the protein molecules, and thereby causes the "salting-in" effect to be much larger than in water".

The solubility of many dipolar ions is increased by the addition of another dipolar ion. On a molar basis, the effect of salt is usually somewhat larger than for dipolar ions. In a protein mixture this might mean that one constituent, more probably a globulin, would be quite soluble in the complex system, but precipitate under identical solvent conditions in the absence of other protein.

The effect of high concentrations of salt on the solubility of proteins is similar to that observed in simpler systems (salting out of simple alcohols). At high salt concentrations, the solubility of the protein decreases with increasing ionic strength. This "salting-out" effect is described by the emperical relationship

$$\log S = \beta - K_s \Gamma/2 \qquad \text{II}$$

where β is the hypothetical solubility at zero ionic strength, and K_s for protein is much larger than for a smaller molecule, and is nearly independent of pH and temperature. β varies with pH and is generally a minimum at the isoelectric point. If β values for two proteins are sufficiently far apart, the solubility at different pH's may render a "salting-out" separation possible. The

"salting-out" procedures, using primarily ammonium sulfate, have been widely used in protein fractionation studies.

The solubility of most proteins is a function of the net charge on the molecule which is in turn influenced by the pH. As the isoelectric pH is approached the net charge approaches zero and the solubility is decreased. On the basis of the effect of ionic strength on solubility, proteins may be placed in two general classes. Class I, in which the pH of minimum solubility is but weakly dependent on the salt concentration, and in which solubility is increased on both sides of the isoelectric point by increased salt concentration, includes β-lactoglobulin and ovalbumin. Class II includes those proteins in which the pH of minimum solubility is strongly dependent on the ionic strength; the solubility is decreased on the acid side of the isoelectric point and increased on the alkaline side by increased salt concentrations. These effects can be interpreted on the basis of anion binding.

If in a protein mixture, the pH is maintained between the isoelectric points of two or more components, formation of protein-protein "salts" may result. If a separation is made under such conditions mixtures will probably be obtained. It is desirable, therefore, that in protein fractionation studies all separations be carried out under such conditions that the net change on all components will

be of the same sign.

The variation of the solubility of proteins with temperature has been observed by many workers. The relationship between the solubility and the temperature may be given as:

$$\frac{d \ln S}{dT} = \frac{\Delta H}{RT} \qquad \text{III}$$

ΔH for most proteins in the "salting-in" range is positive (heat is absorbed) and the solubility increases with increasing temperature. This temperature solubility relationship can be used to advantage in alcohol fractionation procedures. It must be used with caution, however, because of the denaturation which may result at higher temperatures.

Fractionation of the Plasma Proteins

Prior to the development of a fractionation scheme in media of low dielectric constant and low ionic strength, two procedures were employed for preparing plasma fractions. These are in general the same as have been previously mentioned for certain of the egg white proteins. Euglobulins, those proteins which are insoluble at their isoelectric point in the absence of salt but soluble at both higher and lower pH's, have usually been precipitated by dilution of the protein with water and adjustment of the pH to the

isoelectric point. In practically all cases such procedures have given mixtures which were difficult to purify. By suitable control of purification procedures, however, some quite pure preparations have been obtained.

The other method used for the preparation of proteins has been the "salting-out" technique. The principles of this method have been discussed previously. This method has been primarily used for the albumins which could not be separated by dilution techniques. Many salts have been used but ammonium sulfate is most satisfactory because of its high solubility. Sodium sulfate, sodium chloride, and potassium phosphates have also been employed. The classic fractionation of plasma has been carried out so that fibrinogen is obtained at 0.20-0.25 saturation with ammonium sulfate, euglobulin at 0.33 saturation, pseudoglobulin at 0.50 saturation, and albumin either by difference or full saturation. The "salting-out" procedures, in general, do not give clear cut fractions and extensive purification procedures must be employed.

From the fundamental principles of protein solubility discussed above it is seen "that proteins are most sensitive to the action of electrolytes, at low ionic strengths and low dielectric constant" (37, p. 437).

"Salting-out" depends primarily on unspecific factors such as the molecular size and shape and little advantage can be taken of the specific chemical characteristics of the molecule. In general, "salting-out" procedures depend upon four variables: salt concentration, protein concentration, pH, and temperature, and of these the temperature is not particularly useful because of the balance that must be maintained between salt solubility and protein stability. In low dielectric media fractionation procedures, another variable, the dielectric constant, is added and the temperature variable can be used more efficiently.

In the fractionation experiments to be reported, extensive use has been made of the conditions employed in the plasma fractionation work. Cohn, et. al. (29, p. 462) list the limits of the variables used in the Harvard Plasma Fractionation Laboratory:

TABLE VII.

Plasma fractionation variables and their limits.

Variable	Limits Employed
pH	4.4 to 7.4
$\Gamma/2$	0.001 to 0.16
Ethanol, concentration, mole fraction	0 to 0.163
Protein, concentration g./liter	0.2 to 66
Temperature, C.	0 to -10°C.

Several schemes for plasma fractionation have been devised, repeatedly modified, and are subject to still further modification. In Cohn's laboratory the number of major fractions is held low to enable large scale processing. The general plan used for the fractionation of the plasma proteins may be summarized as follows: Fibrinogen is usually taken out first near pH 7 at low ethanol concentrations. The β- and γ-globulins are removed by increasing the alcohol to about 25% while the temperature is lowered and the pH held near 7. The α-globulin is largely precipitated by lowering the alcohol to 18% and the pH to 5.2. The globulins remaining in solution are removed at 40% alcohol, pH 5.8. The supernatent contains mostly albumin which can be removed by lowering the pH to 4.8 while the alcohol concentration is held at 40%. Subsequent sub-fractionation of the major fractions results in several components in a high state of purity.

The successful preparation of the various plasma fractions is of widespread practical importance. The albumin fraction has been used extensively in treatment of shock cases. The γ-globulin fraction contains many of the antibodies present in the plasma that have been used for passive immunization. The stability of certain

of the plasma fractions is greater when in a more purified condition. Several plasma fractions have been utilized as special by-products. Fibrin has found wide usage as foam, film, and plastic material in surgical operations. Uses for some of the other fractions may be found as they become more readily available.

MATERIALS AND METHODS

Egg White

The eggs used in this study were routinely obtained from the College Poultry Farm within four to six hours after laying and were immediately cooled to 1°C. After cooling, the eggs were broken, the whites separated as completely as possible from the yolks, and the chalazae removed with tweezers. The white was treated in a hand homogenizer[1] in a few early experiments but later in a Waring Blender to break up the thick gel structure. Results of these two treatments will be discussed in the section on experimental results and discussion. To blend the egg white without excessive foaming, the switch of the blender was rapidly "flicked" off and on thirty times. This treatment resulted in a homogeneous structureless egg white which was then used as the starting material for the fractionation work.

Alcohol

A commercial grade of 95% ethyl alcohol was used throughout this study without further purification. In

[1] Central Scientific Company, Chicago, Illinois.

order to minimize local high concentrations of alcohol when added to protein solutions, a solution of 50% (by volume at 25°C.) was used for all additions. Alcohol concentrations were commonly expressed as mole fractions in order to eliminate the troublesome volume-temperature relationship. Alcohol concentrations were checked by refractive index measurements with an Abbe refractometer.

Buffers

Buffers were used throughout this study for pH adjustment. By the use of buffers the possibility of local excesses of free acid or alkali is eliminated. Preparation of acetate buffers of desired pH and ionic strength from stock solutions of 10 M reagent grade acetic acid and 4 M reagent grade sodium acetate was facilitated by the use of the D'Ocagne nomogram (21). Phosphate buffers, used for routine electrophoretic runs and pH adjustment in the fractionation procedures, were prepared with the aid of Green's (49) phosphate buffer chart from stock solutions of 1 M reagent grade primary and secondary potassium phosphate. Sodium carbonate-bicarbonate buffers were used when pH's above eight were desired. Calculated pH values were not in good agreement with measured values in this buffer system and it was usually necessary to adjust the pH

by titration with dilute acid or base. For this reason ionic strength values in this system were not accurately known.

Addition of Reagents

Two methods for introducing reagents into protein solutions were used. When small volumes were involved, the dialysis method was employed. This method consists of dialyzing the reagent to be added into the protein solution through an appropriate Visking tubular membrane. In the case of buffers, the pH was followed and when at the proper value, the tube was removed. The time required varied greatly and was dependent upon the volumes employed and the amount of change desired. When ethyl alcohol was to be added, the reagent concentration in the tube was changed periodically until the desired final concentration was obtained. Normally, a twenty-four hour dialysis period was considered sufficient to establish equilibrium. When large volumes (one to three liters) were employed a "capillary addition" was used. In this method the reagents were added slowly with constant agitation through a fine capillary tube until the desired final concentrations were obtained.

Ionic strengths were adjusted when necessary by dialysis

against solutions of appropriate salt concentrations. Alcohol concentrations were always adjusted by the addition, using either of the above methods, of solutions containing no more than 50% (by volume) ethyl alcohol. Alcohol solutions were cooled to their freezing point before all additions. pH adjustments were accomplished through the use of acetate, phosphate, or carbonate buffers, added as previously described, except in isolated instances. All additions were made at temperatures within one to two degrees of the freezing point of the solution.

All of these precautions were taken to prevent the formation of "local excesses" of reagents and increases in temperature. Such effects can lead to serious denaturation of the protein.

Low Temperature Operation

The availability of equipment for low temperature operation was considered of utmost importance in this study. A refrigerated laboratory (7' x 17') was constructed from a refrigerated trailer secured from the War Assets Administration. Cooling of this room was accomplished by the use of a five horsepower compressor (Freon refrigerant) with a constant stream of air over the cooling coils. Temperatures were held at 1.0 ± 1.0 °C. under normal operating conditions.

A refrigerated bath containing an ethylene glycol-water mixture was used for temperatures below those obtainable in the "cold room". This bath, located in the cold room, was equipped with a household refrigerator compressor unit, and was capable of maintaining temperatures as low as -20°C. with a constancy of $\pm 0.5^{\circ}$.

Centrifugation and Clarification

All fractions were removed by centrifugation. Two types of centrifuges were employed throughout this study. When large volumes were to be centrifuged, and the foaming tendencies of the solution were not serious, an air-driven Sharples super-centrifuge[1] was used. This apparatus was capable of bowl speeds up to 50,000 r.p.m. corresponding to a centrifugal force of approximately 50,000 times gravity. The air space surrounding the bowl was refrigerated by circulating the liquid from the bath through copper coils wrapped around the centrifuge housing.

For small volumes (up to one liter), or when foaming in the super-centrifuge was excessive, an International centrifuge[2] was used. The air space surrounding the cups in this centrifuge was refrigerated as in the super-centrifuge.

[1]Type T-304-24

[2]Size 1, Type SB.

Since centrifugation did not usually give absolutely clear supernatants, a Horman pressure filter[1] was used for clarification purposes. Filter aid was not necessary in this apparatus and was never used. Filtration was usually so rapid that no serious temperature increases were encountered.

Drying Protein Preparations

All protein preparations were dried by vacuum sublimation of the frozen solvent. This method of drying is commonly known as lyophilization. A shell of the material to be dried was frozen in a round bottom flask of suitable volume, a vacuum of at least 4.5 mm. of Hg (the vapor pressure of ice at 0°C.) was maintained, and condensation of the vapor effected by immersion of the condenser in a dry ice-alcohol mixture (approximately -70°C.). By this technique dry proteins can be obtained in approximately twenty-four hours with temperatures never rising above 0°C. until the preparation is nearly dry.

Electrophoretic Analysis

The electrophoretic apparatus used in this study was

[1] F. R. Hormann and Co., Inc., Glen Cove, Long Island.

obtained in part from the Klett Manufacturing Company[1], and partially constructed in these laboratories. The construction of the thermostat, cell, cell holder, and light source have been adequately described by Longsworth, et. al. (73, 75, 77).

The optical system employed was a modification of the Philpot-Svensson system which has been discussed by Svensson (131). A Leica 35 mm. camera with focal plane shutter but stripped of all optics has been substituted for the usual plate holder. An important advantage of this modification is that the smaller image enables a shorter objective to film distance and a correspondingly longer cell to objective path. This provides more nearly parallel illumination of the cell. Convenience of operation and economy of film are increased with no apparent loss of pattern quality. In standard runs photographs were taken with a rectangular diagonal slit.

Constant direct current voltage was supplied by a voltage regulated power supply[2].

All electrophoretic analyses were made at 2°C. Temperature control was accurate to about ±0.05° and

[1]New York, New York

[2]Model 1220, manufactured by the Technical Apparatus Co., Boston, Massachusetts.

maintained by the use of a sealed mercury expansion type thermoregulator, operating through a mercury switch relay assembly (with rectifer and transformer).[1]

Electrophoretic analyses were usually carried out at a field strength of 4.5-6.0 volt-cm.$^{-1}$. Electrophoresis experiments were normally continued until maximum resolution was obtained (10,000-15,000 seconds).

Fifty to one hundred ml. of protein solution was dialyzed with mechanical agitation against two liters of buffer for twenty-four to thirty-six hours at 1-2°C. prior to electrophoresis. In some cases shorter periods were used; on the basis of conductance measurements twelve hours would appear to be the minimum dialysis period under these conditions.

Specific conductance was measured on both buffer and protein solutions prior to electrophoresis at the same temperature at which the run was to be carried out. A Leeds and Northrup electrolytic conductivity bridge[2] was used for this purpose. Prior to electrophoresis protein solutions were usually adjusted, with an Abbe refractometer, to a refractive increment (the difference in refractive

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[1] Manufactured by the American Instrument Company, Silver Springs, Maryland.

[2] Alternating current, Wheatstone bridge type, Model 4960.

index between protein and buffer solution) of 0.0020 at 25°C.

Analyses of the electrophoretic patterns were made in the following manner: The negatives were traced on coordinate paper, at a magnification of about three times the actual cell dimensions, under a standard photographic enlarger. The initial boundaries were also recorded photographically and the record superimposed on the tracing of the pattern to be analyzed. The calculation of relative concentrations was somewhat arbitrary in cases where resolution was not complete. The areas were separated according to the procedure described by MacInnes and Longsworth (80). A line was dropped from the minima between the peaks to the base line and the areas measured with a planimeter. This procedure is not as sound, theoretically, as that described by Pedersen (102), but was found to be more reproducible and considerably easier to carry out. The line bisecting the area under each gradient curve was used for mobility measurements. Average values from the ascending and descending patterns were used in most cases.

Miscellaneous Analyses

Nitrogen analyses were made by a modified Pregl micro-Kjeldahl procedure, using a $SeOCl_2$ catalyst. Samples were

usually taken as solutions and appropriate dilutions made before the analysis. Digestion was allowed to proceed until the solution was clear, two drops of 30% hydrogen peroxide added, and then digested for fifteen minutes more. The ammonia was distilled into 5% boric acid and titrated with 0.01 N HCl. The indicator used was the so-called "mixed indicator" consisting of 0.125 grams of methyl red and 0.0825 grams of methylene blue in 100 ml. of 95% ethyl alcohol.

Carbohydrate analyses were carried out by the diphenylamine colorimetric reaction described by Levine (70), using glucose as the standard. Due to the fact that a slight coloration developed upon heating the protein solution in the acetic-hydrochloric acid mixtures, it was necessary to modify this procedure in the presence of large amounts of protein material by using a protein blank.

Sodium chloride was determined to give approximate values of the ionic strength when that salt was the primary inorganic constituent in the system. This determination was carried out by analyzing for chloride by Mohr's method (138, p. 178) and assuming that all the chloride was present in the form of NaCl.

pH measurements were carried out with a Leeds and Northrup, Universal pH assembly[1] equipped with a glass

[1] No. 7661-A1.

electrode. Readings were taken at 20-25°C. on solutions diluted so that the alcohol or protein did not introduce appreciable errors.

EXPERIMENTAL RESULTS AND DISCUSSION

Factors Affecting the Electrophoretic Composition of Egg White

Throughout this study the electrophoretic technique has been used to determine the amounts of the various proteins in the fractions. Preliminary studies prior to the development of a comprehensive fractionation scheme for the egg white proteins demonstrated the necessity of investigating several factors which might influence the electrophoretic composition.

The effect of ionic strength and protein concentration on the apparent electrophoretic composition of several natural protein systems is well established (64,76,131). These results suggested reinvestigating the electrophoretic analyses of egg white reported by Longsworth, et. al. (77) and Bain and Deutsch (13).

Evans, et. al (41) employing isolation techniques, reported that the percentage of ovomucin in stored eggs first increased and then decreased in a twenty-three month cold storage period. Slight changes in the other proteins were also noted. Such changes might necessitate modification of fractionation techniques. Because of this, the effect of the age of the eggs on the electrophoretic composition of

egg white was determined.

It has been reported that the quantity and heat resistance of the thick mucin gel in natural egg white is an inherited characteristic (62, 63). The possibility that such genetic differences could also effect the protein composition of the egg white has been considered and a limited study of several strains of chickens has been completed.

Normal electrophoretic composition of egg white.

The first investigations in this study were designed to determine the electrophoretic composition of egg white and mobility of the protein constituents under control or reference conditions. Such data are essential for qualitative and quantitative identification of components in the various fractions.

Reference pattern with ovomucin included. In previous electrophoretic investigations of egg white, ovomucin has been removed during the process of dilution and dialysis prior to the experiment. It was obvious that if yields were to be computed, electrophoretic analyses of whole white must be available. A series of experiments was carried out to determine what conditions, if any, could be employed so that electrophoretic analyses could be made without the removal of ovomucin. Egg white was blended as described

and diluted ten fold with solutions of varying sodium chloride concentrations to give approximately a 1% protein solution. This protein concentration is generally satisfactory for electrophoretic analyses. When salt concentrations of 0.15 M and above were employed no precipitation of the egg white proteins was observed. Next, the effect of various pH levels was determined. Buffers were prepared over a range of pH 3.9-8.0 (acetate or phosphate) with a total ionic strength of 0.20. In all cases the buffer salts contributed 25% of the total ionic strength, the remainder being NaCl. The egg white solutions diluted to 1% protein in 0.15 M NaCl were then dialyzed against these buffers. No protein precipitated at pH's above 6.5 and optimal electrophoretic resolution was obtained in the range pH 7.6-7.8. This pH range and ionic strength were selected for all routine analytical work on whole egg white.

A typical electrophoretic pattern obtained from analyses of whole egg white at pH 7.8, $\Gamma/2 = 0.20$, protein concentration 1.0% is shown in Figure 1a. Identification of the protein constituents has followed the general plan described by Longsworth, et. al. (77). Identification has been checked, so far as possible, by comparison with mobility measurements on the purified egg white proteins. Bain and Deutsch (13) observed a component which migrated slower

than conalbumin in the egg white of several birds. They did not observe this component in chicken egg white and no evidence for its presence has been found in this study. The component labelled G_2 was called C_2 by Bain and Deutsch. Mobility measurements on purified globulin preparations and the inability to obtain resolution of two conalbumin peaks under these conditions indicates this component to be a globulin and not conalbumin.

A fast component with a mobility greater than ovalbumin has been observed in some experiments. This component has not been identified and has been included with the ovalbumin for measurement of relative concentrations.

The δ- and ϵ-anomalies in phosphate-chloride buffer, pH 7.8, $\Gamma/2 = 0.20$ have been observed to move with an approximate negative mobility of 1.0×10^{-5} cm.^{2}-volt^{-1}-sec.$^{-1}$ in qualitative agreement with Svensson's observations (131).

Identification of lysozyme. If the initial boundary is displaced a sufficient distance in the cell prior to the passage of current, a component with a positive mobility can be observed at pH's above 7.3. An electrophoretic pattern showing this constituent is shown in Figure 1b. This component is similar to Longsworth's G_1, and the proposal that it is identical with lysozyme has previously been discussed. In an attempt to establish the validity of

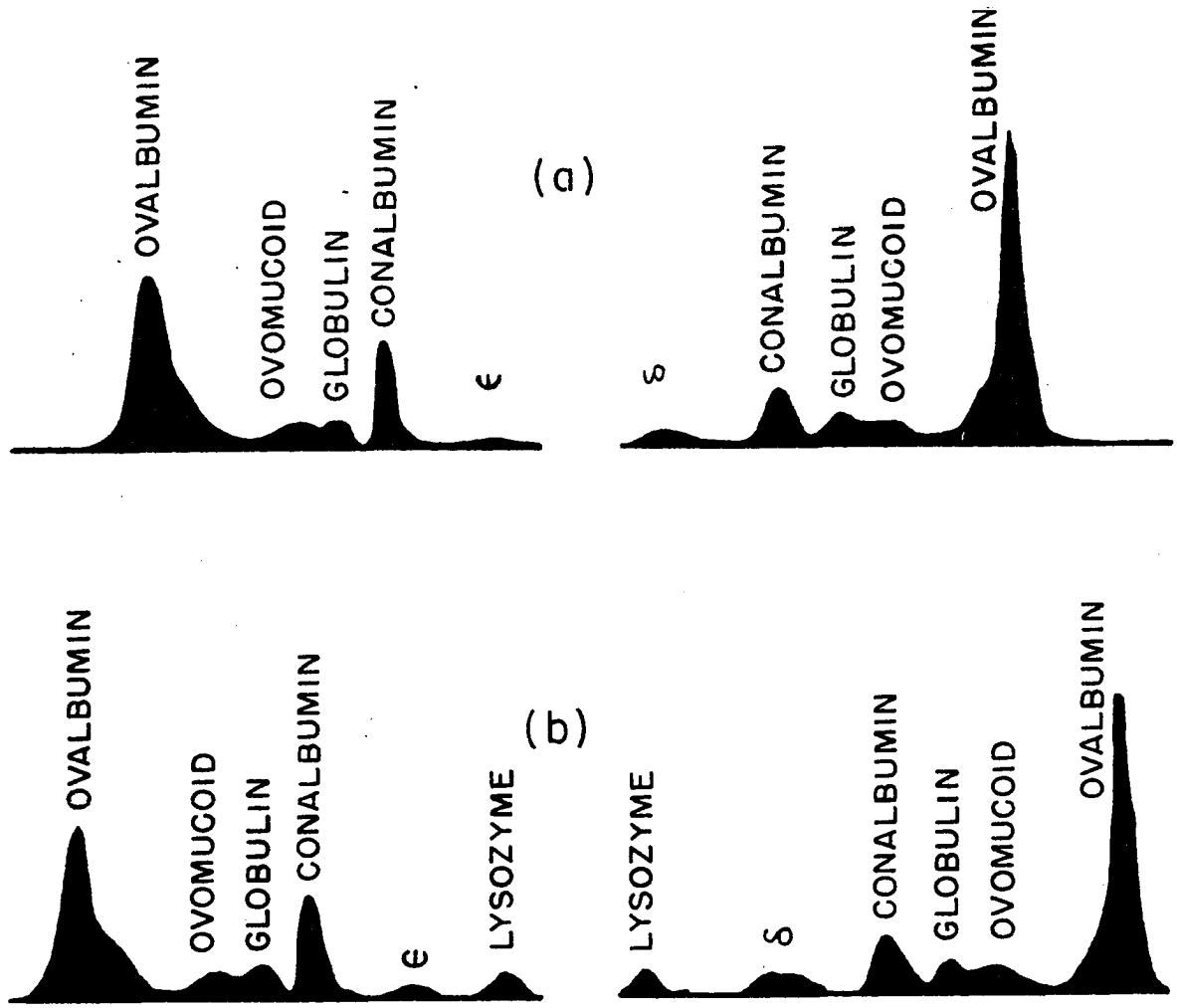


Figure 1. Normal electrophoretic patterns of egg white without lysozyme (a) and with lysozyme (b).

this proposal, the material was isolated from the appropriate region of the electrophoresis cell and tested for lytic activity. Lytic activity in this instance was determined as follows: One ml. of a killed cell suspension of Micrococcus lysodeikticus was placed in a small test tube. One ml. of the solution to be tested (in serial dilution) was added to the tube and incubated at 37°C. for one hour. Two drops of 1 N NaOH were then added and the amount of clearing observed. The end point was taken as that dilution which gave the last clear tube in a series sample. The solution containing the component with the positive mobility, and apparently the same component designated by Longsworth as G_1, was 10,000 times as active as ovalbumin also isolated from the cell. On this basis it is concluded that the egg white protein which has been labelled G_1 is identical with egg white lysozyme; the later name will be used in the remainder of this discussion. The electrophoretic mobility and concentration of this component can be determined but the results must be treated only in a semi-quantitative manner due to the great possibility of interaction between the oppositely charged proteins. In the results that follow, probable errors for lysozyme have not been calculated as this constituent has been observed only in a relatively small number of runs under reference conditions.

Quantitative results and reproducability. A large number of runs were completed under nearly identical conditions so that the probable errors involved in the analyses could be computed. Thirty-one runs were carried out on fresh egg white, without ovomucin removal, at pH 7.7-7.8 in phosphate-chloride buffer, total ionic strength 0.20 (NaCl = 0.15). The protein concentration was 1.0% in all runs. The results of these analyses are shown in Tables VIII and IX. The mean and probable errors of the various measurements are summarized in Table X.

The composition data compare favorably with those reported by Longsworth, et. al. (77), with the exception of the ovomucoid. Bain and Deutsch (13) do not report a figure for ovomucoid but assume that it is probably included in the globulin area. The data in Table X have been used for calculation of yields in all of the fractionation experiments.

The probable errors computed in these experiments are comparable to those usually observed in electrophoretic analyses. A partial breakdown of the errors in electrophoretic analyses can be made. The error involved in tracing the electrophoretic patterns is common to both mobility and concentration measurements, and is about 1-2%. The major error in the

TABLE VIII

Electrophoretic composition of egg white measured under reference co

Run No.	% Ovalbumin			% Ovomucoid			% Globulin			% Conalbumin		
	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.
31	58.8	73.4	66.1	12.1	8.6	10.3	9.2	7.0	8.1	19.8	10.9	15.3
60	65.4	65.8	65.6	9.1	9.0	9.0	10.0	9.0	9.5	15.6	16.2	15.9
61	68.2	67.5	67.9	7.8	8.5	8.2	7.8	8.5	8.1	16.3	15.7	16.0
71	70.5	69.5	70.0	7.5	8.4	7.9	8.1	4.9	6.5	14.0	17.0	15.5
72	67.0	63.2	65.1	7.7	8.0	7.9	11.5	9.9	10.7	15.4	16.0	15.7
73	63.0	68.7	65.9	7.7	7.5	7.6	11.6	6.5	9.0	16.2	17.1	16.7
75	67.5	66.2	66.9	10.2	8.7	9.5	4.1	8.2	6.2	16.3	17.8	17.0
76	71.4	67.5	69.5	6.1	10.6	8.3	8.5	5.5	7.0	14.0	16.9	15.5
77	67.5	65.4	66.5	7.3	8.9	8.1	9.5	8.0	8.8	15.7	17.6	16.6
223	61.3	61.3	61.3	14.9	13.2	14.0	7.0	10.0	8.5	17.0	15.7	16.3
259	65.5	71.4	68.5	7.2	11.8	9.5	10.1	8.9	9.5	16.2	8.3	12.3
260	70.6	68.2	69.4	5.8	10.5	8.2	6.8	10.6	8.7	12.1	10.8	11.4
262	70.6	66.8	68.7	6.9	9.1	8.0	9.5	11.9	10.7	12.9	12.3	12.6
263	65.8	66.4	66.1	11.5	10.2	10.9	8.3	11.4	9.9	14.4	11.9	13.1
264	68.3	70.8	69.5	9.3	7.4	8.4	8.5	10.3	9.4	14.1	11.5	12.8
270	70.7	71.4	71.0	9.8	11.0	10.4	6.7	8.9	7.8	12.9	8.9	10.9
271	70.6	69.5	70.0	10.5	9.9	10.2	7.2	8.7	7.9	11.8	11.9	11.9
273	68.7	67.9	68.3	10.4	12.5	11.5	7.5	9.8	8.7	13.0	9.8	11.4
280	64.8	67.0	65.9	9.2	13.9	11.6	9.6	7.4	8.5	16.4	11.6	14.0
282	66.5	66.9	66.7	9.8	10.4	10.1	9.8	10.1	9.9	13.8	13.8	13.8
283	67.5	63.5	65.5	5.8	12.7	9.3	12.1	9.1	10.6	14.6	14.9	14.7
287	61.6	64.3	64.4	10.8	10.0	10.4	12.1	10.3	11.2	15.5	12.4	13.9
288	66.3	66.5	66.4	11.4	10.5	10.9	10.3	9.8	10.0	12.1	13.2	12.6
289	70.2	65.3	67.8	5.9	12.1	9.0	9.1	8.7	8.9	14.2	13.9	14.0
330	64.0	63.6	63.8	9.2	12.4	10.8	7.1	11.2	9.1	12.7	13.7	13.2
468	71.5	63.0	67.2	6.1	7.5	6.8	6.5	8.6	7.7	13.0	17.9	15.4
509	65.8	64.6	65.2	8.5	10.8	9.7	7.0	9.0	8.0	14.1	15.7	15.9
510	63.5	62.8	63.1		10.7			9.7		12.4	14.4	13.4
512	63.4	63.7	63.6	7.9	10.4	9.1	9.1	9.7	9.4	15.7	16.2	15.9
516	66.5	65.5	66.0	8.6	9.9	9.2	10.3	10.7	10.5	11.6	13.8	12.7
517	63.7	63.2	63.4	10.0	11.1	10.5	9.3	9.5	9.4	13.7	16.2	14.9

TABLE VIII

lc composition of egg white measured under reference conditions

% Ovomucoid			% Globulin			% Conalbumin			% Lysozyme		
Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.
12.1	8.6	10.3	9.2	7.0	8.1	19.8	10.9	15.3			
9.1	9.0	9.0	10.0	9.0	9.5	15.6	16.2	15.9			
7.8	8.5	8.2	7.8	8.5	8.1	16.3	15.7	16.0			
7.5	8.4	7.9	8.1	4.9	6.5	14.0	17.0	15.5			
7.7	8.0	7.9	11.5	9.9	10.7	15.4	16.0	15.7			
7.7	7.5	7.6	11.6	6.5	9.0	16.2	17.1	16.7			
10.2	8.7	9.5	4.1	8.2	6.2	16.3	17.8	17.0			
6.1	10.6	8.3	8.5	5.5	7.0	14.0	16.9	15.5			
7.3	8.9	8.1	9.5	8.0	8.8	15.7	17.6	16.6			
14.9	13.2	14.0	7.0	10.0	8.5	17.0	15.7	16.3			
7.2	11.8	9.5	10.1	8.9	9.5	16.2	8.3	12.3			
5.8	10.5	8.2	6.8	10.6	8.7	12.1	10.8	11.4			
6.9	9.1	8.0	9.5	11.9	10.7	12.9	12.3	12.6			
11.5	10.2	10.9	8.3	11.4	9.9	14.4	11.9	13.1			
9.3	7.4	8.4	8.5	10.3	9.4	14.1	11.5	12.8			
9.8	11.0	10.4	6.7	8.9	7.8	12.9	8.9	10.9			
10.5	9.9	10.2	7.2	8.7	7.9	11.8	11.9	11.9			
10.4	12.5	11.5	7.5	9.8	8.7	13.0	9.8	11.4			
9.2	13.9	11.6	9.6	7.4	8.5	16.4	11.6	14.0			
9.8	10.4	10.1	9.8	10.1	9.9	13.8	13.8	13.8			
5.8	12.7	9.3	12.1	9.1	10.6	14.6	14.9	14.7			
10.8	10.0	10.4	12.1	10.3	11.2	15.5	12.4	13.9			
11.4	10.5	10.9	10.3	9.8	10.0	12.1	13.2	12.6			
5.9	12.1	9.0	9.1	8.7	8.9	14.2	13.9	14.0			
9.2	12.4	10.8	7.1	11.2	9.1	12.7	13.7	13.2	5.0		
6.1	7.5	6.8	6.5	8.6	7.7	13.0	17.9	15.4	3.0	3.0	3.0
8.5	10.8	9.7	7.0	9.0	8.0	14.1	15.7	15.9	4.5		
	10.7			9.7		12.4	14.4	13.4	4.6	2.2	3.5
7.9	10.4	9.1	9.1	9.7	9.4	15.7	16.2	15.9	3.9	1.0	2.5
8.6	9.9	9.2	10.3	10.7	10.5	11.6	13.8	12.7	3.0	3.5	3.2
10.0	11.1	10.5	9.3	9.5	9.4	13.7	16.2	14.9	3.3		

TABLE IX

Electrophoretic mobilities of the protein constituents of egg whi
measured under reference conditions.

Run	Mobilities[1] x 10^5 cm.^{2}-Volt^{-1}-Sec.$^{-1}$												
	Ovalbumin			Ovomucoid			Globulin			Conalbumin			
No.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	A
60	4.89	4.80	4.85	3.46	3.17	3.31	2.73	2.54	2.63	1.96	1.87	1.91	
61	4.99	4.77	4.88	3.23	3.13	3.18	2.54	2.48	2.51	2.14	1.81	1.98	
72	5.36	5.26	5.31	3.76	3.34	3.55	3.00	2.58	2.79	2.15	1.93	2.04	
73	5.35	5.35	5.35	3.90	3.29	3.59	2.85	2.60	2.78	2.12	1.93	2.03	
75	5.10	5.13	5.12	3.27	3.78	3.52	2.54	2.82	2.68	1.79	1.99	1.89	
76	5.42	5.30	5.36	3.62	3.36	3.49	3.16	2.55	2.85	2.06	1.96	2.01	
77	5.53	5.41	5.47	3.82	4.24	4.03	2.82	2.63	2.73	2.06	1.94	2.00	
223	4.70	4.89	4.79	3.11	2.99	3.05	2.40	2.18	2.29	1.59	1.51	1.55	
259	5.70	5.64	5.67	4.20	3.46	3.83	3.15	2.83	2.99	2.38	2.16	2.27	
260	6.08	6.08	6.08	4.53	3.89	4.21	3.42	2.94	3.18	2.47	2.30	2.39	
262	6.19	6.19	6.19	4.60	4.19	4.40	3.66	3.38	3.52	2.82	2.63	2.72	
263	6.25	6.25	6.25	4.59	4.26	4.42	3.79	3.60	3.69	2.65	2.67	2.66	
264	6.45	6.06	6.25	4.89	4.17	4.53	4.03	3.32	3.67	2.94	2.56	2.75	
270	6.16	6.16	6.16	4.54	4.26	4.40	3.69	3.32	3.51	2.65	2.84	2.74	
271	6.23	6.05	6.14	4.53	4.07	4.30	3.69	3.31	3.50	2.64	2.36	2.50	
273	6.30	6.00	6.15	4.35	4.25	4.30	3.80	3.50	3.65	2.90	2.60	2.75	
280	5.70	5.60	5.65	4.15	3.89	4.02	3.10	2.60	2.85	2.30	1.80	2.05	
282	6.40	6.40	6.40	4.60	4.25	4.42	3.50	3.40	3.45	2.70	2.50	2.60	
283	6.50	6.50	6.50	4.65	4.35	4.50	3.70	3.50	3.60	2.90	2.80	2.85	
287	6.10	5.80	5.95	4.20	3.65	3.92	3.30	2.90	3.10	2.30	2.00	2.15	
288	6.40	6.20	6.30	4.55	4.03	4.29	3.50	3.10	3.30	2.50	2.30	2.40	
289	6.50	6.30	6.40	4.70	4.21	4.46	3.70	3.30	3.50	2.80	2.50	2.65	
330	6.15	5.96	6.05	4.05	3.86	3.96	3.26	2.92	3.09	2.32	2.03	2.18	
468	6.06	5.96	6.01	3.97	3.90	3.94	3.30	3.13	3.21	2.42	2.21	2.31	0.
509	5.91	5.91	5.91		3.71			3.08		2.38	2.31	2.35	0.
510	6.18	6.00	6.09	4.23	3.94	4.09	3.52	3.19	3.35	2.46	2.35	2.40	1.
512	6.15	5.73	5.94	4.22	3.79	4.00	3.47	2.95	3.21	2.44	2.12	2.28	1.
516	5.58	5.90	5.74	4.07	4.07	4.07	3.28	3.15	3.21	2.30	2.30	2.30	1.
517	5.94	5.98	5.96	4.33	4.10	4.21	3.58	3.35	3.47	2.66	2.50	2.58	0.

[1] All mobilities negative except for lysozyme.

TABLE IX

ɔtic mobilities of the protein constituents of egg white measured under reference conditions.

Mobilities[1] x 10^5 cm.^{2}-Volt^{-1}-Sec.$^{-1}$											
Ovomucoid			Globulin			Conalbumin			Lysozyme		
ɔc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.	Asc.	Desc.	Ave.
46	3.17	3.31	2.73	2.54	2.63	1.96	1.87	1.91			
23	3.13	3.18	2.54	2.48	2.51	2.14	1.81	1.98			
76	3.34	3.55	3.00	2.58	2.79	2.15	1.93	2.04			
90	3.29	3.59	2.85	2.60	2.78	2.12	1.93	2.03			
27	3.78	3.52	2.54	2.82	2.68	1.79	1.99	1.89			
62	3.36	3.49	3.16	2.55	2.85	2.06	1.96	2.01			
82	4.24	4.03	2.82	2.63	2.73	2.06	1.94	2.00			
11	2.99	3.05	2.40	2.18	2.29	1.59	1.51	1.55			
20	3.46	3.83	3.15	2.83	2.99	2.38	2.16	2.27			
53	3.89	4.21	3.42	2.94	3.18	2.47	2.30	2.39			
60	4.19	4.40	3.66	3.38	3.52	2.82	2.63	2.72			
59	4.26	4.42	3.79	3.60	3.69	2.65	2.67	2.66			
89	4.17	4.53	4.03	3.32	3.67	2.94	2.56	2.75			
54	4.26	4.40	3.69	3.32	3.51	2.65	2.84	2.74			
53	4.07	4.30	3.69	3.31	3.50	2.64	2.36	2.50			
35	4.25	4.30	3.80	3.50	3.65	2.90	2.60	2.75			
15	3.89	4.02	3.10	2.60	2.85	2.30	1.80	2.05			
60	4.25	4.42	3.50	3.40	3.45	2.70	2.50	2.60			
65	4.35	4.50	3.70	3.50	3.60	2.90	2.80	2.85			
20	3.65	3.92	3.30	2.90	3.10	2.30	2.00	2.15			
55	4.03	4.29	3.50	3.10	3.30	2.50	2.30	2.40			
70	4.21	4.46	3.70	3.30	3.50	2.80	2.50	2.65			
05	3.86	3.96	3.26	2.92	3.09	2.32	2.03	2.18			
97	3.90	3.94	3.30	3.13	3.21	2.42	2.21	2.31	0.97	1.37	1.17
	3.71			3.08		2.38	2.31	2.35	0.89	1.98	1.43
23	3.94	4.09	3.52	3.19	3.35	2.46	2.35	2.40	1.47		
22	3.79	4.00	3.47	2.95	3.21	2.44	2.12	2.28	1.38	2.41	1.89
07	4.07	4.07	3.28	3.15	3.21	2.30	2.30	2.30	1.65	0.79	1.22
33	4.10	4.21	3.58	3.35	3.47	2.66	2.50	2.58	0.93		

except for lysozyme.

TABLE X.

Relative composition and mobility of the protein constituents of egg white. Summary of mean and probable errors of the data given in Tables VIII and IX.

	Mobility				Per cent Composition			
	Ascending		Descending		Ascending		Descending	
	Mean	P.E.[1]	Mean	P.E.	Mean	P.E.	Mean	P.E.
Ovalbumin	-5.88	0.38	-5.78	0.33	64.7	2.4	65.1	1.9
Ovomucoid	-4.16	0.32	-3.85	0.28	8.5	1.4	9.9	0.7
Globulin	-3.30	0.32	-3.03	0.26	8.5	1.3	8.9	1.0
Conalbumin	-2.41	0.23	-2.17	0.24	13.9	1.4	13.7	1.9
Lysozyme	+1.22		+1.10		4.5		2.3	

[1] $P.E. = \frac{0.845\ \Sigma(d)}{N}$

computation of relative concentrations is that accompanying the arbitrary separation of areas when resolution is incomplete. A further error in the calculation of mobilities is the location of the first moment of the concentration gradient. This error varies considerably with the complexity of the patterns but averages about 5%. Conductance and current density measurements enter into mobility calculations and can be determined within 1-2% error. The small error involved in pH measurements is not serious since the slope of the pH mobility curves is not great at pH 7.7-7.8. A serious source of error in mobility measurements is

cell leakage. Leakage is difficult to observe if very slow unless the cell is allowed to stand for 12-14 hours. Constant usage of the apparatus makes such a practice impossible. Serious leakage is, of course, observed easily and mobility calculations are never carried out when leakage is suspected.

Protein mobilities are so dependent on pH, ionic strength and protein concentration that they are of limited value in such a complex system. They are extremely useful, however, in the identification of purified components when measured under carefully controlled conditions. Only slight changes in the mobilities of the proteins of egg white have been observed as purification of any constituent was carried out.

Influence of ovomucin.

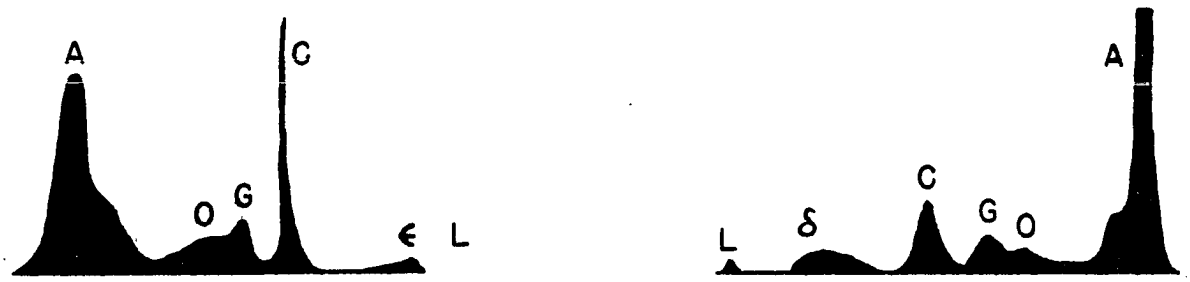
In previous electrophoretic investigations of egg white, ovomucin has been removed prior to the analysis. In the results reported in the preceding section, no component was observed that had not been reported by the earlier workers. Failure to detect ovomucin would not be surprising since the amount in egg white is rather low (less than 2%) and resolution in certain regions of the egg white patterns is poor. Several electrophoresis runs were carried

out under reference conditions on the following preparations:

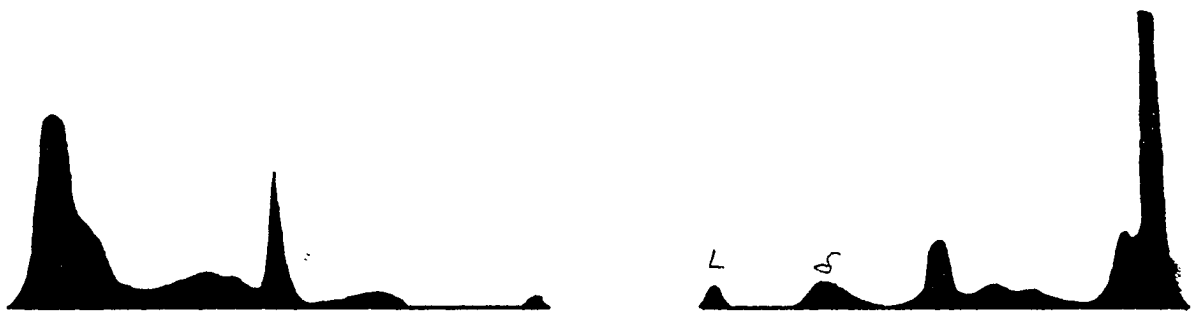
1. Whole egg white.
2. Egg white from which ovomucin had been removed by super-centrifugation. This removal procedure will be described in the fractionation experiments to be discussed.
3. Egg white enriched with ovomucin. This sample was prepared by removing the ovomucin from 250 ml. of egg white in the super-centrifuge and dispersing it in eighty-five ml. of 0.15 M NaCl. Ten ml. of egg white was added to the homogeneous but somewhat opaque suspension yielding a solution that was satisfactory for electrophoretic analysis. This procedure effects a twenty-five fold increase in the relative concentration of ovomucin.

The averaged results from the electrophoretic analyses of these solutions are shown in Table XI and Figure 2. Electrophoretic analyses were attempted at other pH's but it was impossible to keep the ovomucin in solution under any other suitable conditions.

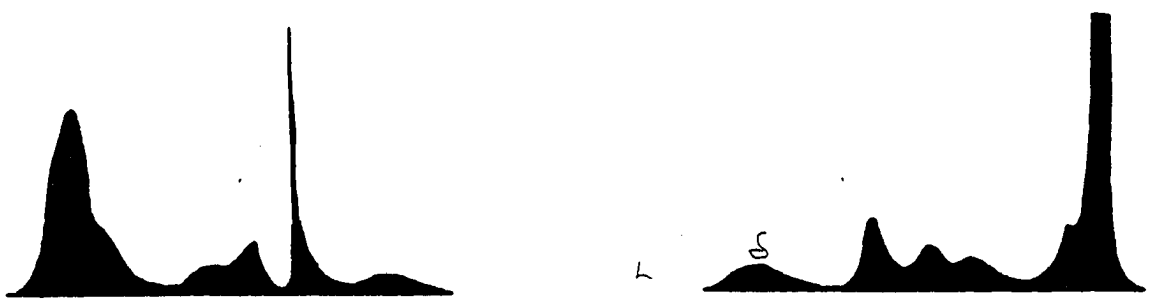
No significant differences can be detected in these experiments but it appears that the globulin area may be



a - NORMAL



b - MUCIN REMOVED



c - MUCIN ADDED

Figure 2. Effect of ovomucin on the electrophoretic patterns of egg white.

somewhat smaller when the ovomucin is removed. Qualitatively the patterns are very similar as no new peaks are observed even in the "high mucin" sample.

TABLE XI.

Comparison of electrophoretic composition of natural, "mucin free", and "high mucin" egg white; pH 7.8, $\Gamma/2 = 0.20$, protein concentration 1.0%. Averages of ascending and descending patterns.

	Natural	Per Cent in "Mucin free"	"High mucin"
Ovalbumin	66.6	66.2	64.5
Ovomucoid	9.5	10.3	10.2
Globulin	9.0	8.1	9.5
Conalbumin	14.2	15.3	15.9

Dependence on pH and ionic strength.

The well established effect of ionic strength on the electrophoretic composition of blood plasma (64,76,131) and the recent report by Cann (25) of a similar effect on the apparent distribution of the ovalbumin component, indicated the need for investigating the composition of egg white over a range of pH and ionic strength values. It has been difficult to obtain satisfactory resolution below pH 5.5 as has been previously mentioned. In only a few

electrophoretic experiments at pH's of 3.9-5.0 has it been possible to satisfactorily separate ovalbumin from the standing boundary anomalies. Longsworth, et. al. (77) assumed that the δ and ϵ -effects were additive and approximately equal in experiments on whole egg white and purified fractions. Several experiments on egg white fractions have been carried out in the same pH range, with the same ionic strength and protein concentration. The results, at least qualitatively, indicate that these assumptions are not valid in the case of whole egg white.

It has been impossible to prevent some precipitation during the preparation of the egg white for electrophoresis below pH 6.5. For these reasons, electrophoretic analyses of whole egg white have been successfully completed only above pH 6.7. The results obtained over a range of pH and ionic strengths on fresh egg white are reported in Table XII. Electrophoretic patterns at four of the pH levels studied are shown in Figure 3. The rather large discrepancies noted between the ascending and descending patterns at the lower pH's can be attributed mainly to poor resolution. The effect of varying the ionic strength at pH 7.7-7.8 is not in agreement with the findings, in other systems, that as the protein to ionic strength ratio approaches zero, the apparent composition of the leading component is decreased.

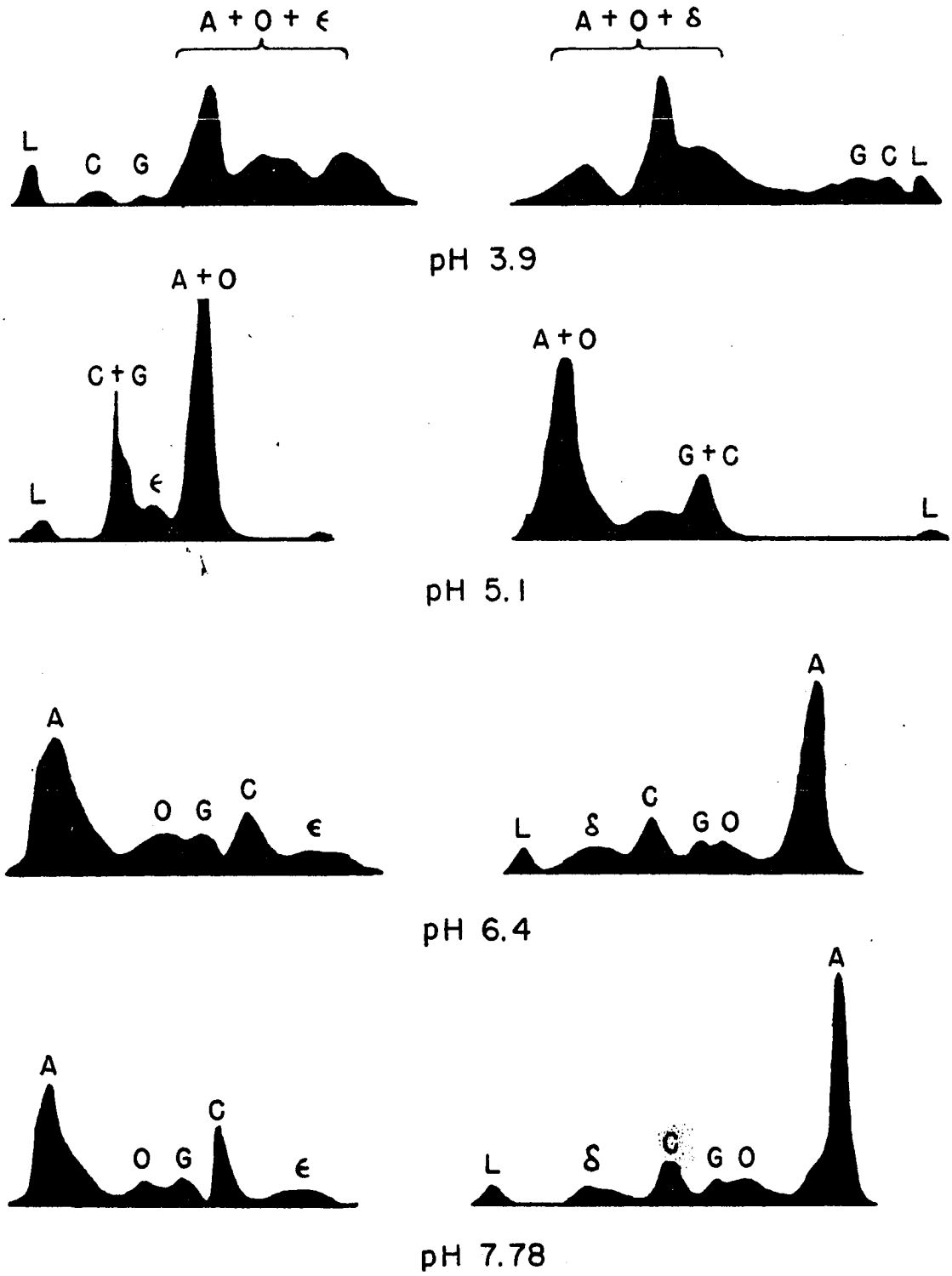


Figure 3. Electrophoretic patterns of egg white at four pH levels.

It is extremely doubtful if the magnitude of the change that is observed is significant. The complexity of the egg white system does not permit any further conclusions to be drawn.

TABLE XII.

The effect of pH and ionic strength on the electrophoretic composition of fresh egg white.

		Per cent									
		Albumin		Ovomucoid		Globulin		Conalbumin		Lysozyme	
pH	Γ/2	Asc.	Desc.	Asc.	Desc.	Asc.	Desc.	Asc.	Desc.	Asc.	Desc.
3.9	0.20	53.4	----	21.2	----	4.8	----	16.5	----	3.8	---
3.9	0.10	60.5	78.0*	14.5	----	6.6	5.3	13.8	----	4.6	3.2
4.5	0.10	71.1*	76.0*	----	----	9.8	5.8	15.0	16.0	4.1	2.2
6.1	0.10	63.6	71.5	8.8	8.2	6.4	3.2	21.2	17.2	---	---
6.6	0.20	63.9	58.8	9.4	10.4	6.6	7.4	16.9	20.4	2.0	3.0
7.8	0.20	66.7	66.5	8.8	10.2	8.8	9.1	14.4	14.0	4.6	2.4
7.8	0.15	66.3	63.4	7.2	10.8	8.7	9.1	14.4	16.7	3.4	2.7
7.8	0.10	61.4	64.7	10.2	9.4	8.4	9.8	15.1	16.1	4.9	3.7
7.8	0.05	61.6	60.6	9.8	12.1	9.0	9.2	19.6	18.0	---	---
8.4**	0.20	67.6	57.7	6.2	9.7	12.4	18.4	13.6	14.3	---	---

* Ovomucoid + Ovalbumin
**Veronal buffer

At pH 3.9 in acetate-chloride buffer, Γ/2 0.20, the differences in the ascending and descending channels are quite marked (Figure 3). All of the proteins of egg white

have been reported as having isoelectric points above 4.0, and as such would be expected to carry a net positive charge at pH 3.9. It is apparent, however, that at least one component is migrating in the opposite direction from the rest of the proteins. This phenomenon has been noted in several experiments, but no satisfactory explanation can be offered. A more critical investigation is suggested but lies outside the scope of the present study.

The influence of ionic strength on the mobility of the egg white proteins is shown in Figure 4. In these experiments the ionic strength due to the phosphate buffer was held constant at 0.05, all changes being made by varying the NaCl concentration. The results are in agreement with those of Koenig, et. al., (64) on the components of bovine plasma, but show even more marked differences than observed in that system. The fact that carefully controlled conditions for routine analytical electrophoresis are required is emphasized by these data.

Genetic differences.

The eggs used in this fractionation study were obtained from the College Poultry Farm where an extensive program of inbreeding is being carried on. If genetic differences,

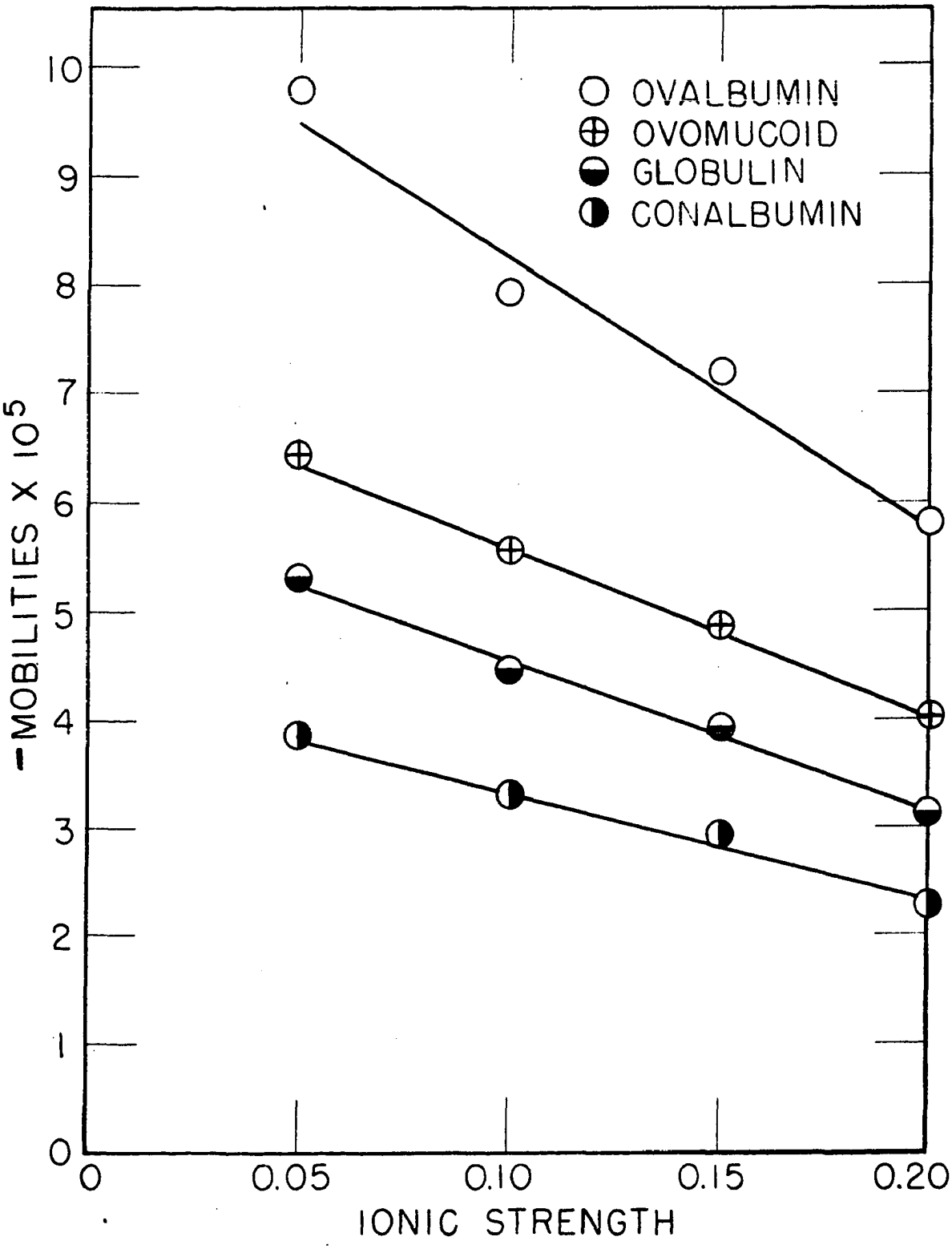


Figure 4. Effect of ionic strength on the electrophoretic mobilities of the egg white proteins.

influencing the character of the egg white protein system, occur, it would be necessary to obtain eggs from the same lot of chickens to compare results. Six groups of chickens of widely different ancestry were chosen to determine what influence inheritable characteristics might have on the electrophoretic composition of the egg white. The six groups chosen for this study were as follows:[1]

Group A Black Australorp
Group B Rhode Island Red
Group C White Leghorn (Inbred line 8)
Group D White Leghorn
Group E Non-deteriorating egg strain
Group F Deteriorating egg strain.

For each electrophoresis experiment the combined whites of at least one dozen eggs were blended. Two or three electrophoresis runs were made on subsequent days on each lot of white. Electrophoresis was carried out in phosphate-chloride buffer, pH 7.8, $\Gamma/2 = 0.20$ (0.15 M NaCl). The electrophoretic results obtained are shown in Table XIII. The large discrepancies in the concentration of ovomucoid and globulin can be accounted for largely by the difficulty

[1] Eggs from the first four groups of chickens were supplied through the courtesy of Dr. A. W. Norkskog of the Iowa State College Poultry Husbandry Department. The two groups of eggs described as deteriorating and non-deteriorating were supplied through the generosity of the U.S.D.A., Agricultural Research Center, Beltsville, Maryland. The non-deteriorating eggs are characterized by a high degree of thick white retention when held at high storage temperatures.

TABLE XIII.

Electrophoretic composition of egg whites of various strains of chickens. Reference electrophoretic conditions.

Group	% Albumin		% Ovomucoid		% Globulin		% Conalbumin	
	Asc.	Desc.	Asc.	Desc.	Asc.	Desc.	Asc.	Desc.
A	67.5	63.5	5.8	12.7	12.1	9.1	14.6	14.9
A	66.5	66.9	9.8	10.4	9.8	10.1	13.8	13.8
A	64.8	67.0	9.2	13.9	9.6	7.4	16.4	11.6
Ave.	66.2	65.8	8.3	12.3	10.5	8.9	14.9	13.4
B	61.6	67.3	10.8	10.0	12.1	10.3	15.5	12.4
B	66.3	66.5	11.4	10.5	10.3	9.8	12.1	13.2
B	70.2	65.3	5.9	12.1	9.1	8.7	14.2	13.9
Ave.	66.0	66.4	9.4	10.9	10.5	9.6	13.9	13.2
C	70.7	71.4	9.8	11.0	6.7	8.9	12.9	8.9
C	70.6	69.5	10.5	9.9	7.2	8.7	11.8	11.9
C	68.7	67.9	10.4	12.5	7.5	9.8	13.0	9.8
Ave.	70.0	69.6	10.2	11.1	7.1	9.1	12.6	10.2
D	70.6	66.8	6.9	9.1	9.5	11.9	14.4	12.3
D	65.8	66.4	11.5	10.2	8.3	11.4	14.1	11.9
D	68.3	70.8	9.3	7.4	8.5	10.3	12.9	11.5
Ave.	68.2	68.0	8.2	8.9	8.8	11.2	13.8	11.9
E	63.5	62.8		10.7		9.7	12.4	14.4
E	63.4	63.7	7.9	10.4	9.1	9.7	15.7	16.2
Ave.	63.4	63.2		10.5		9.7	14.0	15.3
F	66.5	65.5	8.6	8.9	10.3	10.7	11.6	13.8
F	63.7	63.2	10.0	11.1	9.3	9.5	13.7	16.2
Ave.	65.1	64.3	9.3	10.0	9.8	10.1	12.6	15.0
Ave.	66.5	66.2	9.3	10.6	9.3	9.8	13.6	13.2

of resolution in this area. No significant differences can be detected in the protein constituents of the egg white of these groups. It is felt that this problem is worthy of further study when fractionation techniques are available which will permit investigation of purified fractions.

Effect of age of eggs.

Procurement of eggs of known history for fractionation experiments often involves considerable difficulty. If the fractionation of the egg white proteins is ever undertaken on a pilot plant or larger scale operation, this would become an important consideration. Various previously mentioned investigations have indicated that changes in the egg white protein do occur during storage periods. MacPherson, Moore, and Longsworth (84) have shown that there is a gradual transformation of A_1 into A_2 during prolonged storage. Sørensen and Høyrup (123) observed that the yield of crystalline ovalbumin decreases with increased age of the eggs. This experiment was designed to determine whether any such modifications could be detected by electrophoretic analysis of whole egg white.

A series of electrophoresis runs was made at several pH's on eggs stored for fourteen days at 37°C. This

TABLE XIV.

Effect of age of the egg on the electrophoretic analysis of the egg white.

	pH	Buffer	μ/2	NaCl M	P%	% Albumin Asc.	% Albumin Desc.	% Ovomucoid Asc.	% Ovomucoid Desc.	% Globulin Asc.	% Globulin Desc.	% Conalbumin Asc.	% Conalbumin Desc.	% Lysozyme Asc.	% Lysozyme Desc.
F	3.9	Ac	0.1	0	1.50	60.5	78.0*	14.5	----	6.6	5.3	13.8	13.4	4.6	3.2
F	3.9	Ac	0.2	0.15	1.00	53.4	----	21.2	----	4.8	---	16.5	----	3.8	---
S	3.9	Ac	0.1	0	1.50	72.6	75.0	10.3	----	10.3	8.4	5.6	6.4	---	---
F	4.5	Ac	0.1	0	1.50	71.1*	76.0*	----	----	9.8	5.8	15.0	16.0	4.1	2.2
S	4.5	Ac	0.2	0.1	0.75	76.5*	82.4*	----	----	11.8	4.3	11.5	13.4	---	---
F	6.1	Ph	0.1	0	1.50	63.6	71.5	8.8	8.2	6.4	3.2	21.2	17.2	---	---
F	6.6	Ph	0.2	0.15	1.00	63.9	58.8	9.4	10.4	6.6	7.4	16.9	20.4	2.0	3.0
S	6.4	Ph	0.2	0.15	1.00	60.2	62.2	11.2	12.1	8.4	9.2	16.1	16.8	4.0	---
S	6.1	Ph	0.1	0	1.50	90.5	78.5	2.6	9.8	6.7	3.4	5.9	6.0	---	---
S	6.1	Ph	0.2	0.1	0.75	68.8	78.5	10.6	8.2	6.6	4.4	16.5	8.8	---	---
F	7.8	Ph	0.1	0.04	1.50	65.5	69.1	6.6	7.2	9.0	7.2	18.8	16.4	---	---
F	7.8	Ph	0.1	0	1.50	58.8	73.4	12.1	8.6	9.2	7.0	19.8	10.9	---	---
F	7.8	Ph	0.2	0.1	0.75	70.3	76.3	13.5	6.2	5.9	6.6	10.3	10.7	---	---
F	7.7	Ph	0.2	0.15	1.00	71.5	63.0	6.1	7.5	6.5	8.6	13.0	17.9	3.0	3.0
S	7.7	Ph	0.2	0.15	1.00	65.8	64.6	8.5	10.8	7.0	9.0	14.1	15.7	4.5	---

Ac - Acetate
Ph - Phosphate
F - Fresh
S - Stored
*Albumin + Ovomucoid
P - Protein

storage period was sufficient to cause complete breakdown of the thick white. The results of these experiments are reported in Table XIV. The results of the analyses at the low pH's must be considered as doubtful in view of the difficulties previously mentioned.

Comparison of the results from the stored eggs with those given in Tables X and XII for fresh eggs indicate there is no modification in the egg white that can be determined electrophoretically. The results of this study are construed as additional evidence for the hypothesis that the destruction of the thick white gel results from the disintegration of ovomucin fibers. During the blending process prior to electrophoresis any fibers present would be cut into smaller fragments. If this mechanical disintegration gives a product similar to that resulting from the natural deterioration of the thick white, no difference would be expected. If this is the case, the addition or removal of 1-2% of ovomucin as described in the earlier experiment could probably not be detected electrophoretically. This is also substantiated by the previously reported observation (45) that there was no detectable electrophoretic difference in the three layers of egg white.

Miscellaneous Supplemental Experiments

Carbohydrate in egg white and its removal.

During the course of preliminary fractionation experiments it appeared that the carbohydrate content of the fractions, and more especially the carbohydrate to nitrogen ratio, would yield more information as to the nature of the fraction obtained than nitrogen alone. Such information would be of value in supplementing electrophoretic evidence for identification and purity of the fractions.

In the diphenylamine analytical procedure employed, any polysaccharides were hydrolyzed and only hexoses were determined. Since egg white contains 0.45% free glucose (119), several experiments were carried out to determine if this carbohydrate could be readily removed by dialysis. It was apparent that if the hexose in a fraction was to be used for identification purposes, the free hexose would have to be first removed. An alternative to this procedure was to determine the total hexose in a fraction, precipitate the proteins with trichloroacetic acid and then analyze

for the hexose remaining in solution. The difference would then give the amount of protein bound hexose.

Egg white was diluted twenty-five fold, placed in 1/2 inch diameter Visking tubing, and dialyzed against 0.86% NaCl. Hexose and nitrogen were determined at intervals in the total dialyzate, the protein then removed by the addition of an equal volume of 10% trichloroacetic acid, and hexose and nitrogen determined in the filtrate. Typical results are given in curve 2 of Figure 5.

A 500 ml. sample of undiluted egg white was dialyzed in a large Visking tubing (2 1/3 inch diameter) against one liter of 0.86% NaCl. Hexose and nitrogen were determined at intervals. The results are shown in curve 1, Figure 5. The decrease in rate of dialysis is due partially to the formation of a slimy ovomucin film on the dialysis membrane.

It is apparent from these results that the removal of free hexose from the diluted egg white can be accomplished in a relatively short period of time. The efficiency of the dialysis could be materially increased by increasing the membrane surface, by improved agitation, and by constantly changing the diffusate. Considerable nitrogen remains in the trichloroacetic acid filtrate and is believed to be primarily ovomucoid nitrogen as it is known that

this protein is not completely precipitated by this reagent.

The hexose/nitrogen ratio of the whole egg white, 0.484, is in satisfactory agreement with that reported by Sørensen (119), 0.466. After apparent equilibrium was established in the dialysis (curve 2), a ratio of 0.21 was obtained. Sørensen also reported 0.21 for this figure. On this basis the total hexose (as glucose) in whole egg white is 3.62 mg./ml., the free hexose is 4.22 mg./ml. The hexose content of three times recrystallized ovalbumin was found to be 1.9% in agreement with earlier investigations (96, 119).

With the dialysis equipment available it is not feasible to attempt removal of free carbohydrate from egg white prior to starting fractionation. It can be removed readily, however, from small samples for analytical purposes. The procedure involving the trichloroacetic acid precipitation is not satisfactory because of the incomplete removal of the protein.

Study of interaction between some purified proteins of egg white.

In egg white, at its natural pH of 7.8-8.0, one protein, lysozyme, is on the acid side of its isoelectric point and the remaining proteins are on the alkaline side

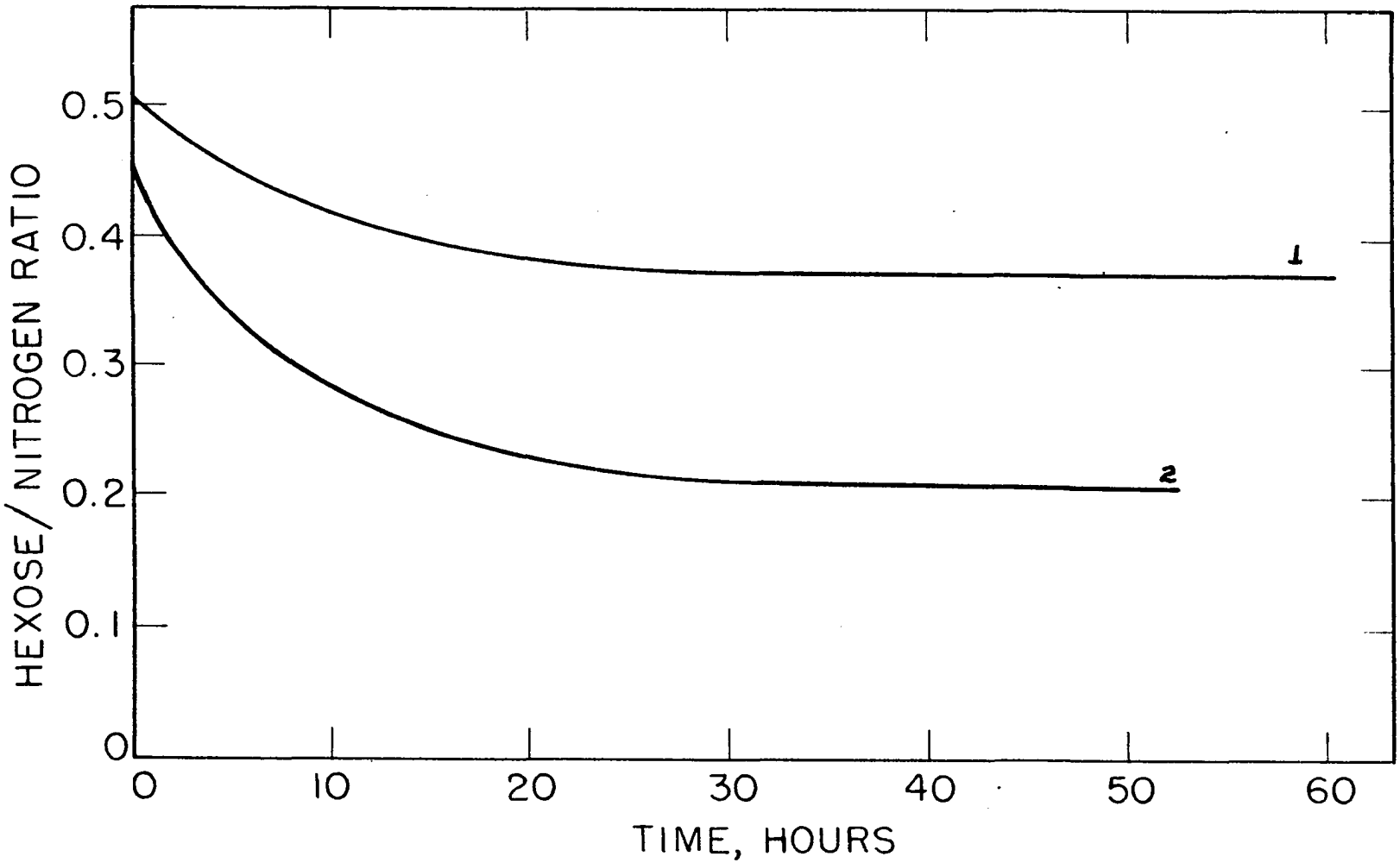


Figure 5. Removal of hexose from egg white by dialysis.

of their isoelectric points. As was previously pointed out, the presence of oppositely charged proteins may lead to extensive interaction. If such interactions do exist they might influence the electrophoretic analysis of the fractions as well as the fractionation efficiency.

Certain combinations of the egg white proteins were examined electrophoretically for evidences of protein-protein interaction. Interaction usually results in marked asymmetries of the electrophoretic patterns and wide variations from the calculated relative composition of the system. The following proteins were available and used for this experiment:

Lysozyme	3x recrystallized, electrophoretically homogeneous
Conalbumin	75% conalbumin, 16% ovalbumin, remainder unidentified
Ovalbumin	3x recrystallized, electrophoretically homogeneous
Ovomucoid	83% ovomucoid and 17% conalbumin

Lysozyme solutions were prepared by dissolving the protein in 0.1 N acetate buffer, pH 4.5. The standard electrophoretic buffer (phosphate-chloride, pH 7.8, $\Gamma/2$ = 0.20) was used to dissolve the other proteins. Appropriate mixtures were prepared and dialyzed against the standard electrophoretic buffer prior to electrophoresis. Electrophoresis was continued for 7-10,000 seconds under a potential gradient of 6.0 volts-cm.$^{-1}$. The initial boundary

was moved to the center of the cell before current was applied so that the components moving in opposite directions could be observed. The results from the electrophoretic runs are given in Table XV. The electrophoretic patterns are shown in Figure 6.

TABLE XV.

Electrophoretic analyses of some mixtures of purified egg white proteins.

Mixture	Mobilities x10^5		Concentration Per cent		Ratio	
	Lysozyme	Other	Lysozyme	Other	Obs.	Calc.
Lysozyme-Conalbumin	+2.45	-2.15	14.7	71.3	0.17	0.24
Lysozyme-Ovalbumin	+1.70	-5.90	4.5	91.0	0.05	0.05
Lysozyme-Ovalbumin	+2.00	-5.90	46.7	52.0	1.1	1.1
Lysozyme-Ovomucoid	+3.04	-2.90	47.1	52.9	1.1	1.1

These results give no indication of interactions in the mixtures studied. The ratios as calculated and observed agree closely. The mixtures were prepared so that the ratios are nearly those that exist in normal egg white with the exception of the one lysozyme-ovalbumin solution. The electrophoretic patterns are normal in that no marked asymmetries

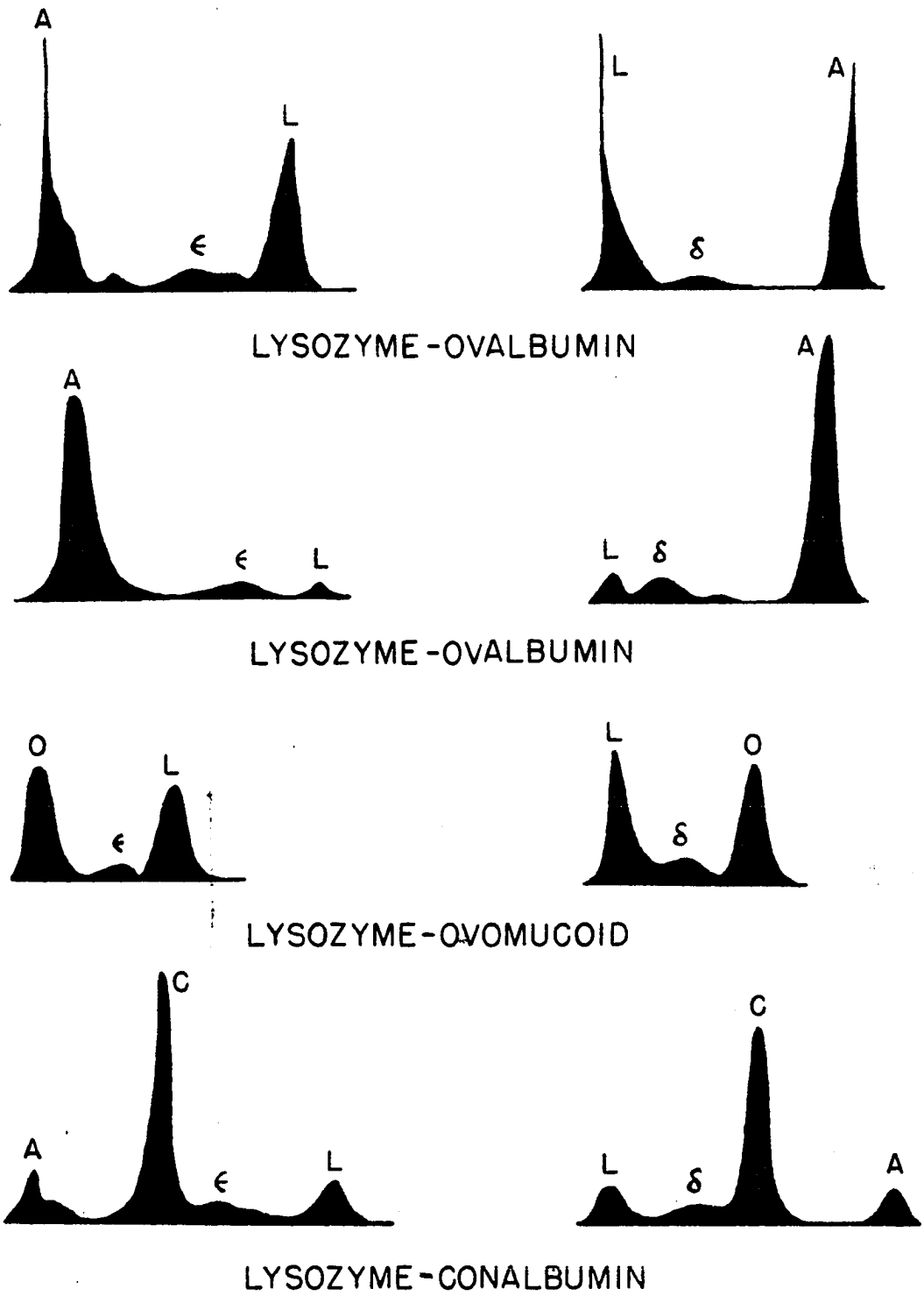


Figure 6. Electrophoretic patterns of some artificial mixtures of egg white proteins.

are noted. While these results do not indicate any interactions, it can not be concluded that there are none in the egg white system under other conditions.

Separation of the Protein Fractions

In the presentation of results and discussion of the fractionation experiments it has been difficult to follow rigidly any single system of naming the fractions obtained. It was convenient during the course of the experimental work to label all fractions with a series of numbers and letters in the order in which they were removed. Due to many modifications, however, a similar fraction may not have the same designation in different experiments, e.g., the fraction consisting primarily of ovalbumin has been called Fraction III in some experiments and Fraction IV in others. A system of nomenclature, on the other hand, in which fractions are labelled by giving them the name of the primary protein constituent is not altogether satisfactory both because of its cumbersomeness and the fact that several fractions consist of about equal amounts of several proteins when first removed. As a result of these difficulties a combination of these nomenclature procedures has been used. In all of the flow sheets the

fractions have been labelled with a series of numbers and letters. This procedure has been devised so that it is immediately apparent whether the fraction is a precipitate or a supernatant. When a fraction is removed from egg white it is given a Roman numeral, for example, III; if this fraction, obtained as a precipitate, is suspended in a solvent which dissolves only part of the fraction, that portion which dissolves is labelled III-2 and the insoluble portion is III-1. If that fraction labelled III-2 is now further fractionated and two portions obtained, the precipitate is called III-2-A and the supernatant is III-2-B. If fraction III-2-B is further fractionated, the precipitate becomes III-2-B-α and the supernatant, III-2-B-β. Thus a precipitate is always given the lower symbol, be it number or letter, and the supernatant the higher.

In the text it has seemed advisable to discuss separately the removal and purification of the protein constituents in the approximate order in which each was removed from egg white.

The symbols which have been used throughout this discussion and in the various figures are as follows:

$\Gamma/2$ = ionic strength

N_2 = mole fraction of ethanol

N = nitrogen in grams/liter except when indicated otherwise

T = degrees Centigrade

$\rightarrow 0$ = indicates the value is approaching zero during dialysis.

All analytical figures for the various proteins in a fraction refer to the grams of nitrogen present. Conversions to a protein basis have not been attempted.

Ovomucin fraction

The first attempts to remove the ovomucin fraction followed the classical procedures previously described. The ionic strength of homogenized egg white was lowered to less than 0.01 by dilution with water or dialysis and the pH adjusted to 6.0. In all cases a heavy, ropy precipitate was obtained upon centrifuging. It was observed that if, prior to centrifuging, the dialysis bag containing the suspended precipitate was carefully transferred to a solution of 0.86% NaCl and dialysis continued, all of the protein redissolved. After removal, however, the precipitate was insoluble in NaCl solutions (0.15-1.0 M) at pH's from 6.5-10.5. It was not dispersed by 10% urea, 1% sodium dodecylbenzenesulfonate, 1% Zepharin (a quarternary ammonium detergent) or reducing agents such as 5% thioglycolic acid and sodium bisulfite. It was extremely difficult to wash other proteins from this precipitate. A positive biuret test was obtained after ten washings with

0.86% NaCl. Electrophoretic analyses of the precipitate were impossible due to its insolubility and analyses of the supernatant gave no indication of the specific removal of any protein. A variable amount of protein was removed in this procedure, usually about 10%. On this basis, it was concluded that this procedure was not satisfactory for the removal of ovomucin.

Since it has been observed that the thick white contains practically all of the ovomucin, and that the outer thin white is formed by the extraction of the soluble proteins by the uterine fluids, an experiment was designed to attempt concentration of the ovomucin by extraction of the thick white with a buffer solution similar to the uterine fluid. One dozen eggs were broken, the yolks separated from the whites, and the thin white removed from the thick with a pipet. The volume of the thick white obtained was 182 ml. The thick white was placed in a two liter flask, covered with 182 ml. of 0.86% NaCl and agitated gently. After twenty-four hours the extracting liquid was removed and added to the thin white. This was repeated four times, after which it was very difficult to remove any further extracting liquid from the thick white. Table XVI shows the results obtained. These results show that 22.7% of the nitrogen remains in the thick white after extraction.

Electrophoretic analyses indicated no differences in the extracted thick white and the thin white plus extracting

TABLE XVI.

Extraction of proteins from the thick white with 0.86% NaCl.

	Grams Nitrogen in	
	Thick White	Thin White + extracting liquid
Before extraction	3.42	2.72
After extraction	0.78	4.69

liquid. The primary limitations in this method of extraction were the disintegration of the thick white so that the extracting liquid could not be completely removed and the dilution of the proteins which made any further fractionation difficult. A second experiment was designed to overcome these difficulties.

Egg white was divided into three lots. Lot A was untreated, Lot B was homogenized, and Lot C was blended in a Waring Blender. Each lot was placed in a bag made of four layers of cheesecloth and "dialyzed" against phosphate-chloride buffer (pH 7.8, $\Gamma/2 = 0.20$). The diffusate was removed at intervals, replaced with fresh buffer, and analyzed for nitrogen. The total amount of nitrogen

diffused through the bag was calculated after measuring the volume of the diffusate. The data obtained are recorded in Table XVII. After the dialysis was stopped it was impossible to remove the egg white completely due to the formation of a typical ovomucin slime inside the bags.

This experiment again indicated that the soluble proteins could not be satisfactorily extracted from the thick white gel. While most of the protein diffused through the cheesecloth bags, there was no apparent selective retention and it was impossible to remove the remaining protein completely from inside the bags.

TABLE XVII.

Removal of protein from thick white by "dialysis" through cheesecloth bags.

		Per cent nitrogen in diffusate of that originally present in	
Time, Hours	Lot A	Lot B	Lot C
3	41.5	81.3	86.1
6	52.2	85.4	93.3
12	59.8	85.8	94.4
22	65.7	86.2	94.9
48	70.8	--	--
72	71.3	--	--

Table XVII shows that the relative rate of "dialysis" increased in the order: untreated, homogenized, blended. This may be partially explained by the assumption that the

ovomucin fibers are smaller when blended which permits a more rapid passage of the protein to the diffusate. This appears to substantiate Slosberg's contention (116) that blending causes a greater disintegration of the ovomucin fibers than homogenizing.

On the basis of the above experiments it was concluded that the ovomucin had not been removed in the dilution experiments because of its insolubility at low ionic strengths, but rather because it was actually insoluble before any treatment was given the egg white. The removal resulted from the precipitation of a portion of the globulin fraction and the subsequent occlusion and clotting of the ovomucin in the precipitate.

It was postulated, therefore, that if sufficient centrifugal force was applied to untreated egg white, the ovomucin might be removed without any additional treatment. To test this hypothesis, 250 ml. of homogenized or blended egg white was centrifuged, batch wise, in the supercentrifuge at 45,000 x G for fifteen minutes. After completion of the centrifugation a slimy, ropy precipitate, characteristic of ovomucin, was recovered from the centrifuge bowl. The precipitate could be removed from the bowl and washed only in a semi-quantitative manner but some typical results are given in Table XVIII.

TABLE XVIII.

Removal of an egg white fraction by super-centrifugation.

Experiment	Percent nitrogen of original	Percent total solids of original
1	0.34	--
2	1.2	1.2
3	1.0	1.0
4	1.1	--
5	1.1	1.1

This fraction has been called the ovomucin fraction but no electrophoretic analyses have been possible due to its insolubility. The data above indicate somewhat less ovomucin in egg white than has been previously reported. It is felt that the figures reported here may be more reliable because no other protein has been rendered insoluble and precipitated along with the ovomucin. The difficulty of removing other proteins once they have been precipitated has already been discussed.

A qualitative difference between ovomucin removal in homogenized and blended egg white was observed. It was easier to remove the supernatant from the super-centrifuge when homogenized egg white was used. With blended egg white, particles of ovomucin appeared in the supernatant and centrifugation usually had to be repeated.

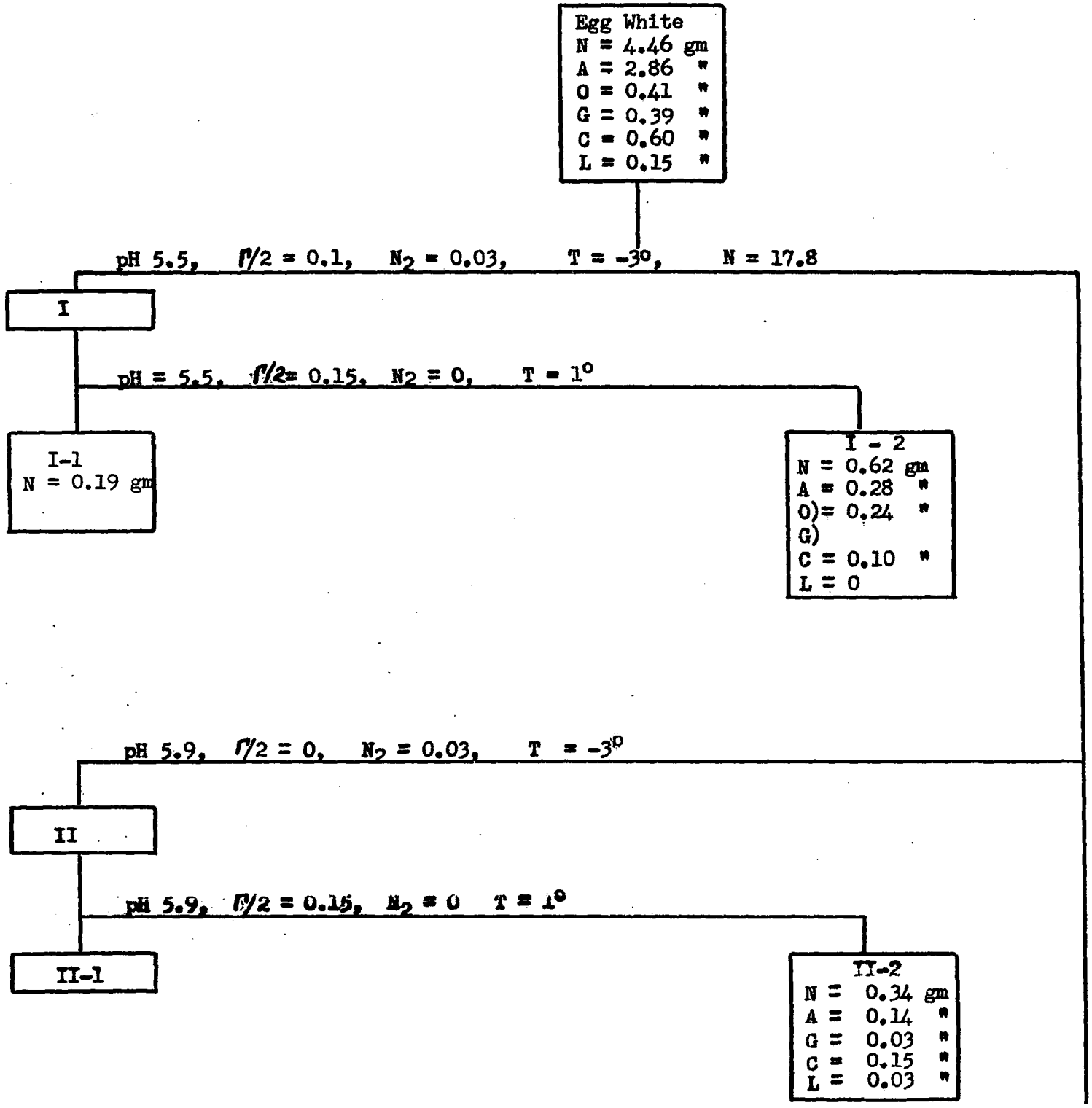
This finding supports the results on the particle size of the ovomucin fibers reported in the extraction experiments.

This method of removal of the ovomucin fraction has several advantages. In subsequent steps in the fractionation procedures considerably less insoluble material was obtained and yields of the soluble fractions were increased. An ovomucin fraction is recovered which is probably more pure than previously obtained. This procedure has been adopted as the first step in the fractionation technique. See Fraction I of Figures 11, 12, 13, 14 and 15.

Lysozyme fraction

The removal of the lysozyme from egg white has followed the general plan described by Alderton, Ward and Fevold (6) for the direct crystallization from egg white. In their procedure, NaCl is added to the egg white to give a final concentration of 5% and the pH is adjusted to 9.0-9.5. Lysozyme crystallizes directly from the egg white upon standing 48-72 hours. Seed crystals were added to initiate crystallization.

Two procedures have been followed in removing the lysozyme fraction from egg white in this comprehensive fractionation scheme. From the standpoint of preventing interactions between oppositely charged proteins it appeared


Egg White
N = 4.46 gm
A = 2.86 "
O = 0.41 "
G = 0.39 "
C = 0.60 "
L = 0.15 "
pH 5.5, Γ/2 = 0.1, N2 = 0.03, T = -3°, N = 17.8
I
pH = 5.5, Γ/2= 0.15, N2 = 0, T = 1°
I-1
N = 0.19 gm
I - 2
N = 0.62 gm
A = 0.28 "
O)= 0.24 "
G)
C = 0.10 "
L = 0
pH 5.9, Γ/2 = 0, N2 = 0.03, T = -3°
II
pH 5.9, Γ/2 = 0.15, N2 = 0 T = 1°
II-1
II-2
N = 0.34 gm
A = 0.14 "
G = 0.03 "
C = 0.15 "
L = 0.03 "

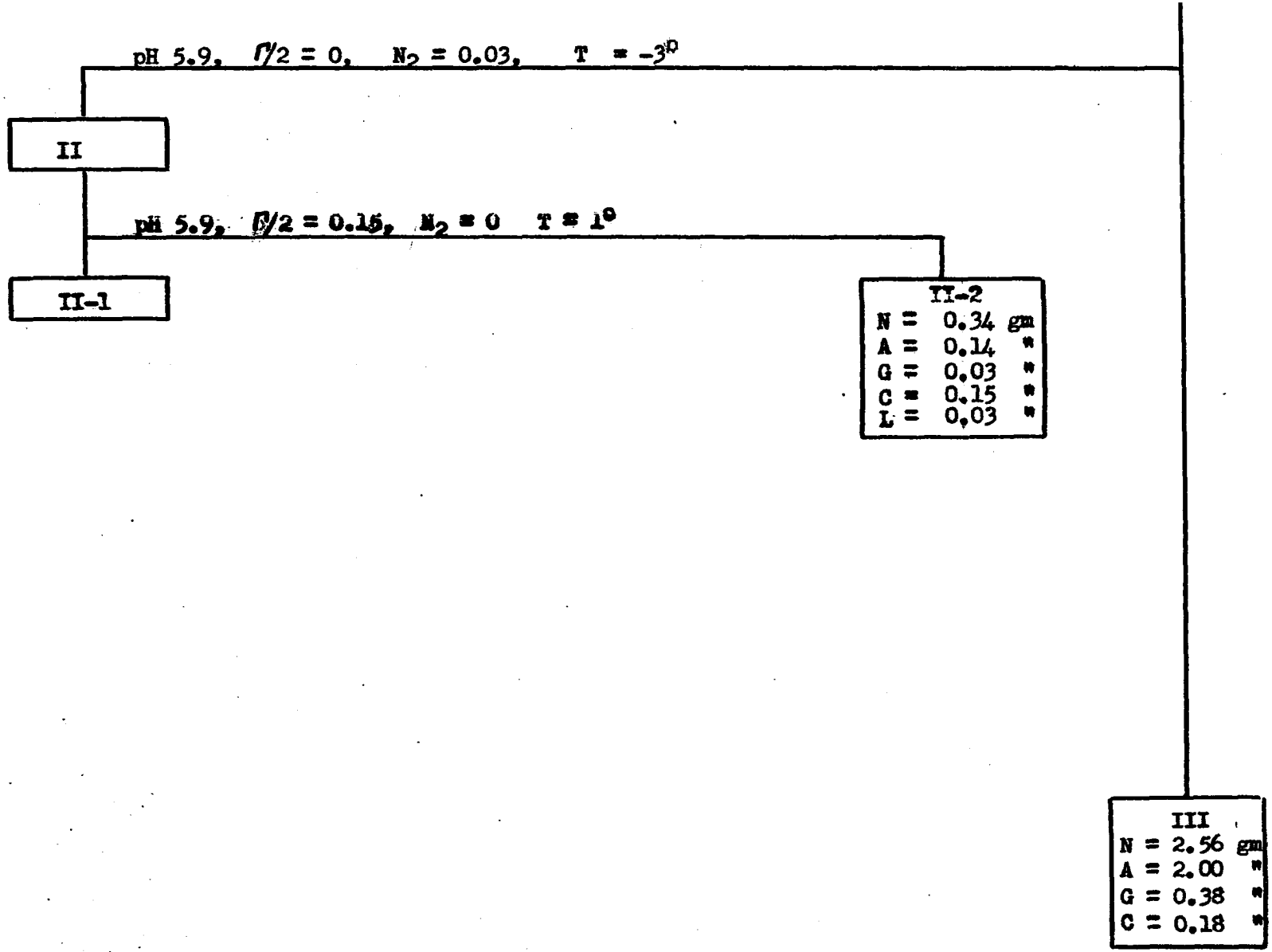
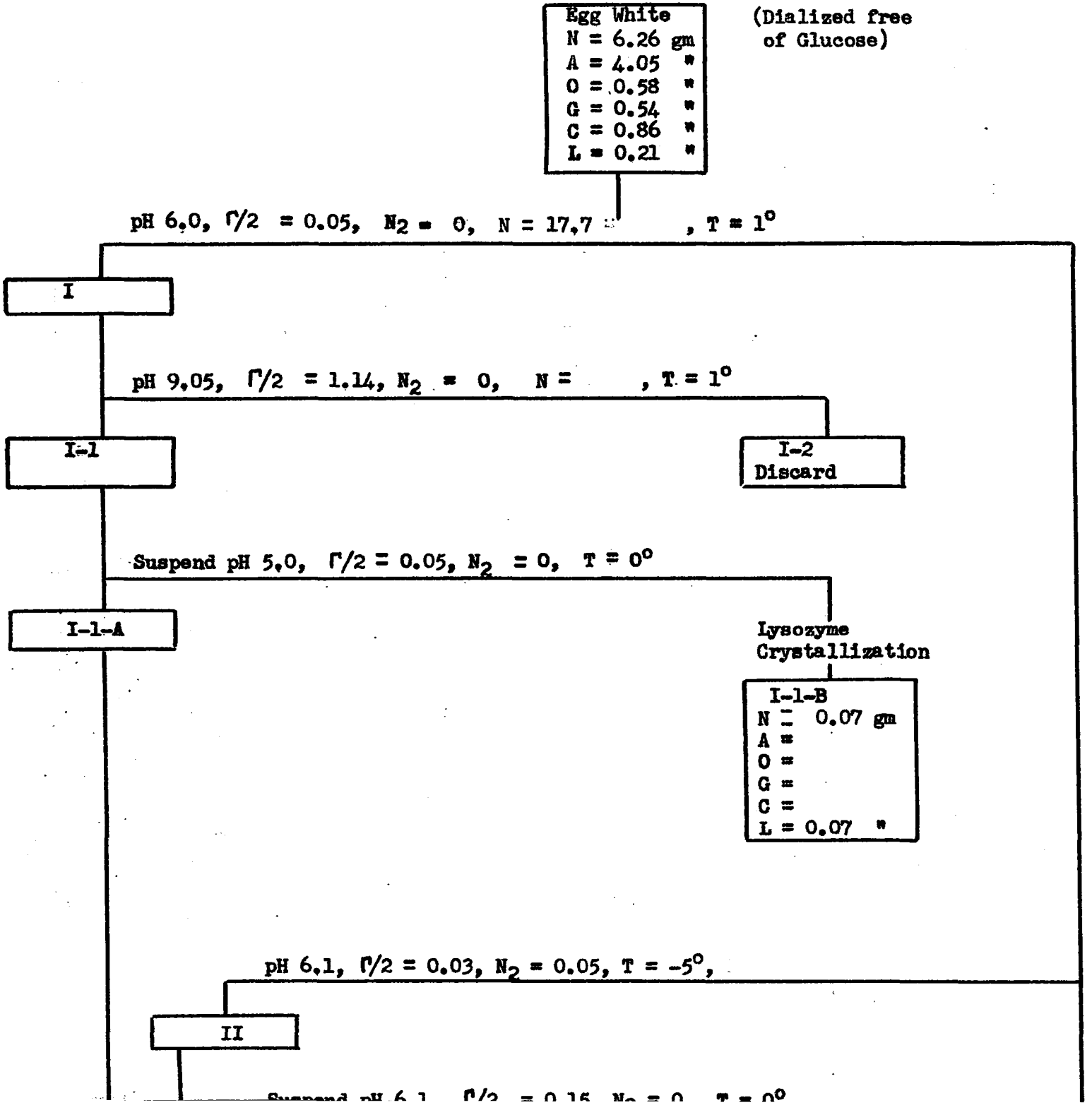


Figure 7. Flow Sheet F-5. This and the following eight figures have been prepared so that precipitates are at the left and supernatants at the right. The data enclosed in boxes are the analytical results for the fraction in grams. N, total nitrogen; A, ovalbumin; O, ovomucoid; G, globulin; C, conalbumin; L, lysozyme. The conditions under which the fractions were removed are given on the horizontal tie lines.


Egg White
N = 6.26 gm
A = 4.05 "
O = 0.58 "
G = 0.54 "
C = 0.86 "
L = 0.21 "
(Dialized free of Glucose)
pH 6.0, Γ/2 = 0.05, N2 = 0, N = 17.7 , T = 1°
I
pH 9.05, Γ/2 = 1.14, N2 = 0, N = , T = 1°
I-1
I-2
Discard
Suspend pH 5.0, Γ/2 = 0.05, N2 = 0, T = 0°
I-1-A
Lysozyme
Crystallization
I-1-B
N = 0.07 gm
A =
O =
G =
C =
L = 0.07 "
pH 6.1, Γ/2 = 0.03, N2 = 0.05, T = -5°,
II

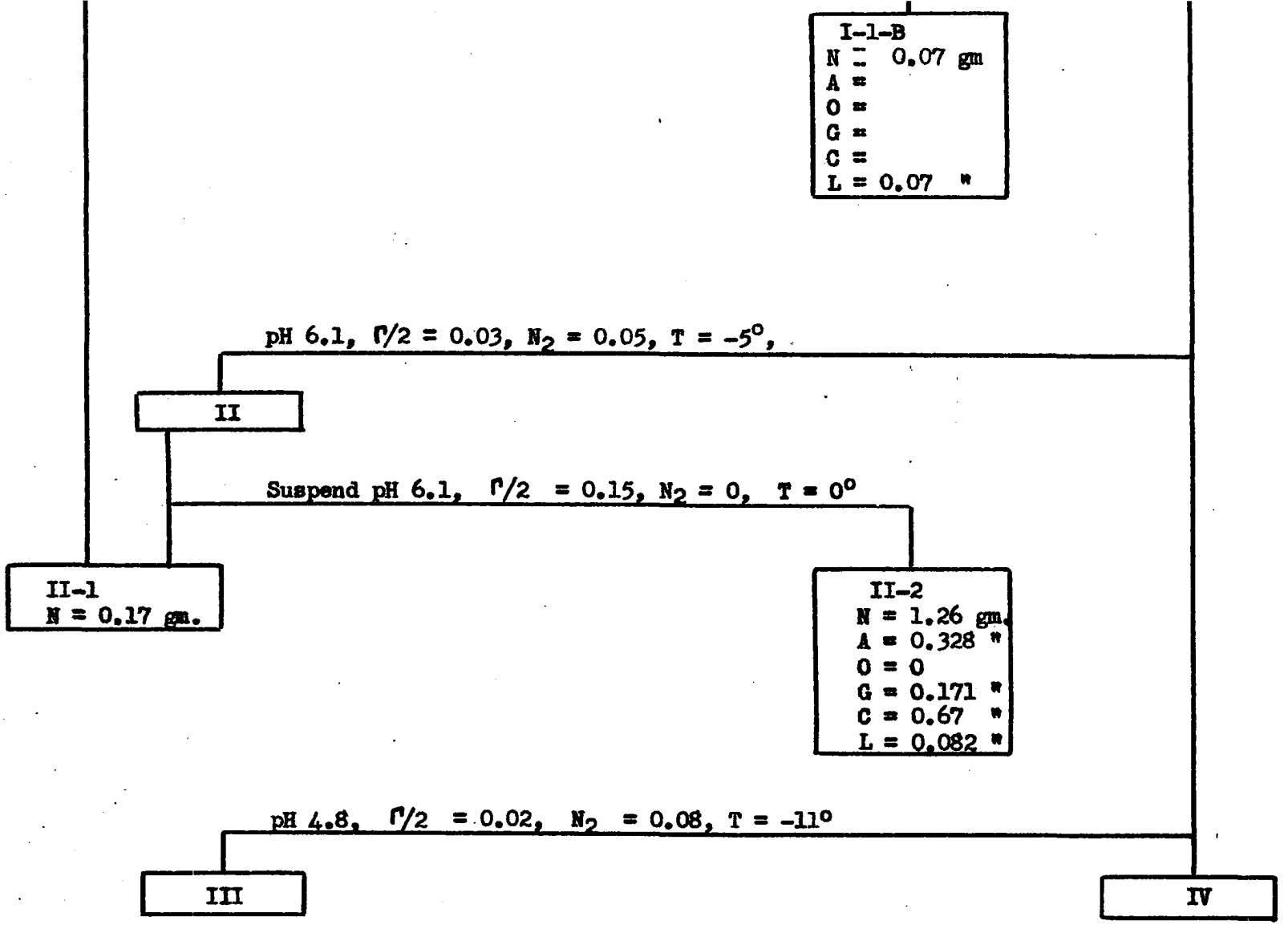
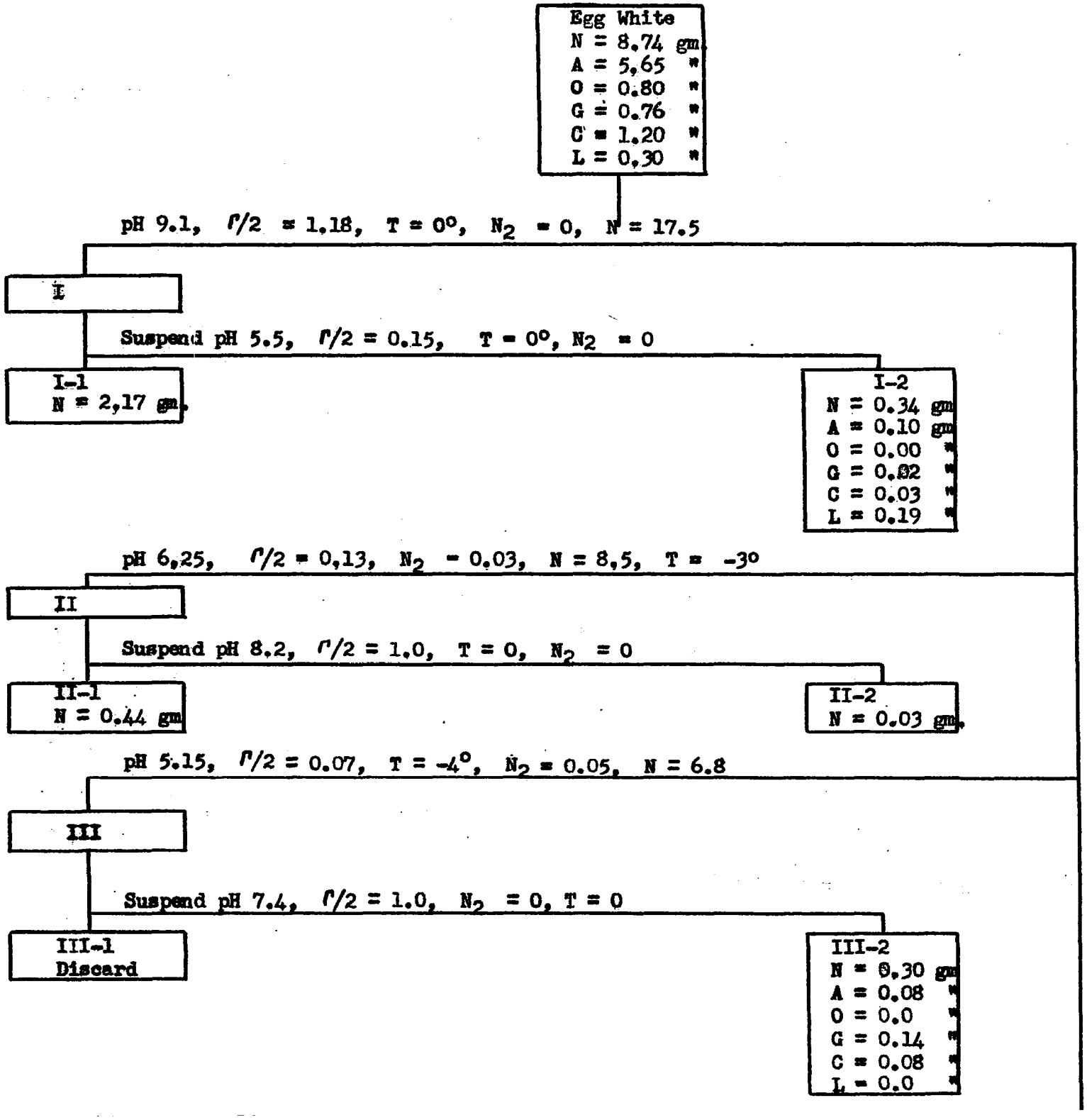


Figure 8. Flow Sheet F-6.


Egg White
N = 8.74 gm.
A = 5.65 "
O = 0.80 "
G = 0.76 "
C = 1.20 "
L = 0.30 "
pH 9.1, Γ/2 = 1.18, T = 0°, N2 = 0, N = 17.5
I
Suspend pH 5.5, Γ/2 = 0.15, T = 0°, N2 = 0
I-1
N = 2.17 gm.
I-2
N = 0.34 gm
A = 0.10 gm
O = 0.00 "
G = 0.02 "
C = 0.03 "
L = 0.19 "
pH 6.25, Γ/2 = 0.13, N2 = 0.03, N = 8.5, T = -3°
II
Suspend pH 8.2, Γ/2 = 1.0, T = 0, N2 = 0
II-1
N = 0.44 gm
II-2
N = 0.03 gm.
pH 5.15, Γ/2 = 0.07, T = -4°, N2 = 0.05, N = 6.8
III
Suspend pH 7.4, Γ/2 = 1.0, N2 = 0, T = 0
III-1
Discard
III-2
N = 0.30 gm
A = 0.08 "
O = 0.0 "
G = 0.14 "
C = 0.08 "
L = 0.0 "

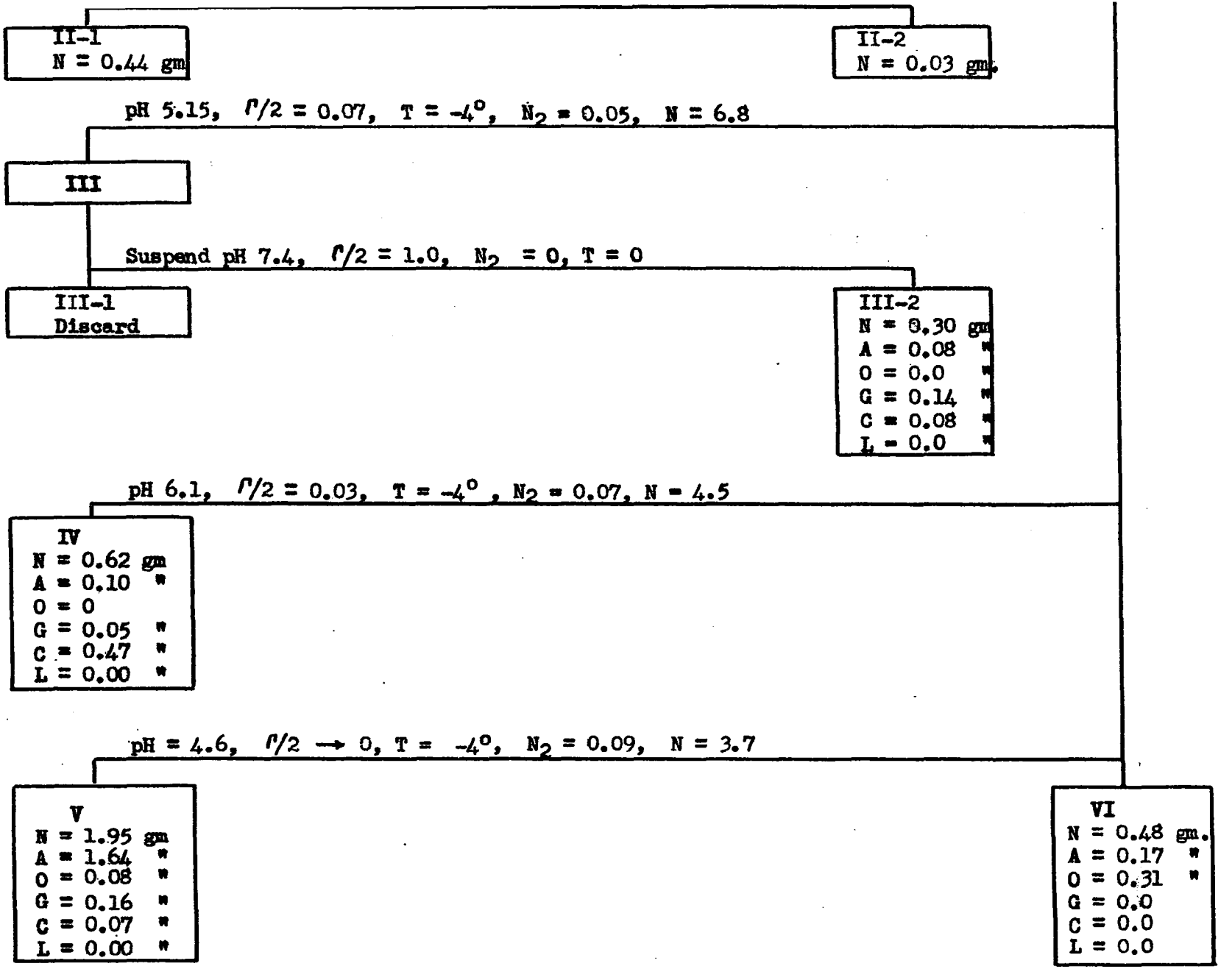


Figure 9. Flow Sheet F-7

desirable to remove the lysozyme fraction first. The supernatant resulting from the lysozyme precipitation was not entirely satisfactory, however, for further fractionation work. Sodium chloride concentrations were too high to permit further fractionation so that a prolonged dialysis was required. Dilution techniques could be employed to reduce the salt concentration, but undesirable protein dilutions also result. The high pH at which the lysozyme precipitations were carried out may be harmful to the other proteins. The time involved in the lysozyme crystallization greatly prolonged the fractionation procedure as no further work was possible until that fraction was removed. In spite of these difficulties, several fractionations have been carried out successfully in which lysozyme has been the first or second fraction to be removed.

The second procedure has been to remove the conalbumin-globulin fraction as will be described in the next section and then to crystallize the lysozyme from the appropriate sub-fractions thus obtained. This method has the disadvantage that interactions are possible during the precipitation and the inclusion of more impurities may result. It has the advantages, however, that large amounts of salt do not have to be removed, all the proteins are not

subjected to high pH's for long periods of time, and the fractionation time can be materially decreased.

In Experiment F-7 (Figure 9), the pH of homogenized egg white was adjusted to 9.1, the ionic strength brought to 1.18 with solid NaCl and the lysozyme allowed to crystallize. Crystallization was observed within twenty-four hours. A fraction (I-2) was obtained which contained 56% lysozyme and accounted for 61% of the original lysozyme in the egg white. The yield figures as calculated here may be somewhat too low because the electrophoretic data for the concentration of lysozyme in egg white are somewhat higher than other reports. Difficulties and errors involved in the electrophoretic analyses for lysozyme in egg white have previously been discussed.

Lysozyme removal in Experiments F-8 and F-9 (Figures 10 and 11) was attempted in the same manner as just described. In F-8 the egg white was blended instead of homogenized, and in F-9 the egg white was blended and the ovomucin fraction removed in the super-centrifuge. In both experiments crystallization was observed within twenty-four hours and the precipitate immediately removed. The crystal form in these experiments was different than in the previous one. The predominant crystal form was a rectangular platelet and upon recrystallization sphero-crystals were

observed. Nitrogen analyses on these fractions (I in Figure 10, II in Figure 11) indicated that only a very small fraction of the original nitrogen had been precipitated. It was apparent that the crystals observed were not lysozyme. Schaible and Bandemer (112) had reported the presence of crystals of a protein calcium phosphate in natural egg white. Positive qualitative tests for calcium and phosphate ions were obtained on the crystalline material in these experiments. Nitrogen was present but it was probably occluded in the precipitate and does not necessarily indicate that protein is an integral part of the crystal.

The explanation for the presence of these crystals is somewhat obscure. First, it appears that when the ovomucin fibers are cut into smaller fragments by blending or removed in the super-centrifuge, the lysozyme crystallizes more slowly. A possible explanation is that ovomucin particles serve as nuclei which facilitate crystallization in untreated egg white. In normal egg white, the precipitation of the lysozyme begins before the "calcium phosphate" crystals are formed and hence the latter are not observed because of the large excess of lysozyme needles. The increased quantity of these "calcium phosphate" crystals under the conditions of the lysozyme precipitation is probably due to a decrease in solubility in

the more alkaline solutions. The identity of this crystalline material cannot be considered well established at this time. It has not been considered as one of the protein components of egg white.

In the preceding two experiments, no further attempts were made to remove the lysozyme fraction before the remainder of the proteins. In Experiment F-12 (Figure 14) the ovomucin fraction was removed as Fraction I in the super-centrifuge. The lysozyme was then removed by adjusting to the following conditions:

pH = 9.3
$\mu/2$ = 1.14
N_2 = 0
N = 17.6 gm./l.
T = 1°C.

The first crystals observed were the platelets of experiments F-8 and F-9. This precipitate was not removed and after seventy-two hours a heavy precipitate of typical lysozyme needles was obtained. This fraction, after washing with 5% NaCl, was electrophoretically pure lysozyme. The yield was 51%.

In fractionation experiments F-6, F-8, F-9, F-10, F-11, and F-13 (Figures 8, 10, 11, 12, 13, and 15) the lysozyme was not removed directly from the egg white but was crystallized from appropriate sub-fractions of the conalbumin-globulin fraction. As will be shown in the

next section, all of the lysozyme in the egg white was precipitated in this fraction. The crystallization was accomplished in the same manner as from whole egg white. The results obtained are shown in Table XIX.

TABLE XIX.

Removal of lysozyme from the conalbumin-globulin fraction.

Experiment	Lysozyme rich fraction	% lysozyme of original	% lysozyme in fraction
F-6	I-1-B	28	100
F-9	III-2-A	74	51
F-10	II-1	19	100
F-11	II-1-B	52	32
F-13	II-2-B-α-1	30	19

It is apparent from these results that the purity and yield of the lysozyme obtained by sub-fractionation of the conalbumin-globulin fraction are considerably lower than when the lysozyme is crystallized directly from the egg white. The purity of the lysozyme from each of these fractions can be readily increased by further crystallization. Due to the distribution of the lysozyme into various sub-fractions however, it is to be expected that yields can not be increased to equal those obtained in the

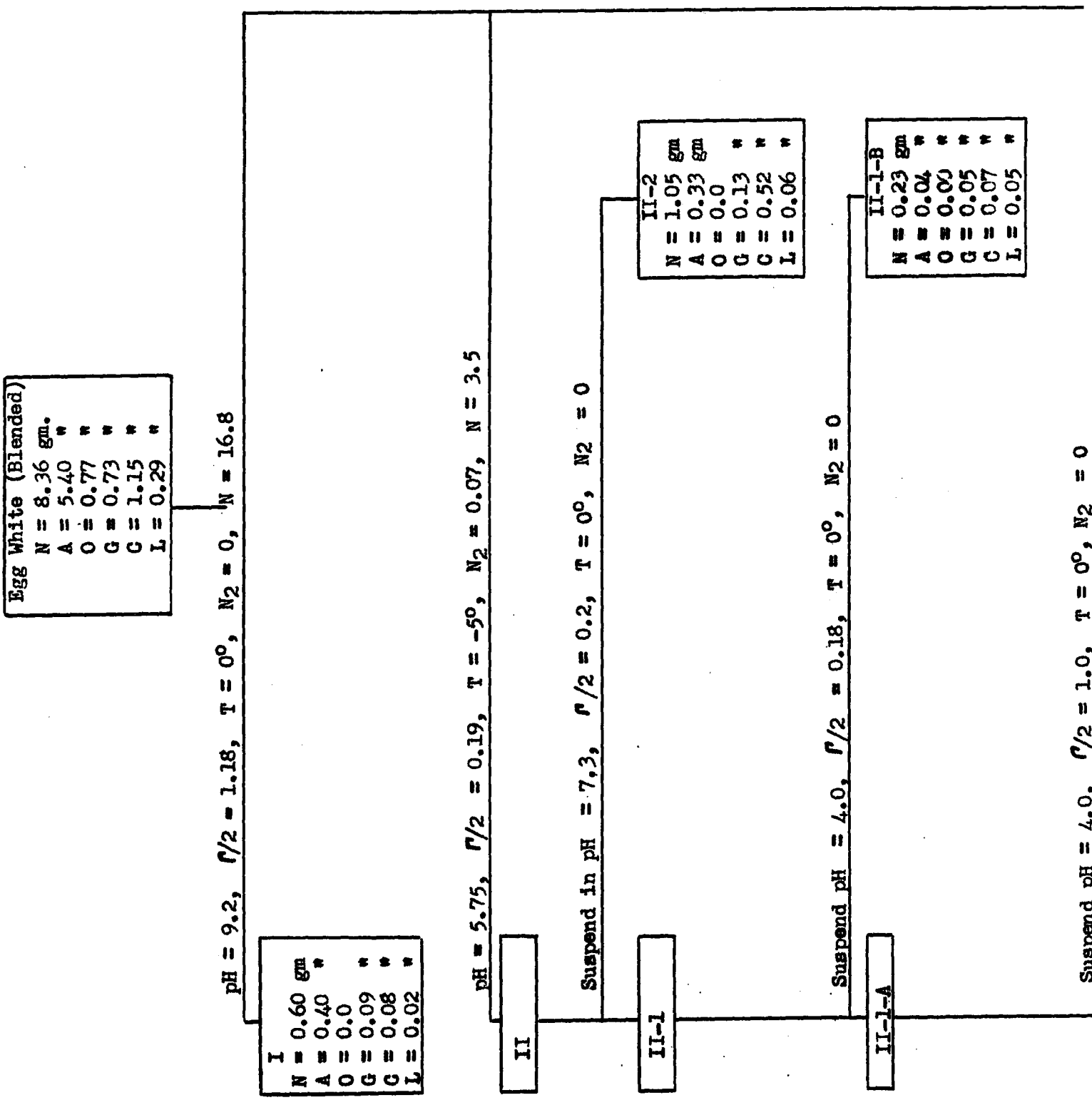
direct crystallization procedure. Lysozyme retains its lytic activity after the ethanol removal of the conalbumin-globulin fraction.

It is apparent that if lysozyme is the desired constituent, no improvement has been made on the isolation procedure already described in the literature. It is quite possible, however, that future investigations will improve the sub-fractionation of the conalbumin-globulin fraction so that a more satisfactory method of removing lysozyme in a comprehensive scheme will become available.

Once lysozyme has been crystallized it can be recrystallized quite readily and nearly quantitatively from solutions at pH 9.5-10.0 with 10% alcohol. All attempts to remove lysozyme from egg white under similar conditions have been unsuccessful. A possible explanation for this difficulty may be that the high salt concentration is needed to dissociate a protein complex.

Conalbumin-globulin fraction

The proximity of the isoelectric points of the conalbumin (6.1) and the globulins (5.5-6.0) and the anomalous solubility behavior of the globulins have rendered a satisfactory separation of these proteins extremely difficult. Due to the low solubility of the globulins, electrophoretic


Egg White (Blended)
N = 8.36 gm.
A = 5.40 "
O = 0.77 "
G = 0.73 "
C = 1.15 "
L = 0.29 "
pH = 9.2, Γ/2 = 1.18, T = 0°, N2 = 0, N = 16.8
I
N = 0.60 gm
A = 0.40 "
O = 0.0
G = 0.09 "
C = 0.08 "
L = 0.02 "
pH = 5.75, Γ/2 = 0.19, T = -5°, N2 = 0.07, N = 3.5
II
Suspend in pH = 7.3, Γ/2 = 0.2, T = 0°, N2 = 0
II-1
II-2
N = 1.05 gm
A = 0.33 gm
O = 0.0
G = 0.13 "
C = 0.52 "
L = 0.06 "
Suspend pH = 4.0, Γ/2 = 0.18, T = 0°, N2 = 0
II-1-A
II-1-B
N = 0.23 gm
A = 0.04 "
O = 0.00 "
G = 0.05 "
C = 0.07 "
L = 0.05 "
Suspend pH = 4.0, Γ/2 = 1.0, T = 0°, N2 = 0

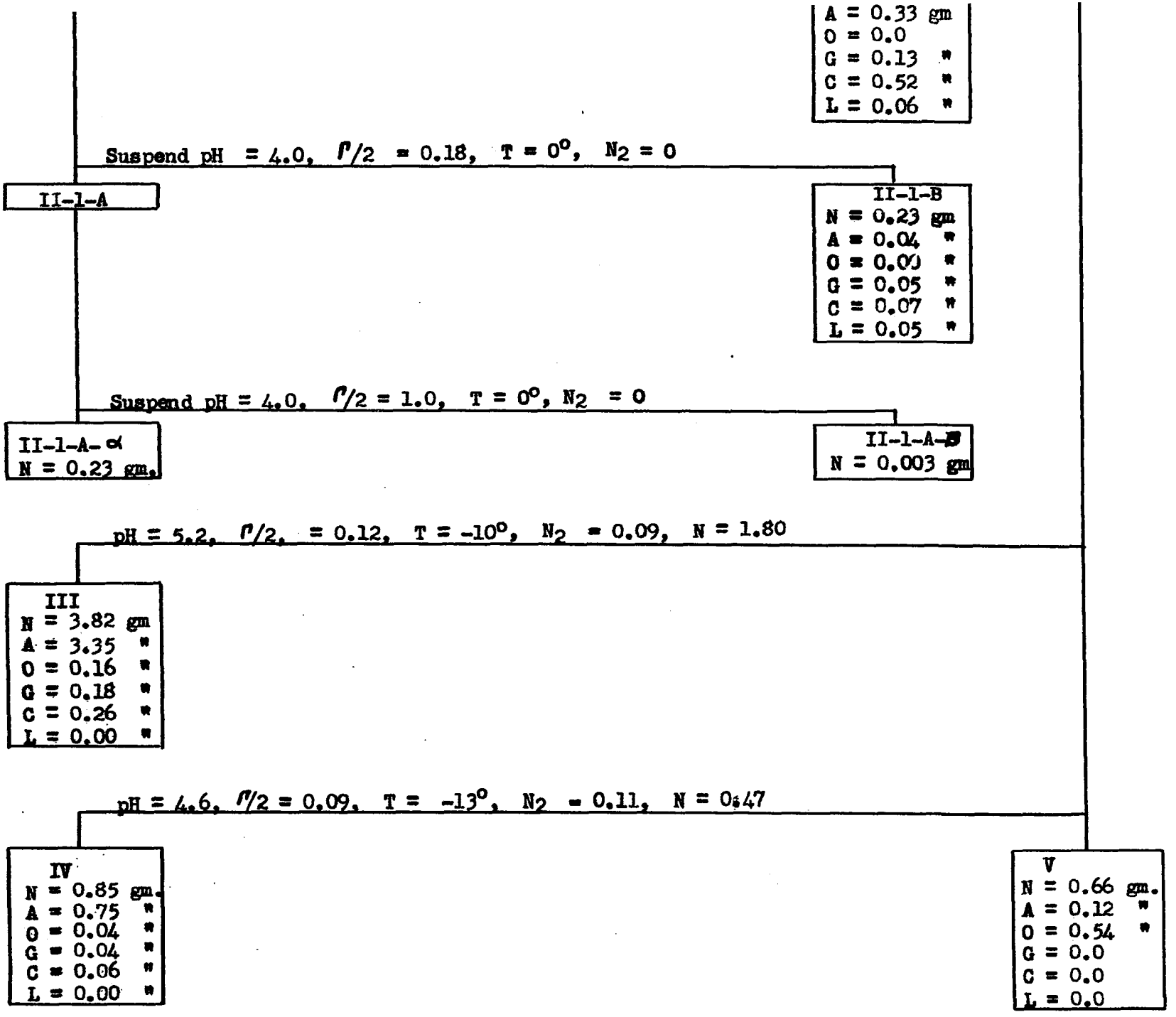
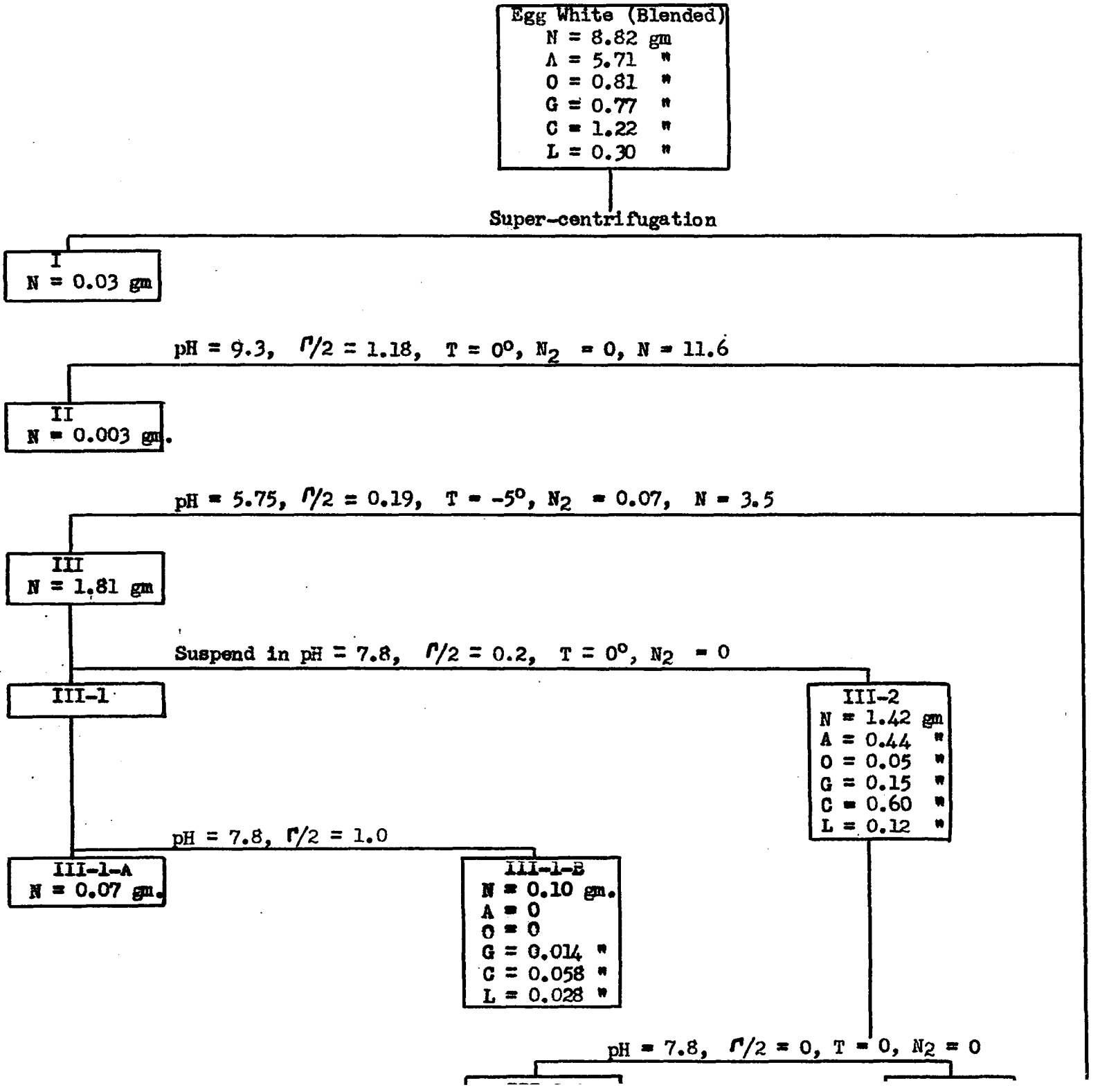


Figure 10. Flow Sheet F-8


Egg White (Blended)
N = 8.82 gm
A = 5.71 "
O = 0.81 "
G = 0.77 "
C = 1.22 "
L = 0.30 "
Super-centrifugation
I
N = 0.03 gm
pH = 9.3, Γ/2 = 1.18, T = 0°, N2 = 0, N = 11.6
II
N = 0.003 gm.
pH = 5.75, Γ/2 = 0.19, T = -5°, N2 = 0.07, N = 3.5
III
N = 1.81 gm
Suspend in pH = 7.8, Γ/2 = 0.2, T = 0°, N2 = 0
III-1
III-2
N = 1.42 gm
A = 0.44 "
O = 0.05 "
G = 0.15 "
C = 0.60 "
L = 0.12 "
pH = 7.8, Γ/2 = 1.0
III-1-A
N = 0.07 gm.
III-1-B
N = 0.10 gm.
A = 0
O = 0
G = 0.014 "
C = 0.058 "
L = 0.028 "
pH = 7.8, Γ/2 = 0, T = 0, N2 = 0

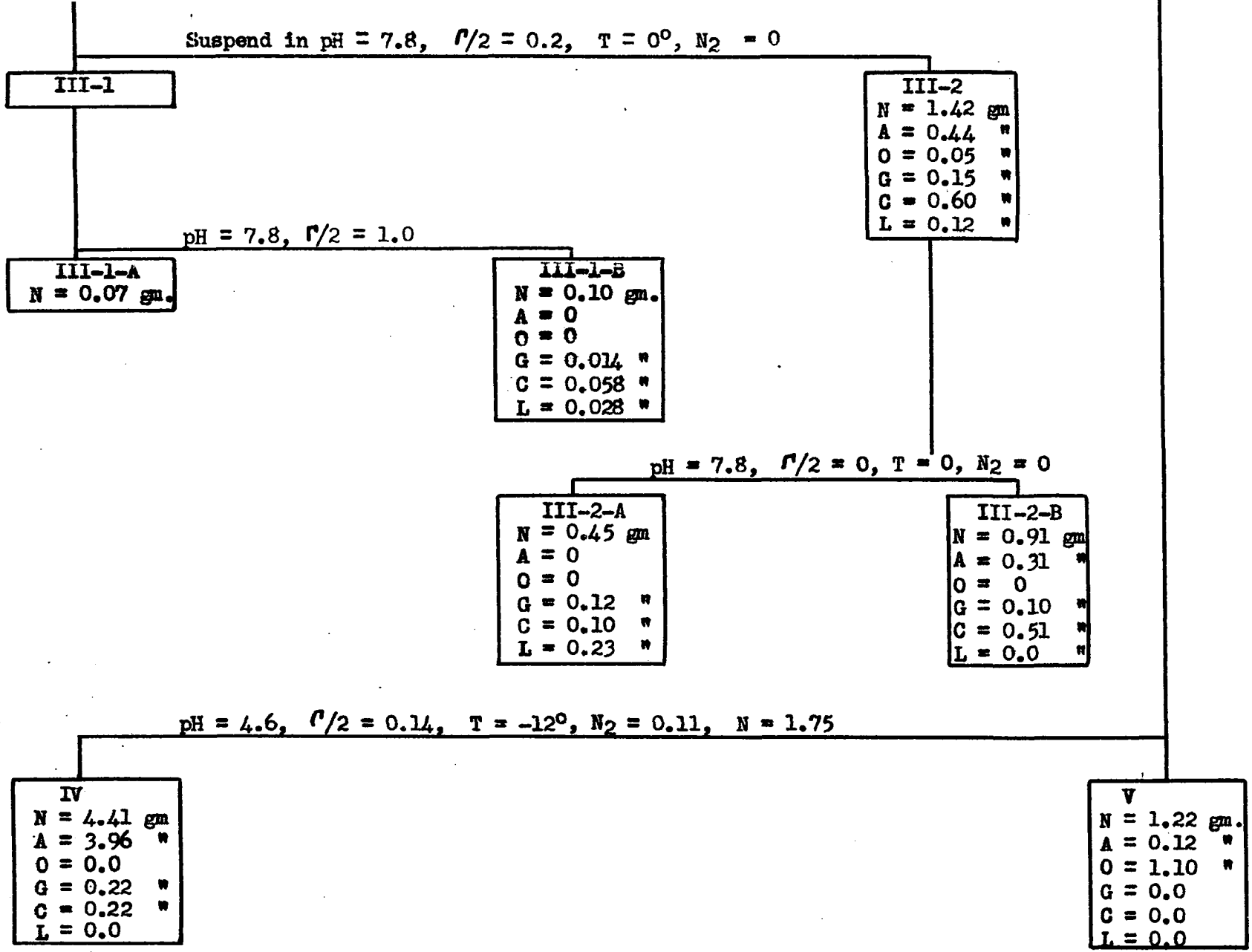


Figure 11. Flow Sheet F-9

analyses of this fraction yield results of dubious value. The electrophoretic mobilities of conalbumin and the globulins have similar values under the reference conditions chosen, making identification and resolution difficult in many electrophoretic experiments. The nature of some of the patterns suggests that protein interactions may exist.

Each fractionation experiment was carried to completion as rapidly as possible so that proteins would not be subjected to damaging conditions for long periods of time. This rapid processing frequently necessitates continuation of the fractionation before analytical data on the last separation is available. This difficulty leads to what appears to be, when all of the data are assembled, pointless "back-tracking" in some of the fractionation schemes.

Early fractionation experiments were planned so that the globulin fraction would be removed first at low ionic strengths. The poor results obtained could be partially explained by the fact that interaction between proteins is increased in the absence of salt. Due to the character of the ovomucin precipitate, separation of the globulin prior to development of the super-centrifuge technique for ovomucin removal was, in general, not satisfactory.

In Experiment F-5 (Figure 7) an attempt was made to remove the globulin fraction as the first step in the fractionation procedure. The following conditions,

$$\begin{aligned} pH &= 5.5 \\ \mu/2 &= 0.1 \\ N_2 &= 0.03 \\ N^2 &= 17.8 \\ T &= -3^{\circ}C., \end{aligned}$$

gave a fraction which contained approximately 60% of the original globulin and 15% of the conalbumin. Upon adjusting the pH to 5.9, and reducing the ionic strength to approximately zero, a second fraction was removed which contained 8% of the globulin and 25% of the conalbumin. In both of these fractions, ovalbumin was the primary impurity, about 50% in each case.

During the removal of the fractions precipitated in the presence of ethyl alcohol, it was noted that some warming of the solution (usually about 5°C.) resulted during the centrifuging operation. When the supernatants were cooled back down to the appropriate temperature, more precipitation occurred. The precipitation was reversible over this narrow temperature range and it was condluded that it was due to an extremely high temperature coefficient of solubility in the aqueous-ethanol solutions.

While this experiment showed marked improvement of the globulin removal over the dilution techniques, it did

not give a good separation of either conalbumin or globulin. Large amounts of insoluble precipitates (Fractions I-1 and II-1 of Figure 7) indicated the desirability of removing the ovomucin prior to subsequent separations.

In Experiment F-6 (Figure 8) an attempt was made to remove the ovomucin prior to separation of the globulin. The pH of egg white was lowered to 6.0 and the ionic strength reduced to 0.05 by dialysis. It was hoped that holding the ionic strength at this level would keep the globulin in solution. No alcohol was employed in this removal. Following the removal of Fraction I, the ionic strength was lowered to 0.03, and alcohol added to a mole fraction of 0.03. A fraction was obtained which was comparatively rich in both globulin and conalbumin (II-2). These results suggested that it might be more advantageous to remove the conalbumin before the globulin. Such a procedure would also have the theoretical advantage that it would not necessitate the removal of a fraction from a solution containing oppositely charged proteins.

Experiment F-7 was designed to embody the improvements suggested by the earlier experiments. Figure 9 shows the detailed flow sheet for the procedures employed. The lysozyme fraction was removed first so that in all

subsequent steps the pH could be maintained on the alkaline side of the isoelectric points of all of the remaining proteins. Fraction II (Figure 9) was removed under the following conditions:

pH = 6.25
$\mu/2$ = 0.13
N_2 = 0.03
N = 8.5
T = -3°C.

Fraction III was removed after the conditions had been modified as follows:

pH = 5.15
$\mu/2$ = 0.07
N_2 = 0.05
N = 6.8
T = -4°C.

From the results of the previous experiments it was expected that Fraction II would contain most of the conalbumin, but when analytical data were available, it was apparent that this fraction contained only a small percentage of the original nitrogen, and that Fraction III-2 contained little if any conalbumin. No satisfactory explanation presented itself for the failure to remove the conalbumin in Fraction II so the conditions were readjusted to those under which its removal had been attempted. The data for Fraction IV show that successful removal of the conalbumin was accomplished. The total over-all yield of nitrogen in this experiment was 76%, but only 43% of the protein was obtained in soluble form so that it could be analyzed electrophoretically.

It was apparent at this stage that better results might be obtained if both conalbumin and globulin were removed in one step. Several fractionation experiments were carried out to determine optimal conditions for this removal. In Experiments F-8 and F-9 the unsuccessful attempts to remove the lysozyme fraction have been described. The conditions for removal of the conalbumin-globulin fraction are described in Figures 10 and 11, and the analytical data obtained summarized in Table XX.

It was of particular interest in these two experiments to observe the effect of the preliminary super-centrifuge treatment. In F-8, 2.9% of the original nitrogen was found in the insoluble, slimy precipitate II-1-A-d, while in F-9, in which super-centrifugation was employed, less than one per cent was found in the similar fraction, III-1-A. This clearly points out one of the advantages in employing this technique.

When Fraction II (F-8) and Fraction III (F-9) were suspended in buffer at pH 7.8, $\Gamma/2 = 0.2$, there was very little selective solution of the fraction, the larger portion going into solution in either case. Extraction of soluble proteins from precipitates has, in general, been unsatisfactory.

In Experiment F-9, a further separation of Fraction III-2 was attempted by lowering the ionic strength.

TABLE XX.

Summary of data for the removal of the conalbumin-globulin fraction.

Experiment	pH	Γ/2	N_2	N	Percent Yield Conalbumin	Percent Yield Globulin	Percent Total Impurities	Remarks
F-8	5.8	0.19	0.07	3.5	51	25	37	Lysozyme not removed
F-9	5.8	0.19	0.07	3.5	54	30	55	Super-centrifuge, Lysozyme not removed
F-10	6.1	0.05	0.07	8.4	65	17	40	
F-10	6.1	0	0	18.8	0	24	47	Super-centrifuge, Lysozyme in fraction
F-11	6.1	0.15	0.07	7.0	72	34	30	Lysozyme in fraction
F-12	6.2	0.15	0.07	8.9	44	40	21	Super-centrifuge
F-13	6.3	0.06	0.07	8.8	92	33	46	Super-centrifuge, all of lysozyme in fraction
L-1	6.1	0.05	0.07	8.8	96	49	42	Super-centrifuge, small lot only, lysozyme in fraction
L-2	7.0	0.05	0.07	8.8	101	49	45	Similar to L-1

Examination of the analytical data points out the difficulties encountered in the electrophoretic analysis of fractions containing a larger proportion of globulins. No satisfactory explanation can be offered for the discrepancies noted, but from other electrophoretic experiments, the data for III-2-A and III-2-B are assumed to be more reliable.

Because of the increased yields of soluble protein and the reduction in the amount of the slimy, insoluble precipitates when the egg white was super-centrifuged as the first step in fractionation procedures, a further attempt to remove the globulin by itself was made in Experiment F-10. Details of this experiment are shown in the flow sheet in Figure 12. Fraction II consisted of globulin in the highest state of purity that had been obtained. The yield was quite low, however, only 29% of the original globulin. Several possible explanations were considered. First, it is possible that extensive protein interaction occurs at the low ionic strength and high protein concentration under which this separation was carried out. Second, the possibility that only about 40-50% of the globulin in egg white was a true euglobulin was considered. The remaining globulin might be a pseudoglobulin, soluble under the conditions of this removal. Later work has supported the second assumption. Lysozyme distributes

itself approximately equally in the several sub-fractions obtained. As previously pointed out, this is a serious disadvantage if the lysozyme fraction is desired. It is possible to crystallize the lysozyme from any of these sub-fractions.

Throughout this study, improvements in technique were made and the yields increased. Filtration was employed to clarify supernatants before any further fractionation. Centrifugation was accomplished without excessive temperature increases. These improvements in technique made it desirable to repeat some of the earlier experiments in an attempt to increase the efficiency of the fractionation. It was observed that as higher alcohol concentrations were employed for the removal of the conalbumin-globulin fraction, the temperature coefficient of solubility appeared to increase. The effect of the temperature on the solubility of the globulins in aqueous solutions at low ionic strength was also quite marked. Opacity of globulin solutions increased as the temperature was reduced, e.g., from 25° to 1°C., and finally a precipitate formed which redissolved upon warming. These solubility relationships have been employed where possible to make separations.

In Experiments F-11 and F-12 (Figures 13 and 14) the ovomucin was removed as Fraction I by super-centrifugation. In F-11 the conalbumin-globulin fraction (II) was removed immediately while in F-12, the lysozyme fraction (II) was removed followed by separation of the conalbumin-globulin fraction (III). While these fractions were removed under nearly identical conditions, only 12% of the original nitrogen was obtained in F-12 while 20% was obtained in F-11. The removal of the lysozyme can account for a part of this difference, but some of it must be due to smaller mechanical losses in F-11. The results of these experiments are summarized in Table XX.

The details of the procedures used for sub-fractionation of the conalbumin-globulin fraction are given in the respective flow sheets. The best results were obtained in F-11 where Fraction II was suspended in a buffer,

$$\begin{aligned} pH &= 6.11, \\ \Gamma/2 &= 0.01, \end{aligned}$$

and dialyzed against water. The pH remains constant during such a dialysis while the ionic strength is lowered. The globulin rich fraction (II-1-B) was further fractionated to yield a globulin solution electrophoretically free of conalbumin (II-1-B-α). This globulin solution was soluble in electrophoretic buffer (pH 7.8, $\Gamma/2 = 0.20$) only to the

extent of about 0.7 gm. N/l at 2°C. It should be pointed out that most of the lysozyme follows the globulin in these fractionation procedures.

Experiments L-1 and L-2 were carried out to determine the effect of pH on the removal of the conalbumin-globulin fraction. These experiments were carried out on small lots of egg white and as such the results can not be compared directly with others obtained on large scale operations. The results of these experiments are summarized in Table XX. It is apparent from these results that the effect of pH on the removal of this fraction is not as pronounced as small changes in alcohol concentration and ionic strength.

Fractionation Experiment F-13 (Figure 15) was designed in an attempt to include all the improvements suggested by the earlier experiments. The details of the conditions for the removal and the sub-fractionation of the conalbumin-globulin fraction (II) are recorded in the flow sheet. The yield of globulin in this fraction again suggested that only a portion of this fraction can be removed under these conditions, probably somewhat less than 50%. The remainder of the globulin has usually appeared in the ovalbumin fractions; this globulin is probably the pseudoglobulin

that has been reported to be present in egg white. It has not been possible to identify the different globulins in the electrophoretic patterns. When purification procedures for the globulins are perfected, satisfactory identification and solubility characteristics may be obtained.

In all of the fractionations carried out the natural yellow color of the egg white has been concentrated in the conalbumin-globulin fraction. Upon sub-fractionation, however, it has been difficult to identify the color constituent, presumably riboflavin, with either the conalbumin or globulin fraction. Bain and Deutsch (14) reported that the riboflavin of the egg white was bound to the conalbumin and could be removed by dialysis only at pH's acid to the isoelectric point. Because of this report, it was tacitly assumed throughout most of this study that the yellow color was indicative of the presence of conalbumin. Anomalous results were obtained in several of the experiments, but were discounted because of the lack of purity of the fractions.

In Fractionation Experiment F-13, nearly all of the color was concentrated in the conalbumin-globulin fraction (II) and upon sub-fractionation was found in the conalbumin rich fraction (II-2). Purification of this fraction

was attempted as shown in the flow sheet, two main fractions (II-2-B-α-1 and II-2-B-α-2) being obtained. Fraction II-2-B-α-1 was insoluble under the conditions described but was readily soluble in 5% NaCl. The yellow constituent was concentrated in this fraction. This was unexpected since II-2-B-α-1 contained only one half as much conalbumin and nearly three times as much globulin as II-2-B-α-2. The yellow fraction (II-2-B-α-1) exhibited an extremely high temperature coefficient of solubility in salt free solutions making it difficult to completely remove all of the colorless water soluble fraction. Thus both on the basis of solubility and electrophoretic analysis, the yellow color was not associated with the protein having typical conalbumin characteristics.

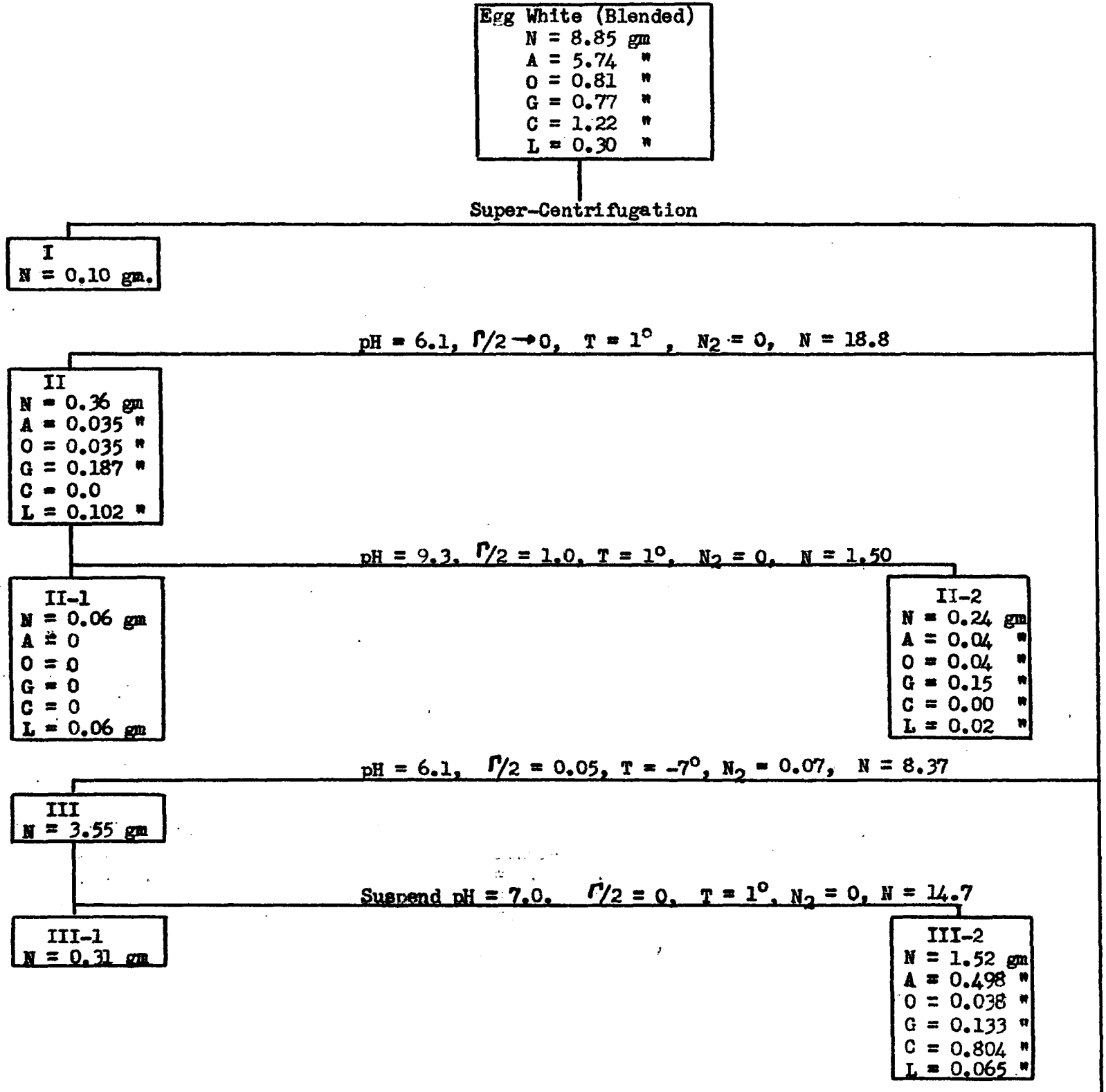
Several possible explanations for this observation may be offered. First, the riboflavin may not be combined with the conalbumin, but rather with a globulin which is not satisfactorily resolved in the electrophoretic analysis. The characteristics of this proposed globulin (a pseudoglobulin) would be very similar to the conalbumin and hence they would not be readily separable under the conditions employed. Second, the solubility characteristics of the conalbumin are not typical of an albumin, but rather of a pseudoglobulin. Since two conalbumins have been proposed

(14, 77) only a portion of the total conalbumin, that binding the riboflavin, may exhibit these globulin tendencies. Third, the electrophoretic analyses indicate an apparent increase in the globulin at the expense of the conalbumin in the sub-fractions. This suggests that as the purity of the fractions is increased, electrophoretic resolution may be improved, permitting identification of the colored constituent.

These results indicate the necessity of carrying out several experiments outside the immediate scope of this study. The interaction of purified conalbumin and riboflavin mixtures should be studied by means of ultraviolet spectrophotometry. Globulin preparations, of higher purity than obtained in this study, should be investigated from the standpoint of solubility and interaction with riboflavin, conalbumin, lysozyme or possibly with other of the egg white proteins.

Ovalbumin fraction

The removal of the ovalbumin fraction has been readily accomplished in media of low dielectric constant and low ionic strength. Much of the success in the removal of this fraction is due to the extensive studies of this protein by many investigators. A means of preparing the protein in


Egg White (Blended)
N = 8.85 gm
A = 5.74 "
O = 0.81 "
G = 0.77 "
C = 1.22 "
L = 0.30 "
Super-Centrifugation
I
N = 0.10 gm.
pH = 6.1, Γ/2 → 0, T = 1°, N2 = 0, N = 18.8
II
N = 0.36 gm
A = 0.035 "
O = 0.035 "
G = 0.187 "
C = 0.0
L = 0.102 "
pH = 9.3, Γ/2 = 1.0, T = 1°, N2 = 0, N = 1.50
II-1
N = 0.06 gm
A = 0
O = 0
G = 0
C = 0
L = 0.06 gm
II-2
N = 0.24 gm
A = 0.04 "
O = 0.04 "
G = 0.15 "
C = 0.00 "
L = 0.02 "
pH = 6.1, Γ/2 = 0.05, T = -7°, N2 = 0.07, N = 8.37
III
N = 3.55 gm
Suspend pH = 7.0, Γ/2 = 0, T = 1°, N2 = 0, N = 14.7
III-1
N = 0.31 gm
III-2
N = 1.52 gm
A = 0.498 "
O = 0.038 "
G = 0.133 "
C = 0.804 "
L = 0.065 "

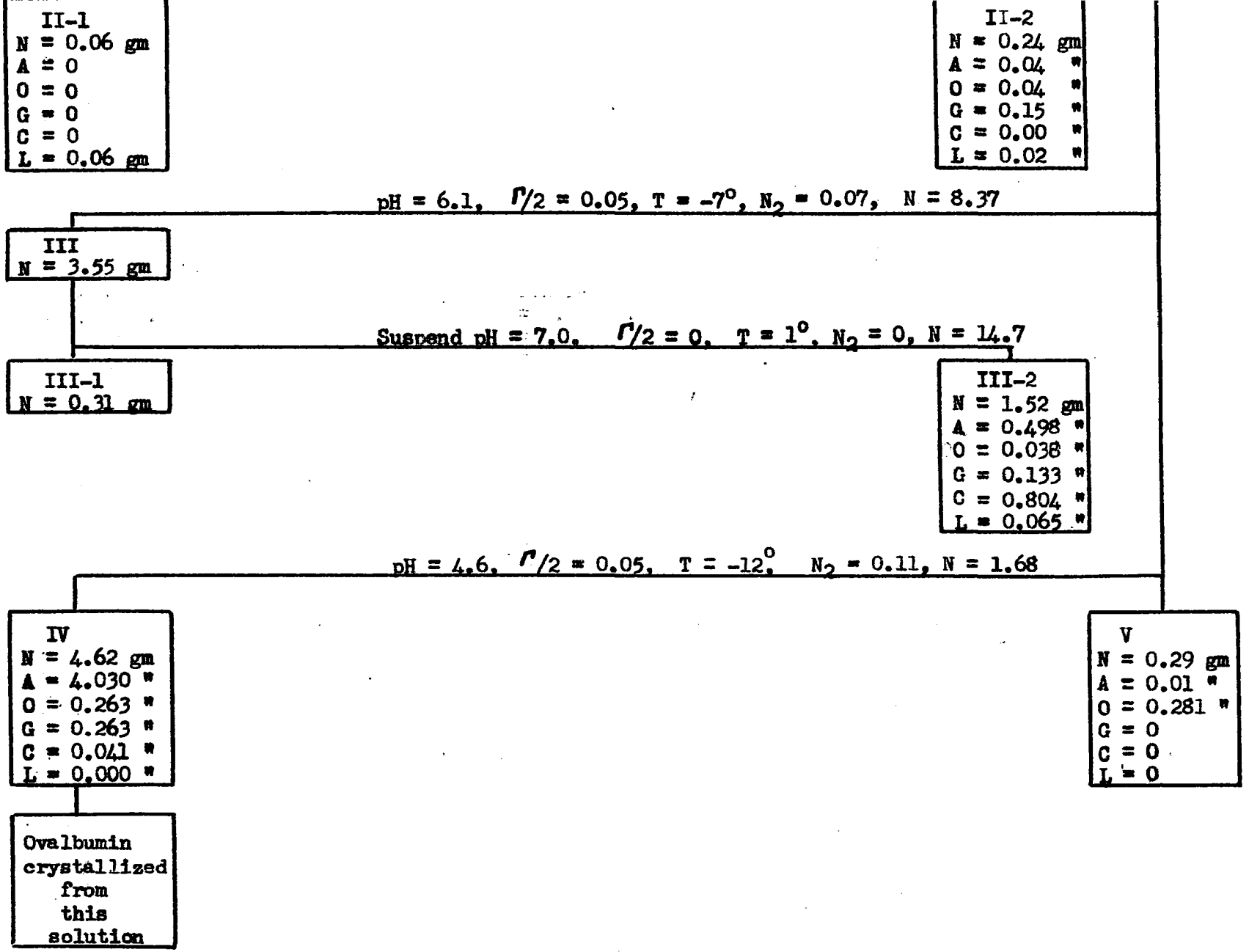
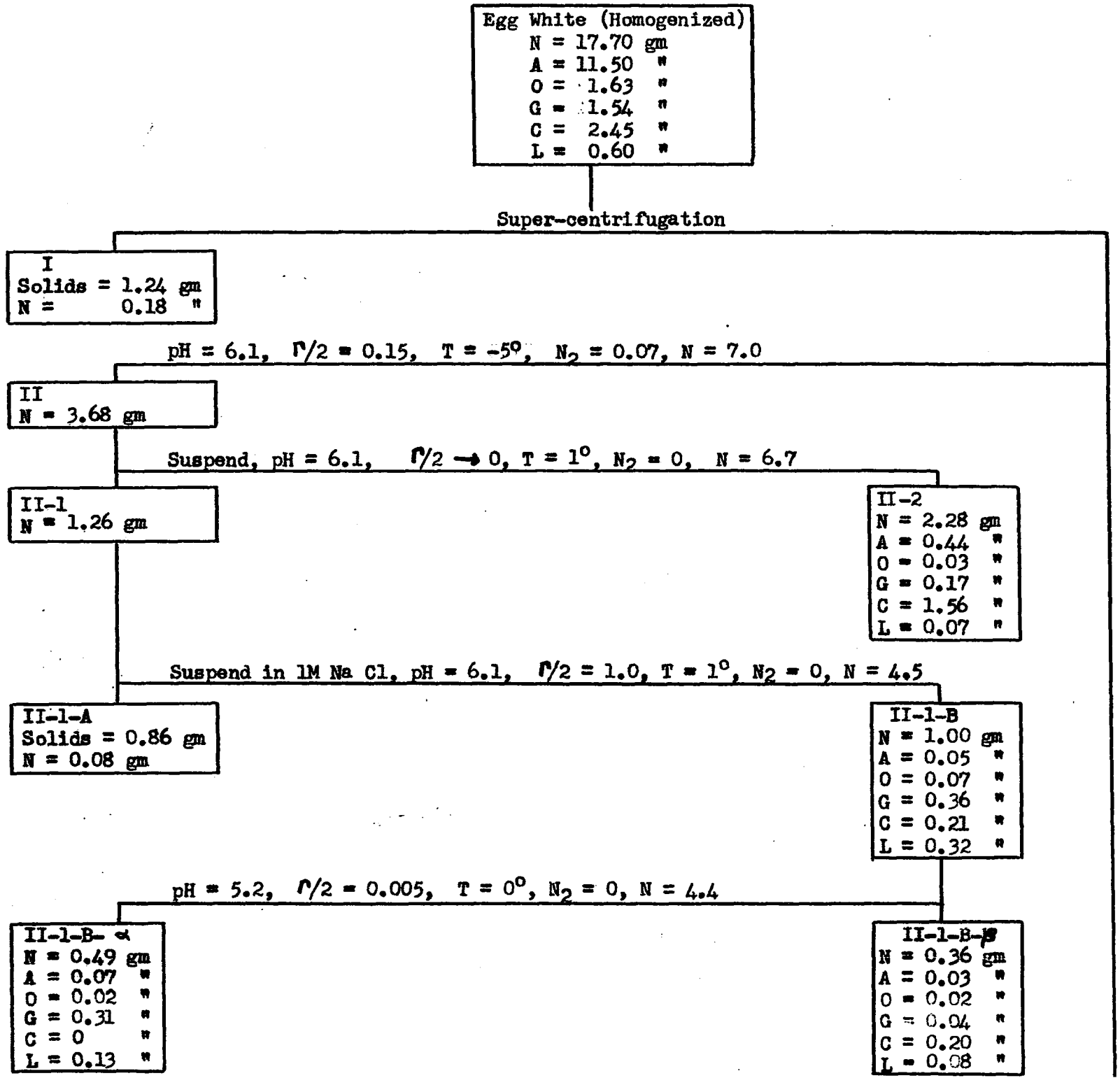


Figure 12. Flow Sheet F-10


Egg White (Homogenized)
N = 17.70 gm
A = 11.50 "
O = 1.63 "
G = 1.54 "
C = 2.45 "
L = 0.60 "
Super-centrifugation
I
Solids = 1.24 gm
N = 0.18 "
pH = 6.1, Γ/2 = 0.15, T = -5°, N2 = 0.07, N = 7.0
II
N = 3.68 gm
Suspend, pH = 6.1, Γ/2 → 0, T = 1°, N2 = 0, N = 6.7
II-1
N = 1.26 gm
II-2
N = 2.28 gm
A = 0.44 "
O = 0.03 "
G = 0.17 "
C = 1.56 "
L = 0.07 "
Suspend in 1M Na Cl, pH = 6.1, Γ/2 = 1.0, T = 1°, N2 = 0, N = 4.5
II-1-A
Solids = 0.86 gm
N = 0.08 gm
II-1-B
N = 1.00 gm
A = 0.05 "
O = 0.07 "
G = 0.36 "
C = 0.21 "
L = 0.32 "
pH = 5.2, Γ/2 = 0.005, T = 0°, N2 = 0, N = 4.4
II-1-B-α
N = 0.49 gm
A = 0.07 "
O = 0.02 "
G = 0.31 "
C = 0 "
L = 0.13 "
II-1-B-β
N = 0.36 gm
A = 0.03 "
O = 0.02 "
G = 0.04 "
C = 0.20 "
L = 0.08 "

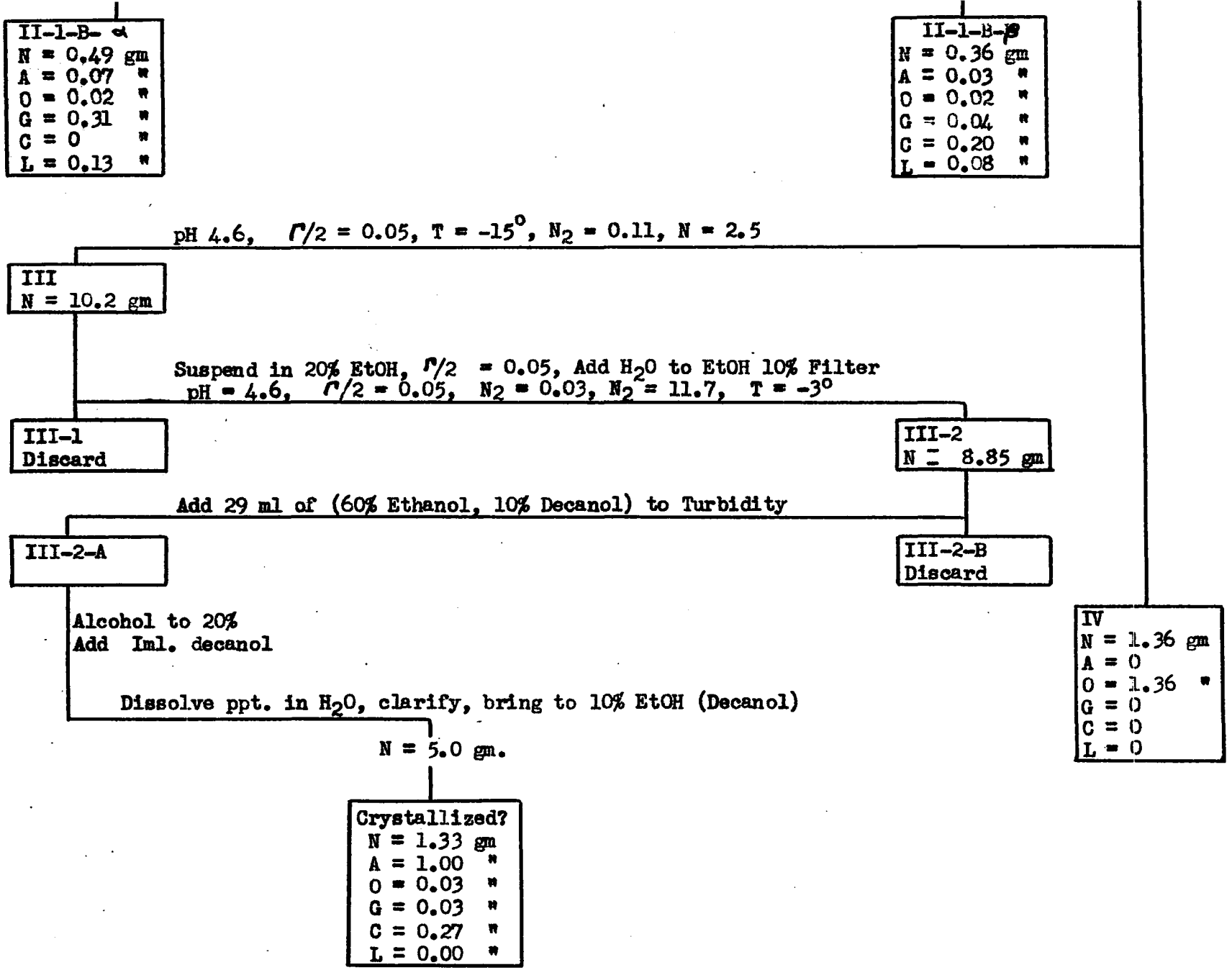
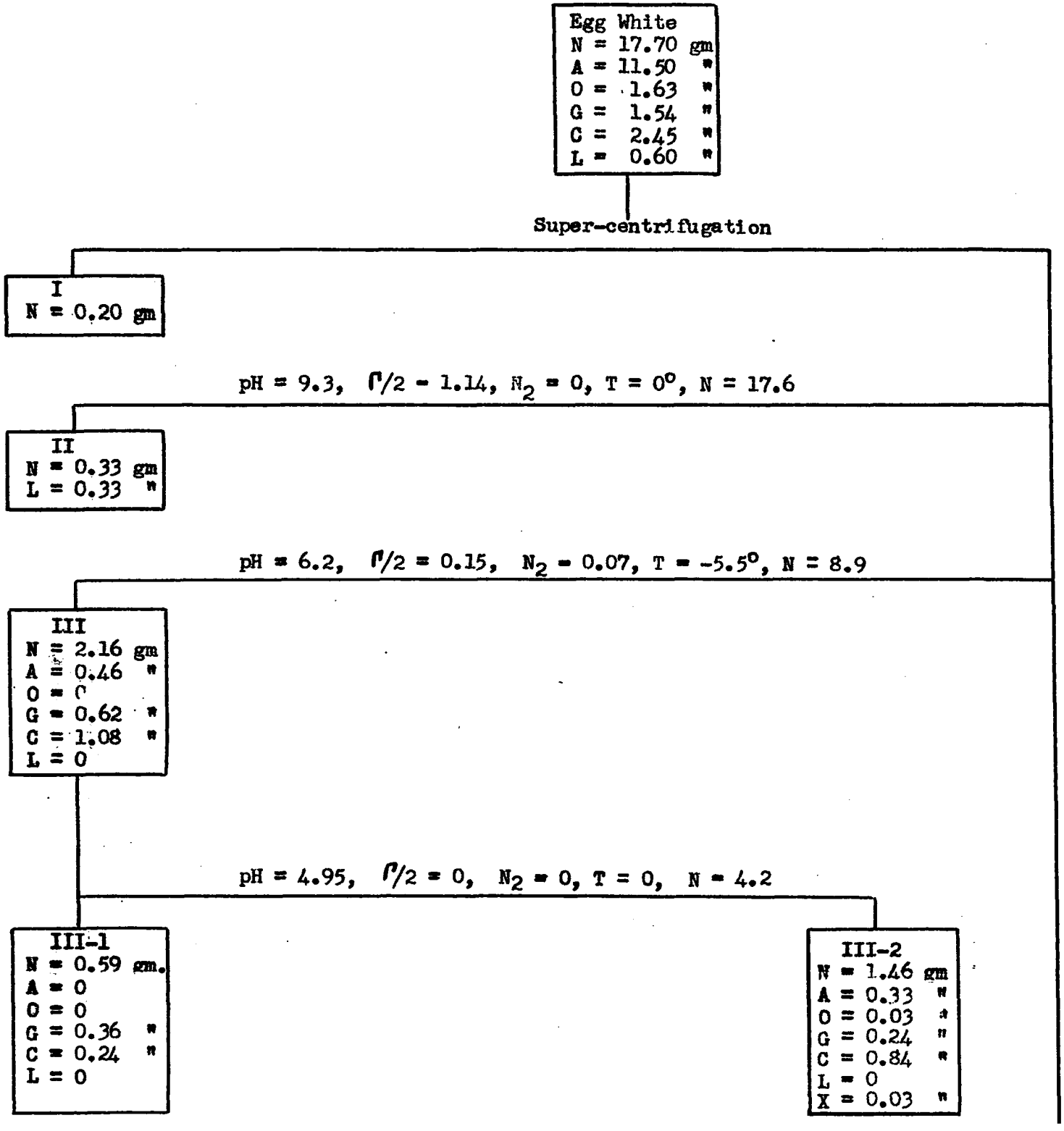


Figure 13. Flow Sheet F-11


Egg White
N = 17.70 gm
A = 11.50 "
O = 1.63 "
G = 1.54 "
C = 2.45 "
L = 0.60 "
Super-centrifugation
I
N = 0.20 gm
pH = 9.3, Γ/2 - 1.14, N2 = 0, T = 0°, N = 17.6
II
N = 0.33 gm
L = 0.33 "
pH = 6.2, Γ/2 = 0.15, N2 = 0.07, T = -5.5°, N = 8.9
III
N = 2.16 gm
A = 0.46 "
O = 0
G = 0.62 "
C = 1.08 "
L = 0
pH = 4.95, Γ/2 = 0, N2 = 0, T = 0, N = 4.2
III-1
N = 0.59 gm.
A = 0
O = 0
G = 0.36 "
C = 0.24 "
L = 0
III-2
N = 1.46 gm
A = 0.33 "
O = 0.03 "
G = 0.24 "
C = 0.84 "
L = 0
X = 0.03 "

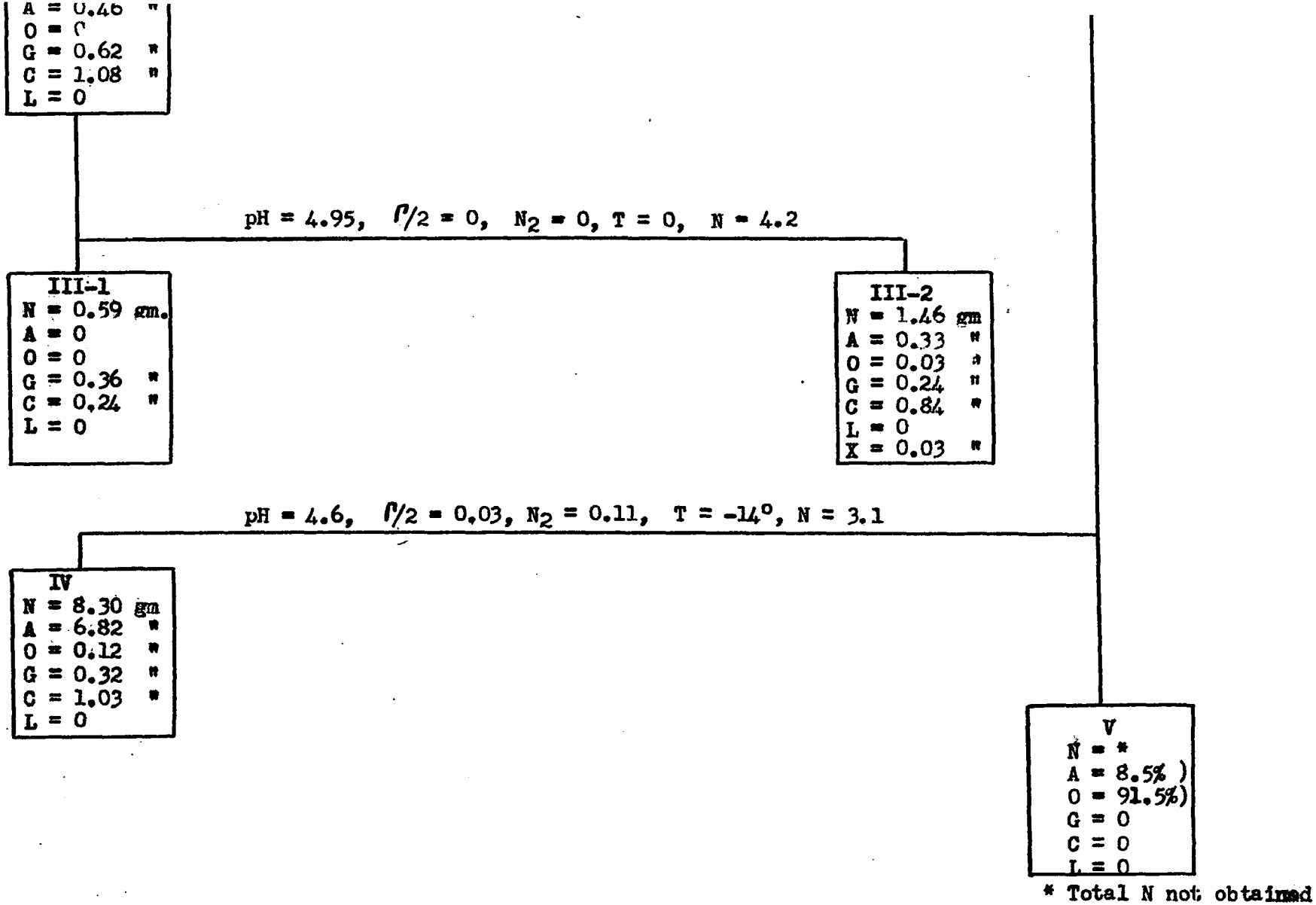


Figure 14. Flow Sheet F-12

crystalline form was available so that pure preparations could be studied in preliminary experiments. The isoelectric point was well established so that isoelectric precipitations could be carried out. Solubility data in ethanol-water mixtures, and the effect of NaCl on the solubility in such mixtures provided a sound basis for removal of the fraction.

The removal of the ovalbumin fraction is described in the flow sheets of the various fractionation experiments (Figures 9 to 15). Some of the results obtained are summarized in Table XXI.

TABLE XXI.

Conditions for the removal and composition of the ovalbumin fraction.

Exp.	pH	$\Gamma/2$	N_2	N	Per cent Albumin In fraction	Per cent Albumin Of original
F-5	Remains in solution				78	70
F-7	4.6	0	0.09	3.7	85	29
F-8	5.2	0.12	0.09	1.8	87	62
F-8	4.6	0.09	0.11	0.5	88	14
F-9	4.6	0.14	0.11	1.8	90	69
F-10	4.6	0.05	0.11	1.7	87	70
F-12	4.6	0.03	0.11	3.1	83	59
F-13	4.6	0.04	0.11	3.4	94	67

The ovalbumin removal was simplified by the fact that the solutions from which it was separated contained only a minor amount of other proteins. Most of the ovomucoid, but only a small fraction of the other proteins remained to contaminate the fraction. From the flow sheets it is apparent that a rather constant amount of ovoglobulin remains in the solution and is precipitated with the ovalbumin. It has not been possible to establish whether this portion of the globulin is soluble under the conditions of the conalbumin-globulin removal or whether the removal of the globulin in that precipitation is incomplete. Further work needs to be done to establish the identity of this constituent. Ovalbumin was, in general, removed at pH 4.6 and at a mole fraction of ethanol of 0.11. The temperature was held as near the freezing point of the solution as possible, -12° to -14°C. It was difficult to obtain satisfactory data as to the effect of ionic strength on the solubility of the ovalbumin because of the difficulty in maintaining suitable temperatures during centrifugation and clarification. Under the conditions of these fractionation experiments ovalbumin, like conalbumin and the globulins, exhibited a high temperature coefficient of solubility. In this study it has not been possible to separate the effects of temperature and ionic strength on

the solubility of the ovalbumin.

Ovalbumin from several of the experiments has been crystallized by the ammonium sulfate procedures. The crystallization procedure used in these studies is similar to those described earlier in this thesis. The ovalbumin fraction was dissolved in water to give a 5-10% protein solution. If necessary, the solution was filtered to clarify, and brought to half saturation with ammonium sulfate. Any precipitate formed was discarded and the solution re-clarified. The pH was adjusted to 4.6-4.7 with acetate buffer. Saturated ammonium sulfate was then added slowly, and with constant agitation until a stirring rod was just visible through 1 1/2 inch of the solution. The solution was then allowed to stand, crystallization taking place within six to eight hours. The crystallization was allowed to continue for twenty-four to thirty-six hours. Re-crystallization was accomplished in the same manner.

Several attempts have been made to crystallize the ovalbumin from ethanol solutions. The method of procedure followed was that outlined for the crystallization of serum albumin at the Harvard Plasma Fractionation Laboratory (37). The ovalbumin fraction paste from the super-centrifuge was used in several experiments and the lyophilized ovalbumin

fraction in others. The ovalbumin was dissolved in water and the pH adjusted to 4.6-4.7. The solutions were clarified by filtration if necessary. Ethanol and decanol were added in varying concentration in several different ways to the ovalbumin solutions. A summary of the conditions that have been employed is given in Table XXII.

TABLE XXII.

Conditions under which the crystallization of ovalbumin has been attempted.

Experiment	Nitrogen gm./l.	Ethanol Per cent	Decanol Per cent
1	10	10	0.1
2	10	20	0.1
3	10	25	0.1
4	5	10	0.1
5	5	20	0.1
6	5	25	0.1
7	10	10	1.0*
8	5	10	1.0*
9	10	20	1.0*
10	5	20	1.0*
11	10	25	1.0*
12	5	25	1.0*

* One per cent decanol was added but was not completely soluble at this concentration in all of the experiments. Solubility does not exceed 0.4-0.5% under these conditions.

It has been difficult to determine to what extent these attempts have been successful. In Experiments 7-12, excess decanol was present and when the preparations were

examined microscopically the decanol-water emulsion prevented positive identification of apparent ovalbumin crystals. Crystals were observed in Experiment 11 after standing about twenty-four hours but a greater portion of the precipitate remained in the amorphous form. In all cases where any crystallization was observed, the crystal form was very irregular. It has not been possible to establish that the crystals observed are ovalbumin and not a crystalline form of the decanol. No crystals have been observed in solutions containing no decanol, but no crystals have been observed in decanol solutions containing no ovalbumin. Further work along this same line is needed.

Ovomucoid fraction

In this study, the ovomucoid has been concentrated in the supernatant remaining after the removal of the ovalbumin fraction. As the purity of this fraction, judged by electrophoretic analysis, has been quite high, no attempt has been made to remove it from solution. Composition data for this fraction in several of the fractionation experiments are given in Table XXIII.

The wide variations in the yield of ovomucoid can be mainly accounted for by the treatments given the earlier fractions. If all fractions are washed thoroughly when

removed, the yield of ovomucoid is improved. Extensive washing of precipitates has not been done routinely however, because of increased losses of the other proteins.

TABLE XXIII.

Composition of the ovomucoid fraction.

Experiment	Per cent Ovomucoid Of Original	In Fraction
F-7	39	65
F-8	70	82
F-9	--	92
F-10	35	97
F-11	84	100
F-12	--	91
F-13	29	89

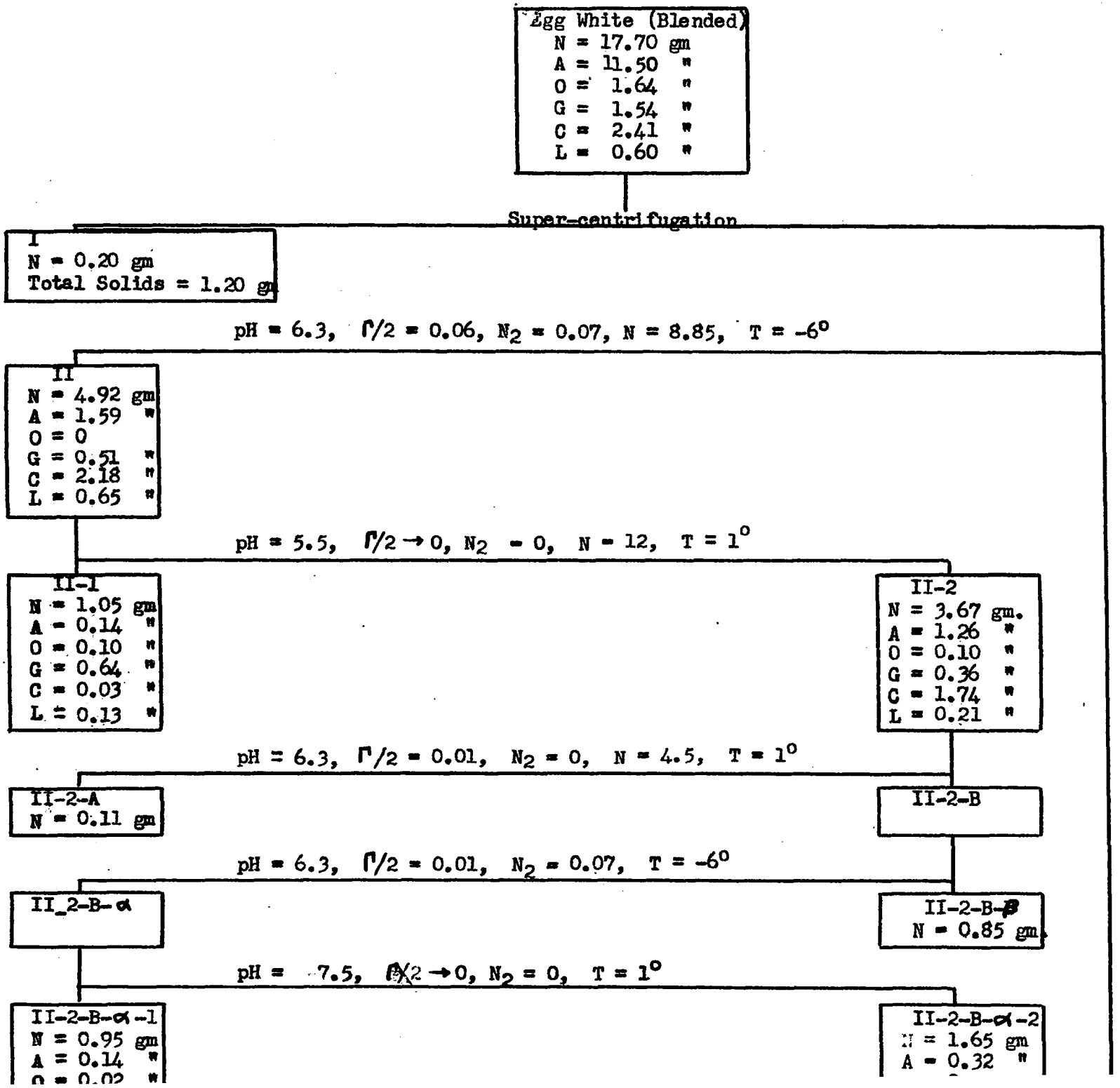
The volume of the supernatant containing the ovomucoid has been about three to four times the volume of the initial egg white. The solutions could not be lyophilized directly because of the high alcohol content, approximately 30%. Solutions richer than 15% alcohol can not be lyophilized satisfactorily because the frozen material melts before the system can be evacuated. Concentration by any means results in an ovomucoid preparation that is quite high in salt. The procedure used to prepare dry ovomucoid

preparations essentially free of inorganic salts, was as follows: The solution was placed in a large Visking tubing and a stream of air blown over the surface. Evaporation was quite rapid, the volume being reduced to one-half to one-third in seventy-two hours. The tube was then placed in water, dialyzed until the alcohol and salt concentration were at the desired level and the solution lyophilized in the usual manner.

Ovomucoid preparations obtained in this study have not been tested for anti-tryptic activity. Further work needs to be done to establish that no reduction in activity has resulted although the conditions employed in these fractionation procedures would appear to be much less harmful than those used in other investigations. In electrophoretic experiments, ovomucoid has exhibited reversible boundary spreading as described by Longsworth, et. al. (77), indicating inhomogeneity.

A Comprehensive Fractionation Scheme

As was previously pointed out, Fractionation Experiment F-13 was designed to include all of the improvements suggested by the earlier experiments. The procedure employed in this experiment has not given as good yields


Egg White (Blended)
N = 17.70 gm
A = 11.50 "
O = 1.64 "
G = 1.54 "
C = 2.41 "
L = 0.60 "
Super-centrifugation
I
N = 0.20 gm
Total Solids = 1.20 gm
pH = 6.3, Γ/2 = 0.06, N2 = 0.07, N = 8.85, T = -6°
II
N = 4.92 gm
A = 1.59 "
O = 0
G = 0.51 "
C = 2.18 "
L = 0.65 "
pH = 5.5, Γ/2 → 0, N2 = 0, N = 12, T = 1°
II-1
N = 1.05 gm
A = 0.14 "
O = 0.10 "
G = 0.64 "
C = 0.03 "
L = 0.13 "
II-2
N = 3.67 gm.
A = 1.26 "
O = 0.10 "
G = 0.36 "
C = 1.74 "
L = 0.21 "
pH = 6.3, Γ/2 = 0.01, N2 = 0, N = 4.5, T = 1°
II-2-A
N = 0.11 gm
II-2-B
pH = 6.3, Γ/2 = 0.01, N2 = 0.07, T = -6°
II_2-B-α
II-2-B-β
N = 0.85 gm
pH = 7.5, Γ/2 → 0, N2 = 0, T = 1°
II-2-B-α-1
N = 0.95 gm
A = 0.14 "
II-2-B-α-2
N = 1.65 gm
A = 0.32 "

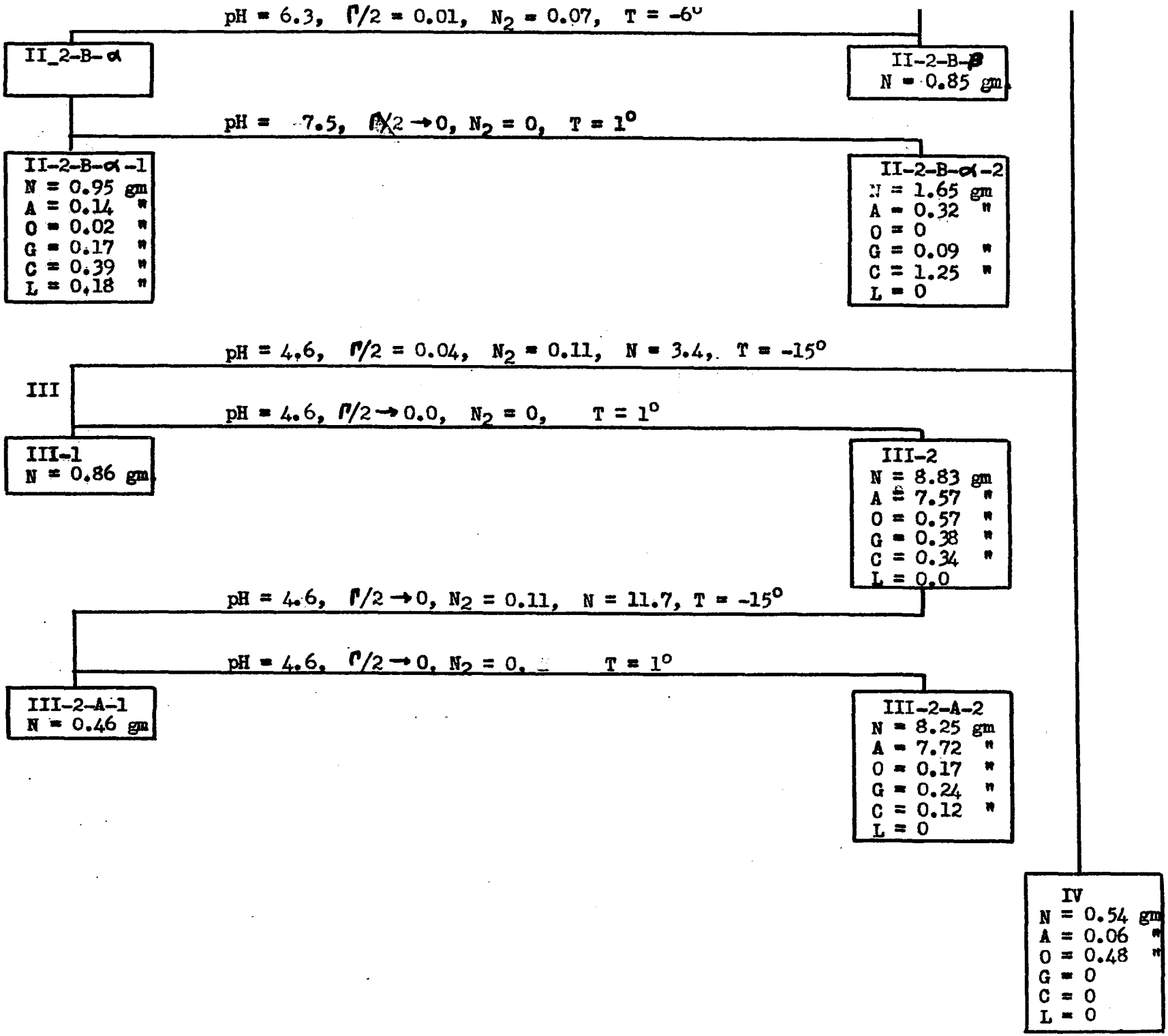


Figure 15. Flow Sheet F-13.

of some fractions as in other schemes, but from the comprehensive standpoint, this procedure is recommended as the most suitable at the present time for the separation of the egg white proteins into fractions.

The details of the procedure are shown in Figure 15 and a summary of the conditions for removal and composition of the various fractions is given in Tables XXIV and XXV. The ovomucin fraction was removed first in the super-centrifuge. This separation is quite satisfactory and is recommended for the preparation of ovomucin. The conalbumin-globulin fraction was highly contaminated with ovalbumin (32.3%) and contained a considerable quantity of the ovomucoid. If excessive losses can be avoided, this step may be improved by thorough washing of the precipitate to remove these contaminants. It is possible that more of the globulin would be removed in this fraction if the ionic strength were lowered to approximately 0.02-0.03. This ionic strength should be sufficient to prevent the extensive protein interactions occurring at very low salt concentrations. A cleaner separation is accomplished when the lysozyme is removed before this fraction, but the disadvantages of this procedure have been discussed. Sub-fractionation of the conalbumin-globulin

TABLE XXIV.

Conditions for the separation of the egg white proteins into fractions.
Fractionation Experiment F-13.

Fraction	pH	$\Gamma/2$	$T^{\circ}C.$	Ethanol N_2	Nitrogen gm./l.	Nitrogen in Fract. gm.	Nitrogen % of Orig.
I	Super-centrifuge				17.70	0.20	1.1
II	6.3	0.06	-6	0.07	8.35	4.92	27.8
II-1	5.5	→0	1	0	12.00	1.05	5.9
II-2-A	6.3	0.01	1	0	4.5	0.11	0.6
II-2-B-β	6.3	0.01	-6	0.07	--	0.85	4.8
II-2-B-α-1	7.5		1	0	--	0.95	5.4
II-2-B-α-2	7.5		1	0	--	1.65	9.3
III	4.6	0.04	-15	0.11	3.4	9.69	54.6
III-1	4.6	→0	1	0	--	0.86	4.8
III-2-A-1	4.6	→0	1	0	--	0.46	2.6
III-2-A-2	4.6	→0	1	0	--	8.25	46.7
IV	Supernatent				--	0.54	3.1
Total yield							84.3

TABLE XXV.

Distribution of the egg white proteins into fractions.
Fractionation Experiment F-13.

Fraction	Ovalbumin (1)*	Ovalbumin (2)**	Ovomucoid (1)	Ovomucoid (2)	Globulin (1)	Globulin (2)	Conalbumin (1)	Conalbumin (2)	Lysozyme (1)	Lysozyme (2)
Egg white	64.9	--	9.2	--	8.7	--	13.8	--	3.4	--
II-1	13.4	1.2	9.5	6.1	61.0	41.5	2.9	1.2	12.4	21.7
II-2-B-α-1	14.7	1.2	2.1	1.2	17.9	11.0	41.0	16.2	19.0	20.0
II-2-B-α-2	19.4	2.8	0	0	5.4	5.8	75.8	52.0	0	0
III-2-A-2	93.7	67.2	2.1	10.4	2.9	15.5	1.5	5.0	0	0
IV	11.1	0.5	88.9	29.3	0	0	0	0	0	0
Total yield		72.9		47.0		73.8		74.4		41.7

* (1) Percent of constituent in the fraction.

** (2) Percent of constituent in the fraction of that originally present in the egg white.

fractions needs to be improved. From the results here, it is suggested that lysozyme should be removed from this fraction prior to any other component. This will give lysozyme preparations in higher yields and eliminate the possibility of further interactions in the system. This should be followed by the separation of the globulin as described for Fraction II-1 (Figure 15). Further subfractionation should be governed by the purity of the fractions obtained.

The ovalbumin separation is satisfactory as described. Increased yields of ovalbumin can be accomplished by inclusion of the supernatents from the Fraction II purification procedures. Further investigations to determine suitable conditions for crystallization of the various protein components from ethanol-water mixtures is suggested.

The procedure whereby ovomucoid is recovered from the supernatant from the ovalbumin separation is satisfactory. Increased yields would result from more extensive washing of earlier fractions and inclusion of supernatants from purification steps. It might be desirable to precipitate ovomucoid from its solutions and eliminate the troublesome concentration procedures.

Several possibilities for improving the comprehensive fractionation of egg white as presented in this thesis can

be suggested:

1. Intensive studies should be carried out to improve the procedures for quantitative estimation of lysozyme, ovomucoid, conalbumin and avidin.
2. Yields would be improved by reworking washings and purification supernatents.
3. Solubility studies on purified globulin should be made.
4. Further electrophoretic experiments at other pH levels might improve analysis of the conalbumin-globulin fraction.
5. Positive identification of the riboflavin binding component might eliminate the discrepancies observed in the conalbumin-globulin sub-fractionation.
6. Due to the high temperature coefficient of solubility exhibited by several of the egg white proteins, a marked improvement would result from the use of centrifugation equipment capable of being maintained at any desired temperature. A high speed centrifuge with a capacity of one liter of solution, and capable (according to the manufacturer's claims[1]) of holding temperatures

1. Ivan Sorvall, Inc., New York, New York.

within one degree at temperatures as low as any employed in these fractionation procedures has recently become available.

In view of the successes in the large scale processing of plasma under similar conditions, it appears quite possible that the procedures described will be applicable to pilot plant production of egg white proteins. Processing of the egg white on a large scale would probably increase yields. The greatest loss of protein results from mechanical losses during centrifugation and clarification. These losses are rather constant and independent of the volume being processed; hence percentage yields increase with increased volume. High speed centrifugation for the removal of ovomucin might be difficult to obtain but continuous operation centrifuges are available for the other separations. The advantages to be gained from production of sizeable quantities of the egg white proteins have been previously discussed.

SUMMARY AND CONCLUSIONS

1. An extensive electrophoretic investigation of the protein composition of egg white has been completed. The data from thirty-one electrophoresis runs, made under similar conditions, have been analyzed statistically. The source and magnitude of the individual errors are discussed.
2. The presence of ovomucin and the age of the egg are shown to have no detectable influence on the electrophoretic patterns of egg white. Evidence is presented to support the view that the ovomucin fibers are cut into smaller fragments during treatment in a Waring Blender than in a hand homogenizer.
3. Changes in pH and ionic strength result in only slight modification of the apparent electrophoretic composition but markedly affect the mobilities of the egg white proteins.
4. No significant differences were observed in electrophoretic analyses of egg white from six lots of chickens with widely different ancestry.
5. Dialysis of egg white results in the removal of a dialyzable carbohydrate, presumably glucose. Under

the conditions employed, dialysis of undiluted egg white proceeds so slowly that it is impractical on a large scale operation.

6. No evidence for interaction was found when appropriate mixtures of lysozyme with conalbumin, ovomucoid, or ovalbumin were studied electrophoretically.

7. A method for the comprehensive fractionation of egg white in media of low dielectric constant and low ionic strength is described and suggestions for its improvement offered. Several advantages are claimed for this method over those previously reported for preparing egg white fractions:

    a. Ovomucin has been removed by super-centrifugation with a minimum of alteration and occlusion of other proteins.

    b. The ethyl alcohol acts as a foam depressant so that the Sharples super-centrifuge can be used for continuous operation.

    c. The advantages previously described for the preparation of the plasma proteins are applicable to the egg white fractionation.

8. Once lysozyme has been crystallized it can be recrystallized with 10% ethanol.

9. An improved method for the preparation of conalbumin is described. The possibility that the riboflavin of egg white is not bound to the main conalbumin component is suggested.
10. Ovalbumin prepared by these procedures is readily crystallized by the use of ammonium sulfate.
11. Evidence is given to support the view that two globulins, with different solubility characteristics, are present in egg white.

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