THE ISOLATION, FRACTIONATION AND ELECTROPHORETIC CHARACTERIZATION OF THE GLOBULINS OF MUNG BEAN (PHASEOLUS AUREUS)

By

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A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1951

ACKNOWLEDGMENT

To Prof. C. D. Ball

Appreciation is gratefully extended for his stimulating guidance and helpful criticism in the accomplishment of this work.

To Dr. H. A. Lillevik

His valuable assistance and advice in this work is likewise deeply appreciated.

To Michigan State College

For granting the opportunity to carry out this study the author is grateful.

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INTRODUCTION

Separation of proteins from biological systems and their purification as chemical substances have been the concern of biological chemists for over a century (1). Data are available on the purified proteins of many of the common seeds of commercial importance. It is surprising that little is known about the proteins of Mung bean, Phaseolus aureus, an economically important crop in Asiatic countries which has become an acknowledged crop in the United States. The object of this work was devoted to: 1) a study of the development of a method in order to produce and reproduce homogeneous fraction or fractions, and 2) particular care of the products to keep them as nearly as possible in their natural state. The preparation of undenatured proteins is in many respects a special art. The procedures when developed are generally simple but the choice of the best conditions from among the variety of possibilities is difficult, and every step involves rigorous attention to detail in order to maintain the protein in an undenatured state.

The large dimensions and unique structure of these highly organized protein molecules render them particularly labile. Exposure to heat, to high acid or alkaline conditions, or to conditions of low dielectric constant brings about changes in the spatial arrangements of the reactive groups which alter, and may completely destroy, native properties. The phenomenon called denaturation may be noted as a change in solubility, as a change in molecular shape, as a change in chemical reactivity or of immunological specificity. The spatial relations between free reactive

groups of the native protein may be distorted or destroyed (2), (3), (4).

The procedure used for the separation of Mung bean globulins was extraction of uniformly ground meal with sodium chloride solution. The extract was diluted in conformity with globulin preparations with distilled water and the precipitated protein was thus obtained. Separation of the precipitate from the solvent was accomplished by ordinary centrifugation. The exposure of protein to the large air interfaces of the Sharples centrifuge was avoided. The foaming produced by Sharples centrifuges indicates denaturation of protein. Since the Mung bean globulins are insoluble at low concentrations of neutral salt solvent, the adjusting of the pH of the extract for precipitation was not necessary. With this procedure a buffer was not used for the extraction and an acid was not employed for the adjusting of the pH of the extract for precipitation. Protein sub-types, such as glutelins and globulins, when they occur together are readily separated since the globulins dissolve in dilute salt solutions whereas the glutelins are insoluble in all neutral solvents. The protein which precipitated was regarded as globulin, whereas the albumins remained in solution (5), (6*), (7), (8).

It was very important to discover that the Mung bean globulins have

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*Protein of the wheat and other cereal seeds contain a similar complement of proteins, i.e., glutenin, gliadin, globulin and albumin. The glutenin and gliadin make up the bulk (90%) of the protein of cereal seeds, whereas the seeds of dicotyledonous plants in general contain globulins as their principal protein components.

their highest solubility in 0.4M NaCl and are insoluble at the concentration of 0.08M NaCl. This soluble-insoluble range permits a purification scheme of the globulins by repeated dispersion in 0.4M NaCl solution and precipitation at a concentration of 0.08M by dilution with distilled water. The soluble-insoluble range of protein must be determined before fractionation or purification can be accomplished.

The aim of this study was the isolation of the protein in a state close to its state in nature. The stability and solubility of protein are usually greater in dilute salt solutions than in water or in dilute acids or bases (9), (10). Mung bean globulins, which were highly soluble in the dilute NaCl solution, were also highly protected from denaturation because the dielectric constant of the medium was high. That the stability of proteins is greater in solutions of greater dielectric constant has long been recognized (10). With this procedure the globulins are brought to a relatively inert solid state by diluting as rapidly as possible to 0.08M NaCl, They are maintained insoluble at low temperature, and in this state reactive groups are protected from each other (protein-protein reaction) and from the enzymes for which they are the substrate (11).

In the course of the study of the purification of the isolated protein by dialysis, the need for a rapid method of membrane-equilibration dilution became apparent. A rotating "outside-liquid" dialysis apparatus was constructed. The separation and purification of Mung bean globulin was undertaken with the membrane-equilibration procedure. With the indirect slow step-wise dilution technique the fractionation of globulin

was made possible. Extensive electrophoretic studies were made for the characterization of the total globulin as well as the isolated fractions.

HISTORICAL

That certain proteins were soluble in saline solutions was first observed by Denis in 1859 (12). This observation was later confirmed by Hoppe-Seyler. Denis noted that when sodium chloride extracts were made of both animal and vegetable tissues, certain protein materials were dissolved.

During the last decade of the nineteenth century Osborne (13) did the greater part of his outstanding work on the isolation of the proteins of seeds. He demonstrated a remarkable appreciation of the fundamental relationship between the solubility of the protein and the salt content, the acidity, and the temperature of the solvent and brought about his separations of the protein components of the extracts from seed by well conceived manipulations of these factors. In 1902 (14) he published one paper on the basic character of the protein molecule in which he showed that edestin, a typical seed globulin, enters into ionic reactions with acids to form true salts. In 1905 (15) he published a solubility curve of edestin in salt solutions which showed both the ascending limb of the salting-in and the descending limb of the salting-out effects. These were pioneering attempts to formulate the principles of solubility upon which all of his protein isolation studies had been predicated.

In 1896 Osborne and Campbell (16) studied six distinct proteids (globulins) from ten different seeds.

In 1897 Osborne and Campbell reported on studies of the proteids of

lupine seed (17), the castor bean (18), the sunflower seed (19), the cow pea (20) and the white podded adzuki bean (21). Dilute salt solution extracted very little globulin material from lupin meal but large quantities were obtained with stronger solutions. It was suggested that the two globulin fractions recovered by dialysis and direct dilution may have been the same but that there was a probable combination of some sort between the globulin and other constituents of the seed.

Comparisons of the castor bean, sunflower seed and the hemp seed globulins, which show similar composition and a property of being partly insoluble and partly soluble in a saturated solution of sodium chloride, led to further study of the castor bean seed globulins. They found that addition of a small quantity of acid caused such changes in the castor bean fraction that had been soluble in saturated salt solution so that it behaved much like the insoluble fractions.

It was the opinion of these authors that the sunflower contained as its principal protein (called proteid by them) the globulin edestin, but that which they recovered was mixed with helianthotannic acid from which they did not succeed in bringing about a complete separation.

The chief protein of the cow pea was found to be a globulin that closely resembled the leguminin of the pea and vetch. Fractional procedures involving redissolving in brine and precipitation by direct dilution and dialysis disclosed a second globulin which resembled phaseolin.

The composition of the globulin extracted from adzuki beans was found to be identical to that obtained from the white bean, Phaseolus vulgaris.

Later in the same year Osborne (22) reported results of further investigation as to the amount and properties of the proteins of the maize kernel. One water-extracted protein, precipitated by dialysis, he indicated as a globulin named maysin. Prolonged dialysis yielded yet another small fraction which resembled edestin.

In 1898 Osborne and Campbell reinvestigated the protein constituents of the pea (23) and vetch (24) after repeated fractional precipitation of the globulins from the seeds of the horse bean (25) and lentil (26) resulted in two fractions, one of which coagulated at 100°C. When the previously reported legumin of the pea was separated from contaminating vicilin, they found that it closely resembled the legumin of vetch.

A careful comparison of the reactions and properties of the proteins of the pea, lentil, horse bean and vetch were reported in a separate paper (27). Legumin is a globulin which dissolved readily in saline solution and was precipitated therefrom either by dialysis, dilution or cooling. Vicilin was a globulin they found associated with legumin in the pea, lemtil and horse bean but none was obtained from vetch. In salt solution it was the more soluble globulin which fact made separation of the two possible. This fraction was completely coagulated at 100°C.

Two varieties of soy beans were studied by Osborne and Campbell (28). They named the chief protein constituent glycinin, a globulin somewhat similar to legumin. The more soluble globulin, which resembled phaseolin in composition, remained in the supernatant after glycinin was removed.

Osborne and Harris (29) were the first to study pure proteins in relation to their solubility in solutions of different salts. They

investigated the action of salts on the globulin edestin obtained from hemp seed. Chlorides of monovalent bases, sodium and potassium and caesium, were found to have very nearly the same solvent power. The divalent bases, barium, strontium, calcium and magnesium dissolved approximately twice as much edestin as the monovalent chlorides. The solubility in general was observed to be dependent on the nature of the metal, the divalent metallic chlorides were found to have more dispersing power. Lithium chloride proved to be the only exception; its solvent power was much less than that of the other monovalent chlorides. The effect of sulfate on the solubility of the globulin was very similar to that of the corresponding chlorides of the divalent metals.

During the years 1916 - 1927 Jones and Johns together and with other investigators studied the proteins from a number of seeds. Two globulins, canavalin and concanavalin, were isolated from the jack bean by Jones and Johns (30). Fractionation of the sodium chloride extracts with variable saturations of ammonium sulfate produced the two globulins.

Johns and Jones (31) extracted air-dried oil-free peanut meal with a 10 percent sodium chloride solution at several different temperatures and found that the yield of protein obtained was not apparently temperature dependent. A major globulin fraction was obtained by partial saturation of the extract with ammonium sulfate and a minor fraction by complete saturation of the filtrate.

The principal protein was recovered from the velvet bean by Johns and Finks (32). The globulin was obtained by dialysis of a sodium chloride extract in parchment bags suspended in running water.

Johns, Finks and Gersdorff (33) reported that the principal protein of the coconut endosperm was a globulin. The globulin was recovered by dialysis for 7 to 10 days of a ten percent sodium chloride extract.

Johns and Waterman (34) studied the proteins of the Mung bean, Phaseolus aureus Roxburgh. They found a five percent sodium chloride solution the most effective extractant. Part of the protein material was removed from the extract by heat coagulation at 40°C., and two other fractions were precipitated at high temperatures (71°C. and 100°C. respectively) which indicated an albumin and two globulin fractions. The globulin fraction was further separated by variations in saturation of ammonium sulfate.

The globulin of the cohune nut was isolated by Johns and Gersdorff (35). Extraction experiments were made with different concentrations of sodium chloride in water, with 70 percent alcohol and with one percent hydrochloric acid and extracting for one hour in each case. Ten percent sodium chloride was found to extract the maximum amount of protein. Dialysis of the extract yielded a higher percentage of globulin than partial saturation with ammonium sulfate.

The soluble salts occurring naturally in the lima bean seed were found by Jones et al. (36) to be sufficient to dissolve 15.15 percent of the protein when water was the extractant. This was practically as much as was obtained by a three percent sodium chloride solution which was determined as the most efficient concentration of the salt. Two globulins were separated by fractional precipitation.

In a study of the proteins of wheat bran by Jones and Gersdorff (37)

a globulin was one of the three recovered. Bran ground to pass a 40 mesh sieve was found to be as effective of extraction as that ground to pass a 100 mesh sieve. Seven globulin preparations were recovered from 4 percent sodium chloride extracts by various methods but direct dialysis against water and acidification with carbon dioxide gas followed by dilute acetic acid was productive of the largest quantity of globulin.

By dialyzing or by addition of ammonium sulfate to saline extracts of white rice flour, Jones and Gersdorff (38) were able to isolate a protein fraction which consisted of two globulins, coagulating at 74° and 90°C. Ammonium sulfate fractionation was not possible since both precipitated too closely together so fractional heat coagulation was the means by which they were separated.

The principal protein of the seed of the silver maple was isolated by Anderson (39) and found to be a globulin. It was extracted with ten percent sodium chloride and precipitated from the extract with saturated ammonium sulfate.

Gortner, Hoffman and Sinclair (40) were the first to emphasize the effect of the particular ion of various salt solutions on the peptization of proteins. Samples of wheat flour were studied at almost constant hydrogen ion concentrations with different salts and a marked lyotropic effect was observed. It was evident that the hydrogen ion concentration per se could not have been playing the major role, and that such peptization differences as they observed must be attributed to some other factor. They found a definite lyotropic effect of the order $KF < KCl < KBr < KI$. The same relative order held for all the salts studied. In conclusion

they remarked that "there is a lyotropic series of ionic effects in an aqueous systen of protein and salts, and that these effects are due to properties of the anion and the cation of the salt and are measurable even at a constant hydrogen-ion concentration."

Bishop (41) observed that the yield of nitrogen as salt soluble from barley flour was increased when the particle size was decreased. Staker (42), in a study of the peptization of seed protein, thought that whenever possible samples should be ground to pass through a 100 mesh sieve.

Crystalline globulin was prepared from seeds of pumpkin and squash by Vickery et al. (43). Air-dried meals were extracted with warm sodium chloride solution. Albumin was removed by heat coagulation. By diluting with four volumes of warm water (60°C.) globulin precipitated when the solution came to room temperature. By further dilution with cold water and on standing in a cold room another fraction of globulin precipitated in crystals.

Quensel (44) extracted barley meal with NaCl buffered with phosphate to pH 7.0. Fractional precipitation of the extract was accomplished with solid ammonium sulfate. The globulins were separated from the albumins, low molecular and poly-dispersed material by dispersion of the precipitate in NaCl solution and subsequent dialysis against distilled water. The method of characterization of the globulin components was based on the ultracentrifugation sedimentation constants, which confirmed the presence of four globulins.

Vassel (45) isolated two globulins, linin and conlinin, from linseed

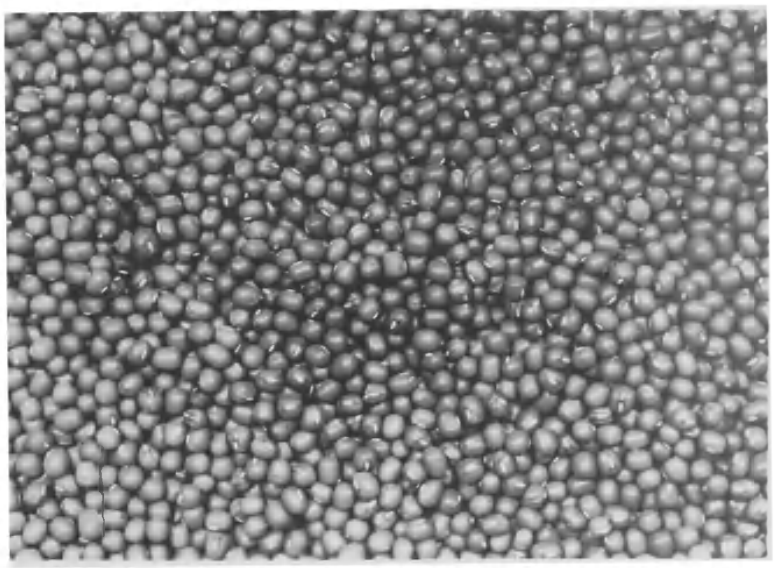
TABLE XXXXI. ELECTROPHORETIC MOBILITY CALCULATIONS

Globulin Fraction	Run No.	pH	d_1 Ascending (cm)	d_2 Descending (cm)	q cross section area of cell (cm)	k_c at 1°C.	R Resistance of protein solution in ohms
$G_2(17)$	114	3.28	2.67	2.40	0.3	.0048937	80
	115	3.90	2.07	1.93	0.3	.0048937	76
	116	4.36	2.16	2.03	0.3	.0048937	68
	120	4.61	1.14	1.09	0.3	.0048937	69
	121	7.15	0.85	0.78	0.3	.0048937	72
	119	7.48	1.65	1.59	0.3	.0048937	72.5
	122	7.78	2.62	2.94	0.3	.0048937	74.5
$G_2(14)$	124	3.27	2.19	2.02	0.3	.0048937	81
	128	3.85	2.21	2.19	0.3	.0048937	72.3
	126	4.44	1.52	1.45	0.3	.0048937	65
	127	4.78	0.92	0.95	0.3	.0048937	69.5
	136	7.27	1.96	1.71	0.3	.0048937	78
	130	7.52	2.27	2.45	0.3	.0048937	77
	129	7.75	1.69	2.14	0.3	.0048937	62.7
$G_3(11)$	97	3.34	2.91	2.60	0.3	.0048937	62.6
	96	3.80	2.80	2.73	0.3	.0048937	70
	99	4.40	1.21	1.07	0.3	.0048937	53.5
	105	6.15	1.87	2.06	0.3	.0048937	74
	104	6.63	2.77	2.85	0.3	.0048937	78
$G_4(07)$	137	3.32	2.64	2.42	0.3	.0048937	76.5
	138	3.90	1.75	1.67	0.3	.0048937	70.3
	139	4.21	1.43	1.41	0.3	.0048937	64
	140	4.52	1.14	1.32	0.3	.0048937	68.5
	152	7.30	1.65	1.67	0.3	.0048937	58.4
	142	7.50	2.27	2.29	0.3	.0048937	74.5
	153	7.78	1.91	1.82	0.3	.0048937	60.5

was dispersed in normal sodium acetate at 40°C., crystals formed as the temperature reverted to room temperature. The isoelectric point of this fraction, pH 5.5, was determined by titration.

Crystalline globulin was recovered from fresh tomato juice by Carpenter (49). The juice was concentrated to a syrup (35-40 percent) by freezing out the water. It was then subjected to dialysis and an amorphous precipitate formed in the cellophane sac. The globulin was dissolved in molar sodium chloride at 50°C. and when five volumes of water at 50° were added, precipitation occurred. After standing at 0°C. for 48 hours, the globulin was found to be crystalline.

A careful study of the literature is important in order that we build for the future on the broad foundation of past experience. At present it is still necessary to treat plant proteins as a group. The next phase of investigation may reveal how they can be treated as individuals, that is, as specific substances.



The Mung bean, Phaseolus aureus, is a summer annual legume, belonging to the field bean family. It is a native of Asia and was known in the United State as early as 1835 under the name of Chicksaw pea, but it has not been until recent years that it has become an acknowledged crop, rapidly gaining in popularity.

The Mung bean seed is about one half the size of soybean seed. It is globose or oblong in shape and most varieties are green in color, but others are yellow, brown and marble black. Photograph by courtesy of the Cornell Seed Co., Saint Louis, Missouri

THE MATERIAL

The seeds* used in these experiments were ground to pass through a 60 mesh sieve. After grinding, the meal was thoroughly mixed so that uniform aliquots could be obtained.

The meal was analyzed for moisture, ash, total nitrogen, lipide and crude fiber, as described in the methods of analysis (50), with some modifications as noted below.

For the moisture determination 10 gram samples were dried at 101°C. to constant weight with a Brabender's moisture tester. It was found that the ground meal contained 8.20 percent water (Table 1 and Figure 1).

In the determination of the ash content (51), 5.000 gram samples of meal were put into each of four weighed and marked porcelain crucibles. The samples were charred over an open flame under a hood, and then transferred to an electric furnace and heated at 650°C. until all carbon was oxidized. The crucibles and contents were cooled in a desiccator and then weighed. The average ash content for the four samples was 3.098 percent (Table 2).

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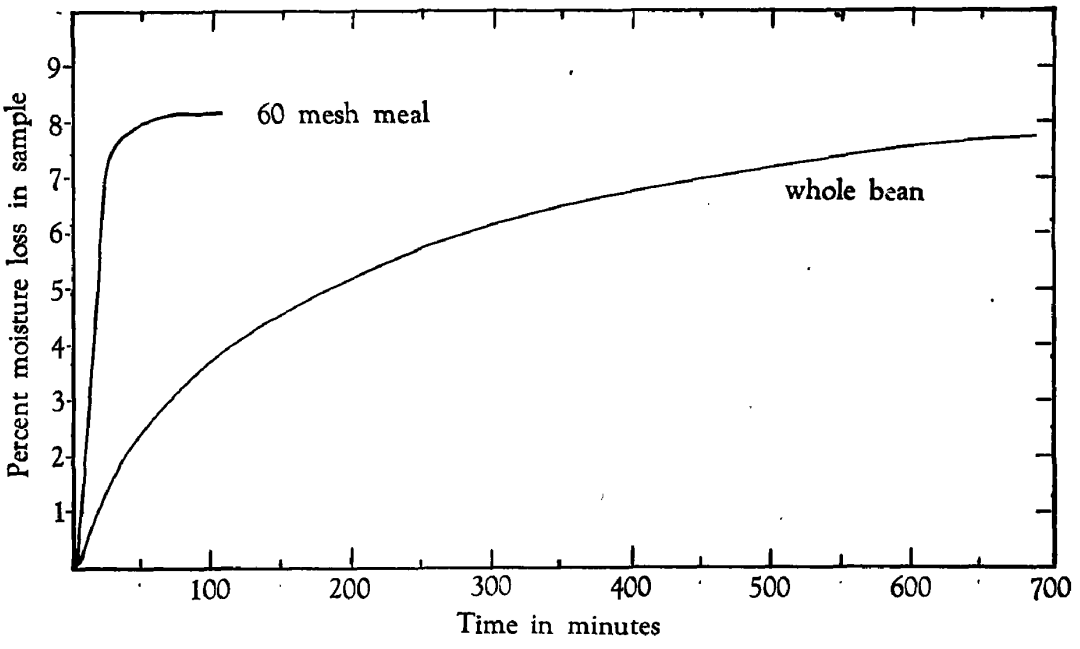
*The seeds were supplied by courtesy of the Johnston Seed Company, Enid, Oklahoma. The sample used was Johnston Jumbo type of the 1948 crop and had not been processed in any way and were in their natural state when received. The seeds were bright in color and fully matured.

TABLE I

DETERMINATION OF MOISTURE CONTENT OF 60 MESH MUNG BEAN MEAL AND WHOLE BEANS WITH BRABENDER MOISTURE TESTER AT 101°C.

Time in Minutes	10 gm. Meal % Water Loss	10 gm. Meal % Water Loss	10 gm. Whole Bean % Water Loss	10 gm. Whole Bean % Water Loss
10	5.3	5.6	0.8	0.7
20	7.1	7.3	1.4	1.2
30	7.5	7.6	1.8	1.6
40	7.8	8.0	2.3	2.2
50	8.0	8.1	2.6	2.5
60	8.1	8.2	2.8	2.8
70	8.2	8.2	3.2	3.1
80	8.2	8.2	3.4	3.3
90	8.2	8.2	3.6	3.5
100	8.2	8.2	3.7	3.6
120			4.2	4.1
140			4.5	4.4
160			4.7	4.7
180			5.0	5.0
200			5.2	5.2
220			5.45	5.4
240			5.7	5.7
260			5.8	5.8
280			6.0	6.0
300			6.2	6.2
320			6.3	6.3
340			6.45	6.4
360			6.55	6.5
380			6.65	6.6
400			6.75	6.7
420			6.8	6.8
440			6.9	6.9
460			7.0	7.0
480			7.1	7.1
500			7.2	7.2
520			7.25	7.25
540			7.3	7.3
560			7.4	7.4
580			7.5	7.5
600			7.55	7.55
640			7.6	7.6
700			7.6	7.6

Figure 1.



The effect of grinding Mung bean through 60 mesh size screen upon the rate of drying to constant weight at 101° C as determined by the Brabender's Moisture Tester.

TABLE II

DETERMINATION OF ASH CONTENT OF MUNG BEAN
(Air Dried)

Sample		1	2	3	4
Wt. crucible	gm.	22.1080	22.6538	22.3886	23.1850
Wt. sample	gm.	5.0000	5.0000	5.0000	5.0000
Total wt.	gm.	27.1080	27.6538	27.3886	28.1850
Wt. crucible and ash	gm.	22.2622	22.8093	22.5441	23.3394
Loss in wt. (Organic matter)	gm.	4.8458	4.8445	4.8445	4.8456
Wt. of ash	gm.	0.1542	0.1555	0.1555	0.1544
% of ash content		3.084	3.11	3.11	3.088

The total nitrogen content of air-dried meal was determined by the Kjeldahl method (52). The results obtained from four 2 gram samples were 3.788 percent total nitrogen of the air-dried meal and 4.127 percent total nitrogen calculated on dry weight of the samples (Table III).

TABLE III

TOTAL NITROGEN CONTENT OF MUNG BEAN MEAL

	Grams Sample	Kjeldahl Titration ml. HCl N 0.1007	% Nitrogen Air-dried Sample	% Nitrogen Calculated on Dry Wt. Basis
1.	2.0000	53.75	3.788	4.127
2.	2.0000	53.65	3.781	4.119
3.	2.0000	53.80	3.792	4.131
4.	2.0000	53.80	3.792	4.131
Average		53.75	3.788	4.127

The lipide content of Mung bean meal was determined. Four 10 gram samples (weighed to within 0.1 mg.) of 60 mesh meal were extracted with 125 ml. 95% ethanol in a Soxhlet Extractor over the aluminum shaving bath at 100°C. This was followed by a 10 hour extraction with ethyl ether and then another 10 hour extraction with 125 ml. of 95% ethanol. The combined extracts were evaporated to dryness and the residue was exhaustively extracted with petroleum ether as described by Dill (53). Table IV shows the average value of 0.809 percent lipide content.

TABLE IV

DETERMINATION OF LIPIDE CONTENT OF MUNG BEAN MEAL BY SOXHLET EXTRACTOR USING 10.0 GRAM SAMPLES

Sample	1	2	3	4
Wt. of beaker gm.	41.8234	47.5004	45.7700	50.5056
Wt. beaker/lipide gm.	41.9049	47.5816	45.8510	50.5856
Wt. of lipide gm.	0.0815	0.0812	0.0810	0.0800

The fiber content of Mung bean meal was determined on four 2-gram samples of air-dried meal (Table V). The procedure (54) is outlined as follows:

1. A thin layer of asbestos was prepared in the bottom of a Gooch crucible connected to a filter flask. Water was added to the crucible to make a uniform and firm layer of asbestos.

2. Two grams of air-dried sample were weighed on a piece of paper and introduced into the crucible.

3. The sample was washed twice with 10 ml. hot 95% ethanol to remove the moisture and some of the lipide.

4. The sample was washed once with cold ethanol to bring the temperature down to room temperature and washed again twice with 10 ml. ethyl ether to remove the major portion of lipide.

5. The crucible and contents were placed in a 600 ml. beaker and 200 ml. of 1.25% H_2SO_4 was added. This was boiled gently for 30 minutes. The beaker was covered with a round bottomed flask which contained water and served as a condenser.

6. At the end of 30 minutes the solution was carefully filtered through a 15 cm. piece of linen cloth fitted into a 4 inch funnel. The residue was rinsed with hot water to wash out the acid. The filtrate was discarded.

7. The residue was transferred from the linen cloth into the 600 ml. beaker using 200 ml. of 1.25% NaOH in a wash bottle to make the transfer. This was boiled for 30 minutes with the reflux condenser as previously described.

8. The material in the beaker was filtered through the same linen cloth and washed with hot water.

9. The residue was transferred to a beaker. The contents in the beaker were then transferred into a Gooch crucible and washed with hot water.

10. The Gooch crucible and its contents were dried for 2 hours at 100°C. then cooled in a desiccator and weighed.

11. The Gooch crucible was ignited for 2 hours at 650°C., cooled in a desiccator and weighed. The difference between the two weights was crude fiber.

TABLE V

DETERMINATION OF FIBER OF MUNG BEAN MEAL

Sample	1	2	3	4
Wt. Gooch crucible, asbestos and fiber (dried)	13.1219	12.8734	14.0304	13.2312
Wt. after ignited 2 hrs. at 650°C.	13.0332	12.7832	13.9434	13.1420
Loss in wt.	.0887	.0902	.0870	.0888

The average of the four samples was 0.0887 gm. crude fiber. The

average percentage of crude fiber on the air-dried sample was 4.435%.

The nitrogen-free extract is composed of sugars, starch and in large part, material classed as plant cellulose and polysacchrides. The nitrogen-free extract was calculated by subtracting the sum of water, ash, protein, fat and crude fiber of the sample from 100. Since the figure was determined by calculation instead of directly, it includes the cumulative errors of the other determinations and thus is not an exact value. From the previous determinations the constituents of a given sample of Mung bean meal are as follows:

Moisture	8.2%
Ash	3.09%
Protein (N x 6.25)	23.69%
Lipide	0.91%
Crude fiber	4.43%
Nitrogen-free extract	59.76%

EXTRACTION

A survey of the literature revealed important information about the action of certain salts on the peptization of seed proteins (29) (40) (42) and some factors which effect peptization (31) (41) (46). The author's attention has been focused on a more systematic investigation of this field of study. It became apparent that the fundamental relationship between the solubility of the Mung bean protein and the nature and amount of salt content of the solvent must first be studied in detail.

Extraction of the Mung bean protein, expressed as total nitrogen, from (60 mesh) Mung bean meal with various concentrations of chlorides, sulfates, phosphates and carbonates of sodium and potassium was carried out and the results of these extractions have been reported in terms of percent of total nitrogen extracted and have been plotted against pμ, the negative logarithm of the ionic strength of the solvent.

The salts c.p. used for extraction are as follows:

NaCl	Na_3SO_4	Na_2HPO_4	Na_2CO_3	Na_2SO_3
KCl	K_2SO_4	K_2HPO_4	K_2CO_3	

One liter of a one molar solution was made of each of the above salts at 25°C. as stock solutions. Molar solutions of HCl and NaOH were also made for purposes of comparison. Appropriate dilutions of these molar solutions were made at 25°C. to prepare M/10, M/100 and M/1000 solutions.

METHOD.- 1. Five grams of the air-dried 60 mesh Mung bean meal previously described was carefully weighed and introduced into a 250 ml. centrifuge

bottle. Twelve glass beads were added as agitators.

2. Fifty ml. of the desired concentration of solvent were added to the reaction bottle. The sample-solvent weight-volume ratio was thus 1:10. Extraction consisted of shaking at a low speed (120 oscillations per minute) for exactly 30 minutes. Six bottles were placed at one time in a six-hole wooden block mounted on the shaking machine.*

3. At the end of 30 minutes the bottles were removed from the shaker and immediately centrifuged for 15 minutes at 2000 r.p.m. The solid material was packed in the bottom of the bottles and the clear liquid extracts were poured into Kjeldahl flasks for total nitrogen determinations (52).

The results obtained from the total nitrogen determination were calculated in terms of percentage of nitrogen extracted per total nitrogen content of sample. Calculations were reported on the dry weight basis. In order to determine the percentage of nitrogen extracted per total nitrogen content of the sample, a series of determinations were made in each case on the extract and in the case of Na_2SO_3 were also made on the corresponding residues.

All the extractions were conducted at room temperature which was approximately 25°C. However, the exact room temperature was recorded on the date when the experiment was done. The solutions were made to volume at 25°C. in a water bath.

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*Cenco-Meinzer Laboratory Shaker

The results of these extraction are shown in Tables VI through XIX and in Figures 2 through 4. In the tables the ionic strength is also expressed as the negative logarithm of the ionic strength and is symbolized by the expression pu.

TABLE VI

SODIUM CHLORIDE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. NaCl	Ionic Strength	Log of Ionic Strength	μ	Kjeldahl Titration 0.2027N HCl	% N per Total N air dried	% N per Total N dry wt.
1.0	1.0	0.0	0.000	42.8	63.10	69.28
.9	.9	- .046	.046	43.3	63.85	69.55
.8	.8	- .097	.097	43.8	64.59	70.36
.7	.7	- .155	.155	44.1	65.04	70.85
.6	.6	- .222	.222	44.7	65.94	71.83
.5	.5	- .301	.301	45.1	66.54	72.48
.4	.4	- .398	.398	45.2	66.69	72.65
.3	.3	- .523	.523	44.5	65.64	71.50
.2	.2	- .699	.699	42.8	63.10	68.74
.1	.1	-1.000	1.000	38.85	57.19	62.19
.09	.09	-1.046	1.046	26.7	39.02	42.51
.08	.08	-1.097	1.097	24.9	36.33	39.58
.07	.07	-1.155	1.155	23.2	33.79	36.81
.06	.06	-1.222	1.222	21.9	31.85	43.69
.05	.05	-1.301	1.301	20.7	30.05	32.73
.04	.04	-1.398	1.398	19.8	28.71	31.27
.03	.03	-1.523	1.523	19.0	27.51	29.97
.02	.02	-1.699	1.699	19.3	27.96	30.45
.01	.01	-2.000	2.000	19.85	28.78	31.35
.009	.009	-2.046	2.046	20.3	29.45	32.08
.008	.008	-2.097	2.097	20.35	29.53	32.17
.007	.007	-2.155	2.155	20.45	29.68	32.33
.006	.006	-2.222	2.222	20.45	29.68	32.33
.005	.005	-2.301	2.301	20.5	29.75	32.41
.004	.004	-2.398	2.398	20.5	29.75	32.41
.003	.003	-2.523	2.523	20.55	29.83	32.49
.002	.002	-2.699	2.699	20.9	30.35	33.06
.001	.001	-3.000	3.000	20.9	30.25	33.06
.0009	.0009	-3.046	3.046	21.3	30.95	33.71
.0008	.0008	-3.097	3.097	21.4	31.10	33.88
.0007	.0007	-3.155	3.155	21.6	31.40	34.20
.0006	.0006	-3.222	3.222	21.6	31.40	34.20
.0005	.0005	- 3.301	3.301	21.6	31.40	34.20
.0004	.0004	-3.398	3.398	21.6	31.40	34.20
.0003	.0003	-3.523	3.523	21.7	31.55	34.36
.0002	.0002	-3.699	3.699	21.6	31.40	34.20
.0001	.0001	-4.000	4.000	21.6	31.40	34.20
water blank				21.5 0.6	31.23	34.04

TABLE VII

POTASSIUM CHLORIDE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. KCl	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.2072N HCl	% N per Total N air dried	% N per Total N dry wt.
1.0	1.0	0.0	0.000	41.5	61.16	66.62
.9	.9	- .046	.046	42.3	62.35	67.92
.8	.8	- .097	..097	43.6	64.30	70.04
.7	.7	- .155	.155	42.2	65.19	71.02
.6	.6	- .222	.222	44.5	65.64	71.50
.5	.5	- .301	.301	44.2	65.19	71.02
.4	.4	- .398	..398	44.1	65.04	70.85
.3	.3	- .523	.523	43.0	63.40	69.06
.2	.2	- .699	.699	41.85	61.68	67.19
.1	.1	-1.000	1.000	41.8	61.60	68.54
.09	.09	-1.046	1.046	39.8	58.61	63.85
.08	.08	-1.097	1.097	38.2	56.22	61.24
.07	.07	-1.155	1.155	36.1	53.08	57.82
.06	.06	-1.222	1.222	30.2	44.26	48.21
.05	.05	-1.301	1.301	25.0	36.48	39.75
.04	.04	-1.398	1.398	22.8	33.19	36.16
.03	.03	-1.523	1.523	21.3	30.95	33.71
.02	.02	-1.699	1.699	19.4	28.11	32.82
.01	.01	-2.000	2.000	18.5	26.76	29.15
.009	.009	-2.046	2.046	18.5	26.76	29.15
.008	.008	-2.097	2.097	18.6	26.91	29.32
.007	.007	-2.155	2.155	18.8	27.24	29.64
.006	.006	-2.222	2.222	18.9	27.36	29.80
.005	.005	-2.301	2.301	19.3	27.96	30.45
.004	.004	-2.398	2.398	19.6	28.41	30.94
.003	.003	-2.523	2.523	20.0	29.00	31.59
.002	.002	-2.699	2.699	20.4	29.60	32.24
.001	.001	-3.000	3.000	20.8	30.20	32.90
.0009	.0009	-3.046	3.046	21.0	30.50	33.22
.0008	.0008	-3.097	3.097	21.1	30.65	33.39
.0007	.0007	-3.155	3.155	21.1	30.65	33.39
.0006	.0006	-3.222	3.222	21.2	30.80	33.55
.0005	.0005	-3.301	3.301	21.2	30.80	33.55
.0004	.0004	-3.398	3.398	21.3	30.95	33.71
.0003	.0003	-3.523	3.523	21.3	30.95	33.71
.0002	.0002	-3.699	3.699	21.4	31.10	33.88
.0001	.0001	-4.000	4.000	21.4	31.10	33.88
water				21.5	31.25	34.04
blank				0.6		

TABLE VIII

SODIUM SULFATE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 24.6°C.

Molar Sol. Na_2SO_4	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.2027N HCl	% N per Total N air dried	% N per Total N dry wt.
1.0	3.0	0.477	-.477	32.4	47.55	51.79
.9	2.7	0.431	-.431	33.4	49.04	53.42
.8	2.4	0.380	-.380	34.55	50.76	55.30
.7	2.1	0.322	-.322	35.55	52.26	56.93
.6	1.8	0.255	-.255	36.4	53.53	58.31
.5	1.5	0.176	-.176	37.9	55.77	60.75
.4	1.2	0.079	-.079	38.6	56.82	61.90
.3	.9	- .046	.046	39.4	58.02	63.20
.2	.6	- .222	.222	44.75	66.01	71.91
.1	.3	- .523	.523	44.75	66.01	71.91
.09	.27	- .569	.569	45.4	66.99	72.97
.08	.24	- .620	.620	44.85	66.16	72.07
.07	.21	- .678	.678	43.75	64.52	70.28
.06	.18	- .745	..745	43.1	63.55	69.22
.05	.15	- .824	.824	42.4	62.50	68.09
.04	.12	- .921	.921	41.4	61.01	66.45
.03	.09	-1.046	1.046	38.0	55.92	60.92
.02	.06	-1.222	1.222	32.6	47.85	52.12
.01	.03	-1.523	1.523	26.65	38.95	42.43
.009	.027	-1.569	1.569	25.6	37.38	40.72
.008	.024	-1.620	1.620	25.2	36.78	40.06
.007	.021	-1.678	1.678	24.7	36.00	39.6
.006	.018	-1.745	1.745	24.1	35.14	38.27
.005	.015	-1.824	1.824	24.0	34.98	38.11
.004	.012	-1.921	1.921	23.5	34.24	37.30
.003	.009	-2.046	2.046	23.5	34.24	37.30
.002	.006	-2.222	2.222	22.3	32.44	35.34
.001	.003	-2.523	2.523	22.0	33.00	34.85
.0009	.0027	-2.569	2.569	21.6	31.40	34.20
.0008	.0024	-2.620	2.620	21.6	31.40	34.20
.0007	.0021	-2.678	2.678	21.5	31.25	34.04
.0006	.0018	-2.745	2.745	21.5	31.25	34.04
.0005	.0015	-2.824	2.824	21.5	31.25	34.04
.0004	.0012	-2.921	2.921	21.4	31.10	33.88
.0003	.0009	-3.046	3.046	21.5	31.25	34.04
.0002	.0006	-3.222	3.222	21.5	31.25	34.04
.0001	.0003	-3.523	3.523	21.4	31.10	33.88
water blank				21.5 0.6	31.25	34.04

TABLE IX

POTASSIUM SULFATE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. K_2SO_4	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.2027N HCl	% N per Total N air dried	% N per Total N Dry wt.
0.5	1.5	0.176	-.176	39.3	57.87	63.03
.4	1.2	0.079	-.079	41.4	61.01	66.45
.3	0.9	-0.046	.046	43.2	63.65	69.34
.2	0.6	- .222	.222	44.9	66.24	62.16
.1	.3	- .523	.523	45.0	66.39	62.32
.09	.27	- .569	.569	44.3	65.34	71.18
.08	.24	- .620	.620	44.4	65.49	71.34
.07	.21	- .678	.678	43.9	64.74	70.53
.06	.18	- .745	.745	42.8	63.10	68.74
.05	.15	- .824	.824	42.6	62.80	68.41
.04	.12	- .921	.921	40.5	59.66	64.99
.03	.09	-1.046	1.046	36.8	54.13	58.96
.02	.06	-1.222	1.222	30.2	44.26	48.21
.01	.03	-1.523	1.523	24.6	35.88	39.09
.009	.027	-1.569	1.569	24.9	36.33	39.58
.008	.024	-1.620	1.620	22.3	32.45	35.34
.007	.021	-1.678	1.678	22.2	32.29	35.18
.006	.018	-1.745	1.745	21.5	31.25	34.04
.005	.015	-1.824	1.824	21.5	31.25	34.04
.004	.012	-1.921	1.921	21.5	31.25	34.04
.003	.009	-2.046	2.046	21.4	31.10	33.88
.002	.006	-2.222	2.222	21.3	30.95	33.71
.001	.003	-2.523	2.523	21.1	30.65	33.39
water				21.0	30.50	33.22
blank				0.6		

TABLE X

DISODIUM PHOSPHATE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. Na_2HPO_4	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.1989N HCl	% N per Total N air dried	% N per Total N dry wt.
0.7	2.1	0.322	-0.322	48.8	70.72	77.04
.6	1.8	0.255	-0.255	49.0	71.01	77.36
.5	1.5	0.176	-0.176	51.2	74.24	80.87
.4	1.2	0.079	-0.079	52.2	75.71	82.47
.3	.9	- .046	0.046	52.3	75.86	82.63
.2	.6	- .222	.222	52.3	75.86	82.63
.1	.3	- .523	.523	52.3	75.86	82.63
.09	.27	- .569	.569	51.8	75.12	81.83
.08	.24	- .620	.620	51.3	74.39	81.03
.07	.21	- .678	.678	51.15	74.17	80.79
.06	.18	- .745	.745	51.1	74.10	80.71
.05	.15	- .824	.824	51.0	73.95	80.16
.04	.12	- .921	.921	50.2	72.78	79.28
.03	.09	-1.046	1.046	49.8	72.19	78.64
.02	.06	-1.222	1.222	49.1	71.16	77.52
.01	.03	-1.523	1.523	48.4	70.13	76.40
.009	.027	-1.569	1.569	48.2	69.84	76.08
.008	.024	-1.620	1.620	48.0	69.55	75.76
.007	.021	-1.678	1.678	47.8	69.25	75.44
.006	.018	-1.745	1.745	47.4	68.73	74.87
.005	.015	-1.824	1.824	47.2	68.37	74.48
.004	.012	-1.921	1.921	45.85	66.39	72.32
.003	.009	-2.046	2.046	42.4	61.33	66.81
.002	.006	-2.222	2.222	36.7	52.99	57.72
.001	.003	-2.523	2.523	29.5	42.40	46.19
.0009	.0027	-2.569	2.569	26.1	37.41	40.75
.0008	.0024	-2.620	2.620	25.7	36.82	40.11
.0007	.0021	-2.678	2.678	25.0	35.80	38.99
.0006	.0018	-2.745	2.745	24.4	34.92	38.04
.0005	.0015	-2.824	2.824	24.0	34.33	37.40
.0004	.0012	-2.921	2.921	24.1	34.48	37.56
.0003	.0009	-3.046	3.046	23.7	33.89	36.92
.0002	.0006	-3.222	3.222	23.0	32.86	35.80
.0001	.0003	-3.523	3.523	22.6	32.28	35.16
water				22.1	31.54	34.36
blank				0.6		

TABLE XI

DIPOTASSIUM PHOSPHATE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. K_2HPO_4	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.1989N HCl	% N per Total N air dried	% N per Total N dry wt.
0.7	2.1	0.322	-.322	46.2	66.91	77.88
.6	1.8	0.255	-.255	48.4	70.13	76.40
.5	1.5	0.176	-.176	50.5	73.22	79.76
.4	1.2	0.079	-.079	51.4	74.54	81.19
.3	.9	- .046	+.046	51.5	74.68	81.35
.2	.6	- .222	.222	51.3	74.39	81.03
.1	.3	- .523	.523	51.2	74.24	80.87
.09	.27	- .569	.569	51.2	74.24	80.87
.08	.24	- .620	.620	50.8	73.39	79.95
.07	.21	- .678	.678	50.2	72.78	79.28
.06	.18	- .745	.745	50.1	72.63	79.11
.05	.15	- .824	.824	49.9	72.34	78.80
.04	.12	- .921	.921	49.3	71.46	77.84
.03	.09	-1.046	1.046	48.9	70.87	77.20
.02	.06	-1.222	1.222	49.0	71.01	77.36
.01	.03	-1.523	1.523	48.15	69.77	76.00
.009	.027	-1.569	1.569	47.6	69.11	75.28
.008	.024	-1.620	1.620	46.7	67.64	73.68
.007	.021	-1.678	1.678	47.2	68.37	74.48
.006	.018	-1.745	1.745	46.85	67.86	73.92
.005	.015	-1.824	1.824	46.9	67.93	74.01
.004	.012	-1.921	1.921	43.9	63.53	69.21
.003	.009	-2.046	2.046	38.4	55.46	60.41
.002	.006	-2.222	2.222	32.1	46.21	50.34
.001	.003	-2.523	2.523	25.8	36.97	40.27
.0009	.0027	-2.569	2.569	25.3	36.24	39.47
.0008	.0024	-2.620	2.620	24.8	35.50	38.67
.0007	.0021	-2.678	2.678	24.6	35.21	38.36
.0006	.0018	-2.745	2.745	24.0	34.33	37.39
.0005	.0015	-2.824	2.824	23.6	33.74	36.76
.0004	.0012	-2.921	2.921	23.2	33.16	36.12
.0003	.0009	-3.046	3.046	22.6	32.28	35.16
.0002	.0006	-3.222	3.222	22.4	31.98	34.84
.0001	.0003	-3.523	3.523	22.3	31.84	34.68
water				22.1	31.54	36.36
blank				0.6		

TABLE XII

SODIUM CARBONATE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. Na_2CO_3	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.1989N HCl	% N per Total N air dried	% N per Total N dry wt.
0.7	2.1	0.322	-.322	51.4	74.54	81.19
.6	1.8	0.255	-.255	52.3	75.86	82.63
.5	1.5	0.176	-.176	52.9	76.74	83.59
.4	1.2	0.079	-.079	53.2	77.18	84.07
.3	.9	- .046	+.046	53.4	77.47	84.39
.2	.6	- .222	.222	53.6	77.76	84.71
.1	.3	- .523	.523	54.0	78.35	85.35
.09	.27	- .569	.569	54.1	78.50	85.51
.08	.24	- .620	.620	54.4	78.89	85.93
.07	.21	- .678	.678	54.4	78.89	85.93
.06	.18	- .745	.745	53.8	78.06	85.03
.05	.15	- .824	.824	53.65	77.84	84.79
.04	.12	- .921	.921	53.5	77.57	84.50
.03	.09	-1.046	1.046	53.4	77.42	84.33
.02	.06	-1.222	1.222	53.3	77.27	84.17
.01	.03	-1.523	1.523	53.2	77.18	84.07
.009	.027	-1.569	1.569	51.9	75.25	82.27
.008	.024	-1.620	1.620	51.35	74.41	81.06
.007	.021	-1.678	1.678	51.0	73.90	80.51
.006	.018	-1.745	1.745	50.6	73.31	79.86
.005	.015	-1.824	1.824	50.2	72.72	79.22
.004	.012	-1.921	1.921	49.6	71.90	78.32
.003	.009	-2.046	2.046	48.8	70.72	77.04
.002	.006	-2.222	2.222	48.1	69.69	75.92
.001	.003	-2.523	2.523	40.6	58.67	62.91
.0009	.0027	-2.569	2.569	38.4	55.46	60.41
.0008	.0024	-2.620	2.620	36.4	52.52	57.22
.0007	.0021	-2.678	2.678	34.5	49.74	54.18
.0006	.0018	-2.745	2.745	32.3	46.51	50.67
.0005	.0015	-2.824	2.824	30.2	43.43	47.31
.0004	.0012	-2.921	2.921	28.6	41.08	44.75
.0003	.0009	-3.046	3.046	26.6	38.14	41.55
.0002	.0006	-3.222	3.222	25.6	36.68	39.95
.0001	.0003	-3.523	3.523	24.2	34.62	37.72
water				22.7	32.42	35.32
blank				0.6		

TABLE XIII

POTASSIUM CARBONATE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. K_2CO_3	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.1989N HCl	% N per Total N air dried	% N per Total N dry wt.
0.7	2.1	0.322	-.322	50.0	72.48	78.95
.6	1.8	0.255	-.255	51.5	74.68	81.35
.5	1.5	0.176	-.176	53.0	76.88	83.76
.4	1.2	0.079	-.079	53.1	77.03	83.91
.3	.9	- .046	.046	53.4	77.47	84.39
.2	.6	- .222	.222	53.5	77.62	84.55
.1	.3	- .523	.523	54.0	78.35	85.35
.09	.27	- .569	.569	54.2	78.64	85.67
.08	.24	- .620	.620	54.4	78.94	85.99
.07	.21	- .678	.678	54.3	78.79	85.83
.06	.18	- .745	.745	53.8	78.06	85.03
.05	.15	- .824	.824	53.6	77.76	84.71
.04	.12	- .921	.921	53.3	77.32	84.23
.03	.09	-1.046	1.046	53.0	76.88	83.75
.02	.06	-1.222	1.222	52.8	76.43	83.26
.01	.03	-1.523	1.523	52.6	76.30	83.11
.009	.027	-1.569	1.569	52.5	76.15	82.95
.008	.024	-1.620	1.620	52.4	76.00	82.79
.007	.021	-1.678	1.678	52.0	75.42	82.15
.006	.018	-1.745	1.745	51.6	74.83	81.51
.005	.015	-1.824	1.824	51.0	73.95	80.55
.004	.012	-1.921	1.921	50.1	72.63	79.11
.003	.009	-2.046	2.046	48.8	70.72	77.04
.002	.006	-2.222	2.222	47.3	68.52	74.64
.001	.003	-2.523	2.523	41.65	60.23	65.61
.0009	.0027	-2.569	2.569	39.8	57.51	64.83
.0008	.0024	-2.620	2.620	38.2	55.17	60.10
.0007	.0021	-2.678	2.678	36.5	52.67	57.38
.0006	.0018	-2.745	2.745	33.05	47.61	51.86
.0005	.0015	-2.824	2.824	31.25	44.97	48.98
.0004	.0012	-2.921	2.921	30.4	43.72	47.63
.0003	.0009	-3.046	3.046	28.4	40.79	44.43
.0002	.0006	-3.222	3.222	26.5	38.00	41.39
.0001	.0003	-3.523	3.523	24.8	35.50	38.67
water				22.7	32.42	35.32
blank				0.6		

TABLE XIV

SODIUM SULFITE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 Gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

Molar Sol. Na_2SO_3	Ionic Strength	Log of Ionic Strength	pμ	Kjeldahl Titration 0.2027N HCl	% N per Total N air dried	% N per Total N dry wt.
1.0	3.0	0.477	-.477	40.60	59.81	65.15
.9	2.7	0.431	-.431	43.80	64.59	70.36
.8	2.4	0.380	-.380	45.85	67.66	73.71
.7	2.1	0.322	-.322	47.55	70.20	76.47
.6	1.8	0.255	-.255	49.10	72.52	79.00
.5	1.5	0.176	-.176	49.60	73.27	79.81
.4	1.2	0.079	-.079	51.10	75.51	82.26
.3	.9	- .046	.046	51.80	76.53	83.37
.2	.6	- .222	.222	51.75	76.48	83.31
.1	.3	- .523	.523	51.40	75.96	82.75
.09	.27	- .569	.569	51.30	75.81	82.58
.08	.24	- .620	.620	51.10	75.51	82.26
.07	.21	- .678	.678	51.10	75.51	82.26
.06	.18	- .745	.745	50.60	74.76	81.44
.05	.15	- .824	.824	50.60	74.76	81.44
.04	.12	- .921	.921	50.40	74.46	81.12
.03	.09	-1.046	1.046	50.10	74.02	80.63
.02	.06	-1.222	1.222	48.70	71.92	78.35
.01	.03	-1.523	1.523	48.40	71.47	77.86
.009	.027	-1.569	1.569	47.30	69.83	76.07
.008	.024	-1.620	1.620	46.40	68.48	74.60
.007	.021	-1.678	1.678	42.70	62.95	68.57
.006	.018	-1.745	1.745	39.35	57.94	63.11
.005	.015	-1.824	1.824	37.65	55.40	60.53
.004	.012	-1.921	1.921	32.75	48.07	52.37
.003	.009	-2.046	2.046	29.60	43.36	47.23
.002	.006	-2.222	2.222	26.40	38.58	42.02
.001	.003	-2.523	2.523	23.70	34.54	37.62
.0009	.0027	-2.569	2.569	23.40	34.09	37.13
.0008	.0024	-2.620	2.620	23.15	33.71	36.73
.0007	.0021	-2.678	2.678	22.55	32.82	35.75
.0006	.0018	-2.745	2.745	22.10	32.14	35.02
.0005	.0015	-2.824	2.824	21.70	31.55	34.36
.0004	.0012	-2.921	2.921	21.70	31.55	34.36
.0003	.0009	-3.046	3.046	21.60	31.40	34.20
.0002	.0006	-3.222	3.222	21.60	31.40	34.20
.0001	.0003	-3.523	3.523	21.50	31.33	34.12
water				21.40	31.10	33.88
blank				0.6		

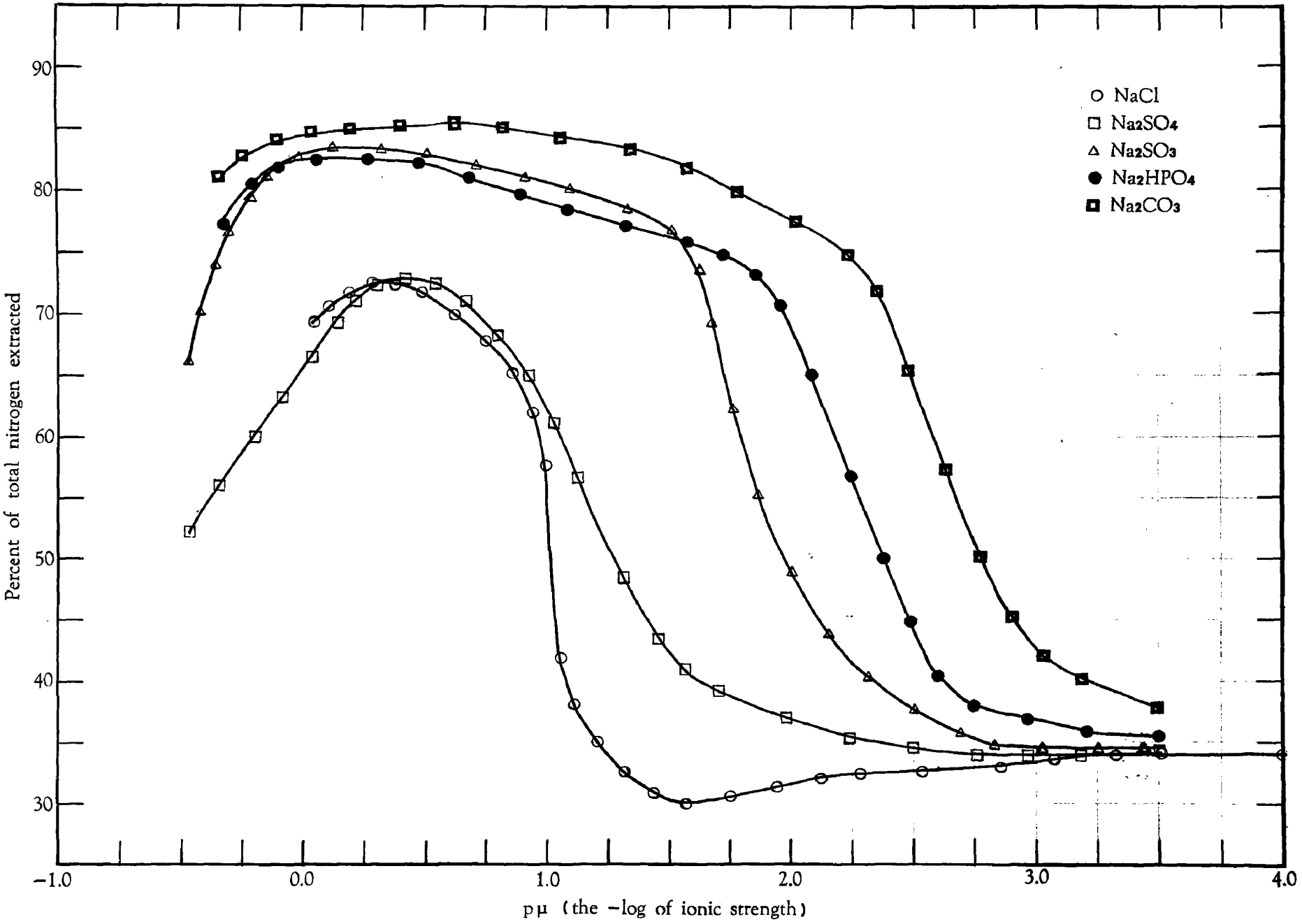
TABLE XV

THE DETERMINATION OF NITROGEN IN THE RESIDUE FROM 18 OF THE SAMPLES EXTRACTED WITH Na_2SO_3 COMPARED WITH THE CORRESPONDING RESULTS OF NITROGEN IN THE EXTRACTS

Molar Solution Na_2SO_3	Kjeldahl Titration 0.2027N HCl	% N per Total N dry wt. Residue	% N per Total N dry wt. Extract
0.9	24.4	1.471	2.670
0.8	22.2	1.335	2.797
0.7	20.6	1.236	2.902
0.6	19.0	1.137	2.998
0.5	18.5	1.106	3.029
0.4	16.9	1.007	3.122
0.3	15.9	0.946	3.165
0.2	16.25	0.9675	3.162
0.1	16.8	1.001	3.140
0.09	16.8	1.001	3.134
0.08	17.0	1.014	3.122
0.07	17.1	1.020	3.122
0.06	17.45	1.041	3.091
0.05	17.4	1.038	3.091
0.04	17.5	1.044	3.079
0.03	18.1	1.082	3.060
0.02	19.4	1.162	2.973
0.01	19.8	1.187	2.955

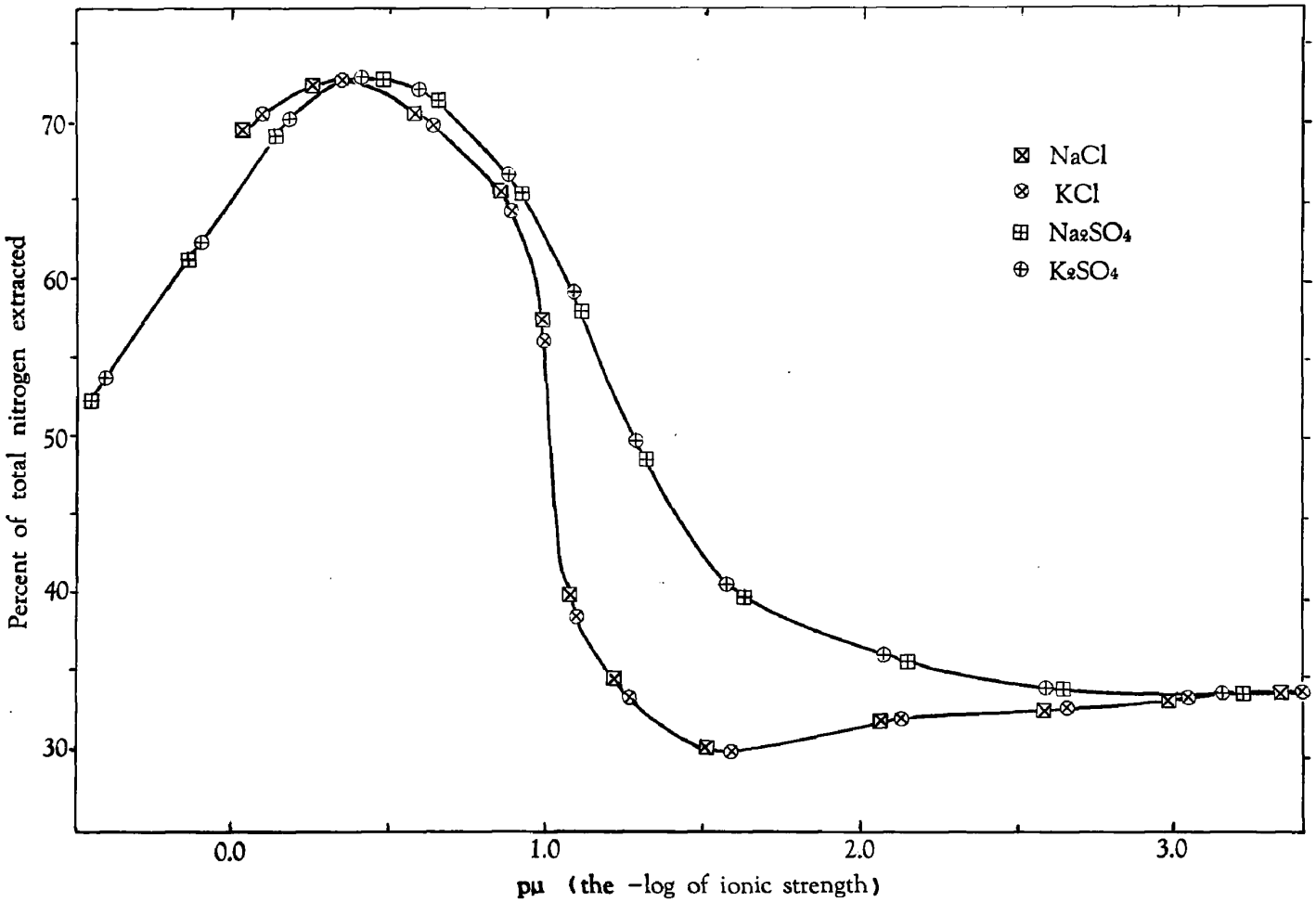
Average value of percentage of total nitrogen in 18 samples 4.134 (sum of average of columns 3 and 4).

Figure 2.



The effect of varying ionic strength as contributed by different salts upon the peptization of protein N from Mung bean. The negative logarithm or pμ, is plotted vs amount extracted.

Figure 3.



This figure shows the effect of ionic strength as contributed by solutions of the salts NaCl, KCl, Na_2SO_4 and K_2SO_4 upon peptization of Mung bean protein at 25° C.

TABLE XVI

HYDROCHLORIC ACID AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

No.	Molar Solution HCl	Kjeldahl Titration 0.1989N HCl	% N per Total N air dried	% N per Total N dry wt.
1.	0.7	36.3	52.384	57.063
2.	0.6	37.1	53.558	58.342
3.	0.5	38.1	55.024	59.939
4.	0.4	39.2	56.639	61.698
5.	0.3	40.5	58.547	63.776
6.	0.2	42.2	61.039	66.491
7.	0.1	43.8	63.387	69.049
8.	0.09	44.3	64.122	69.849
9.	0.08	44.0	63.682	69.370
10.	0.07	43.0	62.214	67.771
11.	0.06	40.1	57.959	63.136
12.	0.05	31.2	43.431	47.310
13.	0.04	14.1	24.688	26.893
14.	0.03	6.9	9.242	10.067
15.	0.02	6.8	9.095	9.907
16.	0.01	7.6	10.270	11.187
17.	0.009	7.8	10.562	11.505
18.	0.008	8.0	10.857	11.826
19.	0.007	8.6	11.737	12.785
20.	0.006	8.8	12.030	13.104
21.	0.005	10.1	13.938	15.183
22.	0.004	11.9	16.580	18.061
23.	0.003	13.1	18.340	19.978
24.	0.002	13.9	19.515	21.258
25.	0.001	17.3	24.503	26.691
26.	0.0009	18.0	25.503	27.780
27.	0.0008	18.6	26.410	28.769
28.	0.0007	18.5	26.263	28.609
29.	0.0006	19.6	27.875	30.365
30.	0.0005	19.8	28.171	30.365
31.	0.0004	20.6	29.346	31.967
32.	0.0003	21.1	30.078	32.764
33.	0.0002	21.3	30.373	33.086
34.	0.0001	22.4	31.986	34.843
35.	Water	22.9	32.721	35.643
36.	blank	0.6		

TABLE XVII

SODIUM HYDROXIDE AS PEPTIZATION AGENT

The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25° C.

No.	Molar Solution NaOH	Kjeldahl Titration 0.1989N HCl	% N per Total N air dried	% N per Total N dry wt.
1.	0.1	57.0	82.758	90.150
2.	0.09	57.0	82.758	90.150
3.	0.08	57.0	82.758	90.150
4.	0.07	55.9	81.143	88.391
5.	0.06	55.9	81.143	88.391
6.	0.05	54.8	79.530	86.634
7.	0.04	54.7	79.383	86.473
8.	0.03	54.6	79.235	86.212
9.	0.02	54.6	79.235	86.212
10.	0.01	51.7	74.980	81.677
11.	0.009	51.0	73.952	80.557
12.	0.008	51.0	73.952	80.557
13.	0.007	51.0	73.952	80.557
14.	0.006	50.35	72.998	79.518
15.	0.005	49.0	71.017	77.360
16.	0.004	48.5	70.285	76.563
17.	0.003	46.8	67.787	73.842
18.	0.002	39.1	56.492	61.538
19.	0.001	28.1	40.351	43.915
20.	0.0009	27.7	39.764	43.316
21.	0.0008	26.95	38.662	42.115
22.	0.0007	25.45	36.462	39.719
23.	0.0006	24.85	35.582	38.760
24.	0.0005	24.0	34.333	37.399
25.	0.0004	23.8	34.041	37.081
26.	0.0003	23.2	33.161	36.123
27.	0.0002	22.55	32.207	35.083
28.	0.0001	22.85	32.647	35.563
29.	water	21.05	30.007	32.687
30.	blank	0.6		

TABLE XVIII

HYDROCHLORIC ACID AS PEPTIZATION AGENT

1.17 gm. NaCl (final concentration, 0.4M) added to each 5 gm. sample. The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

No.	Molar Solution HCl	pH of HCl Solvent	pH of Extract	Kjeldahl Titration 0.1989N HCl	% N per Total N dry wt.
1.	0.7	0.37	0.38	33.4	51.421
2.	0.6	0.43	0.38	34.4	54.024
3.	0.5	0.50	0.42	35.3	55.461
4.	0.4	0.55	0.53	36.1	56.738
5.	0.3	0.70	0.64	36.8	57.858
6.	0.2	0.90	0.90	37.5	58.977
7.	0.1	1.13	1.65	37.7	59.246
8.	0.09	1.20	1.78	37.9	59.617
9.	0.08	1.23	2.00	38.4	60.415
10.	0.07	1.33	2.38	37.5	58.977
11.	0.06	1.40	2.78	33.0	51.785
12.	0.05	1.50	3.20	30.0	46.989
13.	0.04	1.64	3.67	27.4	42.833
14.	0.03	1.72	4.07	25.0	38.999
15.	0.02	1.87	4.48	23.2	36.119
16.	0.01	2.13	5.00	35.8	56.259
17.	0.009	2.18	5.06	36.6	57.539
18.	0.008	2.22	5.10	38.5	60.576
19.	0.007	2.29	5.20	39.8	62.656
20.	0.006	2.33	5.30	41.5	65.372
21.	0.005	2.38	5.30	40.8	64.252
22.	0.004	2.50	5.42	42.4	66.810
23.	0.003	2.63	5.50	42.5	66.970
24.	0.002	2.90	5.61	43.7	68.887
25.	0.001	3.15	5.71	44.2	69.686
26.	0.0009	3.23	5.76	44.7	70.487
27.	0.0008	3.30	5.76	45.2	71.284
28.	0.0007	3.41	5.76	44.8	70.644
29.	0.0006	3.52	5.76	44.6	70.325
30.	0.0005	3.68	5.76	44.5	70.165
31.	0.0004	3.88	5.80	44.4	70.007
32.	0.0003	4.32	5.80	44.4	70.007
33.	0.0002	5.80	5.80	44.4	70.007
34.	0.0001	6.50	5.82	44.4	70.007
35.	water	7.15	5.82	44.1	69.528
36.	blank	7.10	5.82	0.6	

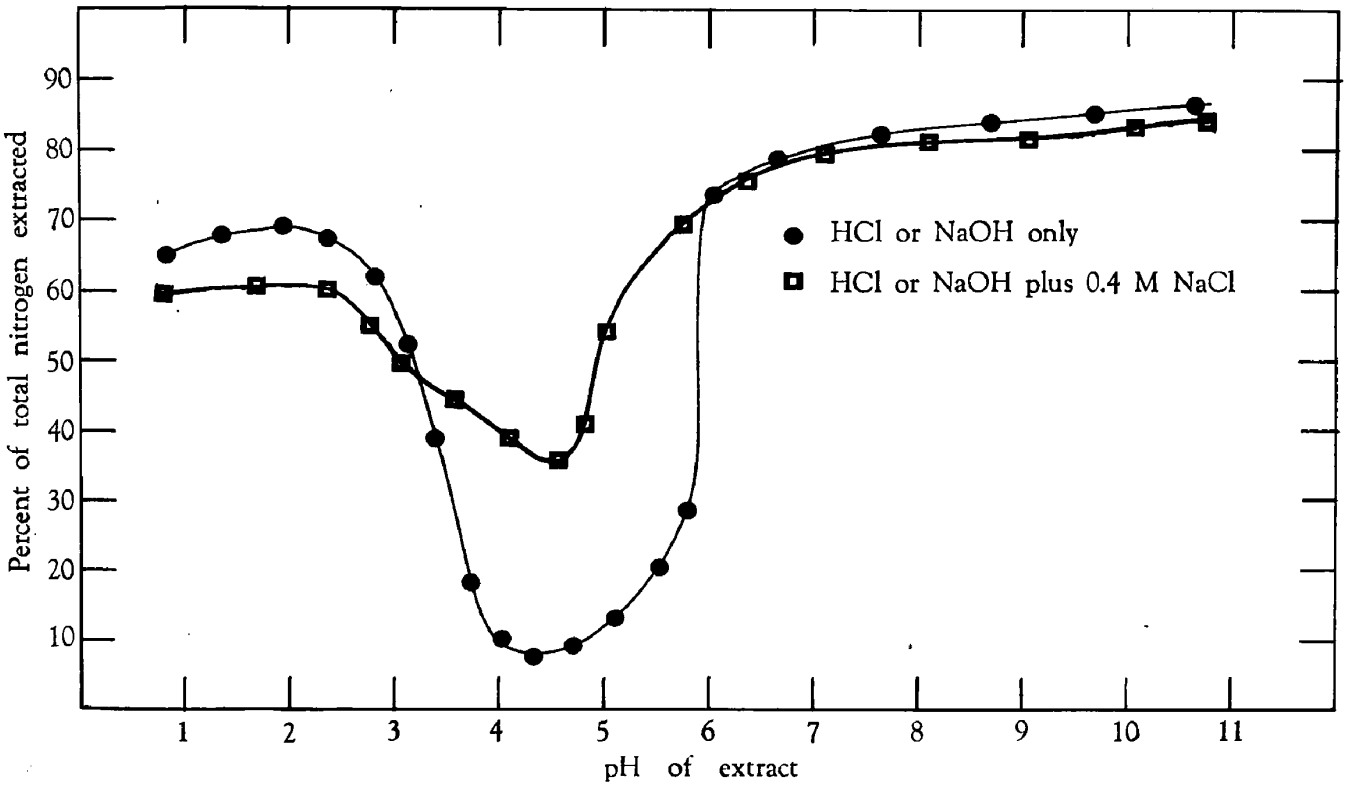
TABLE XIX

SODIUM HYDROXIDE AS PEPTIZATION AGENT

1.17 gm. NaCl (final concentration, 0.4M) added to each 5 gm. sample. The sample-solvent ratio was 1:10 (5 gm. of 60 mesh Mung bean meal was added to 50 ml. of the solvent.) Extraction of protein was made by shaking for 30 minutes at 25°C.

No.	Molar Solution NaOH	pH of NaOH Solvent	pH of Extract	Kjeldahl Titration 0.1989N HCl	% N per Total N dry wt.
1.	0.1	11.5	10.60	53.4	84.392
2.	0.09	11.5	10.52	54.0	85.350
3.	0.08	11.54	10.38	54.0	85.350
4.	0.07	11.54	10.20	54.4	85.991
5.	0.06	11.54	10.02	53.4	84.392
6.	0.05	11.54	9.80	52.8	83.433
7.	0.04	11.52	9.30	52.55	83.031
8.	0.03	11.50	8.84	52.35	82.713
9.	0.02	11.40	8.12	51.5	81.356
10.	0.01	11.28	6.73	50.9	80.397
11.	0.009	11.24	6.61	49.8	78.638
12.	0.008	11.18	6.50	50.0	78.956
13.	0.007	11.08	6.36	48.4	76.399
14.	0.006	11.00	6.22	49.3	77.839
15.	0.005	10.90	6.16	47.9	75.001
16.	0.004	10.80	6.03	47.4	74.800
17.	0.003	10.66	5.93	46.9	74.002
18.	0.002	10.62	5.88	46.1	72.722
19.	0.001	10.42	5.82	46.0	72.564
20.	0.0009	10.38	5.81	45.3	71.445
21.	0.0008	10.30	5.80	45.4	71.605
22.	0.0007	10.20	5.80	45.4	71.605
23.	0.0006	10.10	5.80	45.9	72.404
24.	0.0005	10.00	5.77	45.9	72.404
25.	0.0004	9.88	5.74	46.1	72.722
26.	0.0003	9.67	5.74	46.1	72.722
27.	0.0002	9.20	5.74	46.1	72.722
28.	0.0001	8.20	5.74	46.1	72.722
29.	water	6.92	5.74	45.4	71.605
30.	blank			0.6	

Figure 4.



Various concentrations of HCl and NaOH were used as peptization agents. The pH of the extract of each concentration was measured and plotted vs the percentage of total nitrogen extracted.

THE STUDY OF SOME FACTORS WHICH EFFECT THE PEPTIZATION OF MUNG BEAN PROTEIN

All the neutral solvents, sulfates and chlorides, employed as extracting agents in this study have peptization capacities that are nearly equal, 72 percent nitrogen per total nitrogen content (Tables VI, VII, VIII and IX). Practically identical curves were obtained from sodium and potassium chlorides by plotting their concentrations versus the perdentage of nitrogen extracted (Figures 2 and 3). It was very interesting to note that with those two salts the "steep slope" of the curve was nearly vertical. The significance of the "steep slope" is self-explanatory: that this protein in such a solvent has a very narrow solubility range. Since the solubility of NaCl in aqueous solution has less variation due to changes in temperature than KCl* (55), sodium chloride at a concentration of 0.4 M was considered the more suitable peptization agent for the study of Mung bean proteins.

In experiments involving the peptization of Mung bean protein, it was necessary to standardize the following factors:

A. Time and sample-solvent ratios "S-S-R"

B. Particle size and sample-solvent ratios

C. Mechanical shaking, hand-stirring and successive extractions

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*Grams salt in 100 grams water

NaCl	35.6 at 0°C.	or	39.6 at 100°C.
KCl	28.5 at 0°C.	or	56.6 at 100°C.

D. Temperature and oil-free and non-oil free condition of the samples

(A) The Determination of the Effect of the Extracting Time on the Degree of Peptization of Nitrogen (% N per total N) of Mung Bean Meal with Five Different Sample-solvent Ratios, hereafter Termed "S-S-R".

The 0.4M NaCl solution was used as the peptizing agent. Each extraction was made on six samples using five different sample-solvent ratios and one with water.

No. 1. 5.0 gm. sample to 100 ml. 0.4M NaCl

No. 2. 7.5 gm. sample to 100 ml. 0.4M NaCl

No. 3. 10.0 gm. sample to 100 ml. 0.4M NaCl

No. 4. 12.5 gm. sample to 100 ml. 0.4M NaCl

No. 5. 15.0 gm. sample to 100 ml. 0.4M NaCl

No. 6. 10.0 gm. sample to 100 ml. water

Four extraction periods were employed: shaking (120 oscillations per minute) for 10, 20, 30 and 40 minutes at 25°C. After centrifugation the clear liquids were poured into Kjeldahl flasks for total nitrogen determinations.

Calculations were made from titration data as to:

1. Mgm. N extracted from the samples.

2. Percentage N per air-dried samples.

3. Percentage N per Total N content of air-dried samples.

4. Percentage N per Total N content on dry weight basis.

The results of this work are shown in Table XX.

TABLE XX

EFFECT OF EXTRACTING TIME ON THE DEGREE OF PEPTIZATION WITH FIVE DIFFERENT SAMPLE-SOLVENT RATIOS

With samples 1-5 100 ml. of 0.4M NaCl were used and with sample No. 6 100 ml. of distilled water was used. Temperature was 25°C.

No.	60 Mesh Sample Grams	Kjeldahl Titration 0.1989N HCl	Mgm. N	% N per Sample	% N/T N air-dried	% N/T N dry wt. basis
			Shaking time 10 minutes			
1.	5.0	45.8	12.763	2.55	67.26	73.26
2.	7.5	66.2	18.439	2.45	64.78	70.56
3.	10.0	83.5	23.258	2.32	61.28	66.75
4.	12.5	97.5	27.160	2.17	57.25	62.36
5.	15.0	104.7	29.154	1.94	51.21	55.78
6.	10.0	41.35	11.519	1.15	30.35	33.06
			Shaking time 20 minutes			
1.	5.0	47.6	13.221	2.64	69.67	75.89
2.	7.5	69.1	19.245	2.56	67.61	73.64
3.	10.0	88.6	24.670	2.46	65.00	70.80
4.	12.5	105.5	29.390	2.35	61.95	67.48
5.	15.0	118.2	32.911	2.19	57.81	62.97
6.	10.0	43.45	12.099	1.20	31.80	34.64
			Shaking time 30 minutes			
1.	5.0	47.85	13.255	2.66	70.22	76.49
2.	7.5	70.1	19.527	2.60	68.60	74.72
3.	10.0	90.0	25.285	2.52	66.62	72.57
4.	12.5	110.05	30.647	2.45	64.60	70.37
5.	15.0	127.2	35.422	2.36	62.22	67.77
6.	10.0	44.25	12.316	1.23	32.45	35.34
			Shaking time 40 minutes			
1.	5.0	47.9	13.345	2.66	70.32	76.60
2.	7.5	70.85	19.726	2.63	69.30	75.49
3.	10.0	92.9	25.865	2.58	68.15	74.23
4.	12.5	113.8	31.691	2.53	66.80	72.76
5.	15.0	133.25	31.107	2.47	65.18	71.00
6.	10.0	44.55	12.411	1.24	32.70	35.62

From Table XX the results have been calculated in terms of percentage nitrogen per total nitrogen content of air-dried samples (Table XXI).

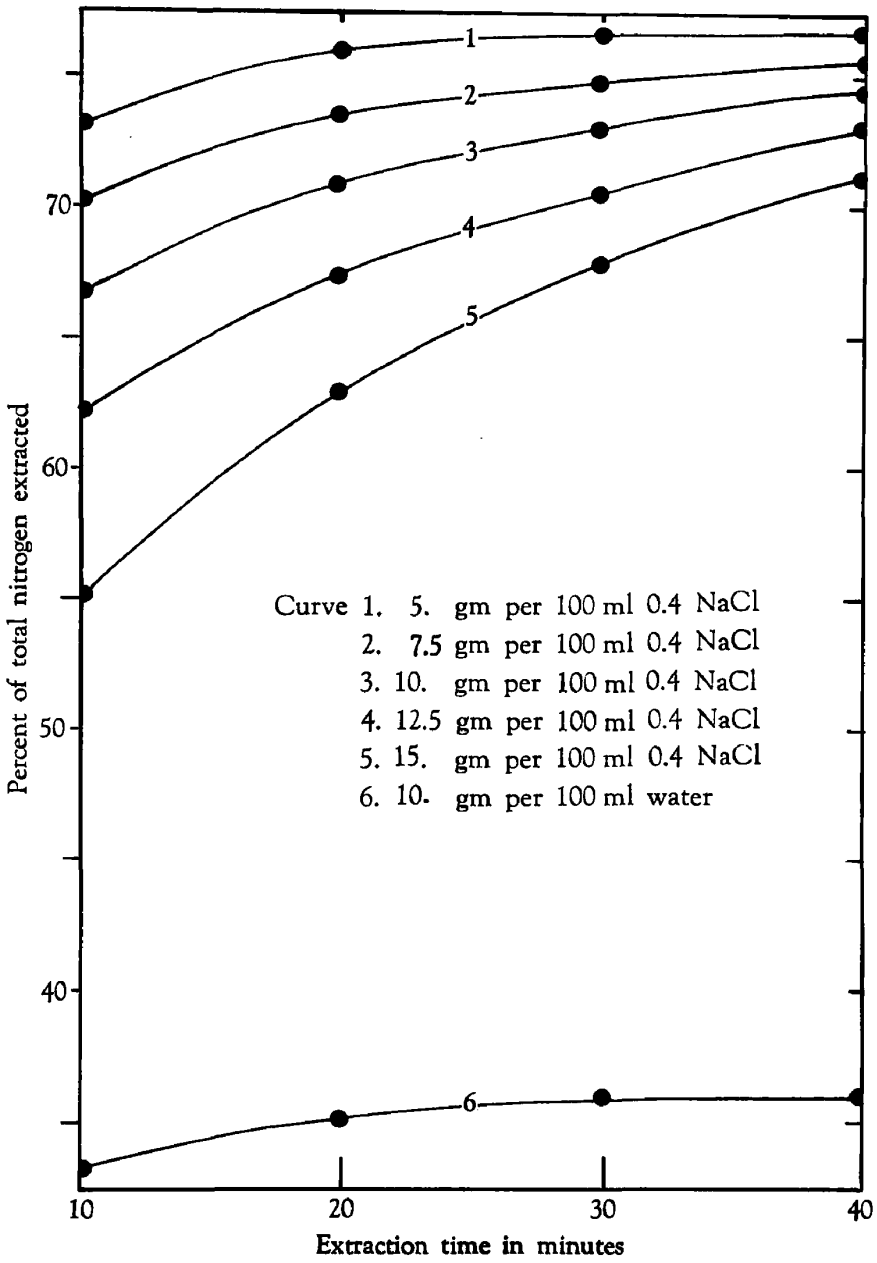
TABLE XXI

PERCENTAGE NITROGEN PER TOTAL NITROGEN CONTENT OF AIR-DRIED SAMPLES

No.	1	2	3	4	5	6
Sample-solvent Ratio	5:100	7.5:100	10:100	12.5:100	15:100	10:100 water
10 Min.	67.2	64.7	61.2	57.2	51.2	30.3
20 Min.	69.6	67.6	65.0	61.9	57.8	31.8
30 Min.	70.2	68.6	66.6	64.6	62.2	32.4
40 Min.	70.3	69.3	68.1	66.8	65.1	32.7

These experiments demonstrate that in all instances an increase in extracting time increases the amount of total nitrogen peptized from the samples. Of the five sample-solvent ratios employed, the one of 5:100 gave the highest value of peptization and that of 15:100 gave the lowest. When the S-S-R is 5:100, time had less influence upon the peptization as shown by an increase of only 3 percent with the increase in time from 10 minutes to 40 minutes. On the other hand, time had a larger influence upon the peptization when the S-S-R is 15:100, in which case there was a 14 percent increase when the same extension of time was employed (Table XXI and Figure 5).

Figure 5.



The effect that various sample to solvent ratios have upon extraction time, using 0.4 M NaCl. Mung bean sample was ground to 60 mesh.

(B) The Determination of the Effect of Particle Size and Time on the Degree of Peptization. Twenty mesh and 40 mesh Mung bean meal samples were used. Sixty mesh Mung bean meal was previously determined. Each extraction was made by shaking (120 oscillations per minute) 5 samples with 0.4M NaCl at 5 different sample-solvent ratios (S-S-R): 5.0:100, 7.5:100, 10:100, 12.5:100 and 15.0:100 and one 10 gm. sample with 100 ml. water. After centrifugation the clear liquids were used for total nitrogen determination. Calculations from the Kjeldahl titration data (Table XXII) were expressed in terms of:

1. Mgm. nitrogen determined from the sample.
2. Percentage of nitrogen per air-dried sample.
3. Percentage of nitrogen per total nitrogen content of air-dried sample.
4. Percentage of nitrogen per total nitrogen content on dry weight basis.

TABLE XXII

EFFECT OF PARTICLE SIZE AND TIME ON THE DEGREE OF PEPTIZATION WITH FIVE DIFFERENT S-S-R

With samples 1-5 100 ml. of 0.4M NaCl were used and with sample 6 100 ml. of distilled water was used. Temperature was 25°C.

No.	Wgt of Sample in Grams	Kjeldahl Titration 0.1989N HCl	Mgm. N Extracted	% N per Sample	% N/T N air-dried	% N/T N dry wt. basis
Shaking time 20 minutes, 40 mesh Mung bean meal.						
1.	5.0	30.5	8.494	1.69	44.76	48.75
2.	7.5	45.1	12.556	1.67	44.11	48.05
3.	10.0	59.0	16.430	1.64	43.29	47.15
4.	12.5	71.8	19.996	1.59	42.15	45.91
5.	15.0	83.2	23.170	1.54	40.70	44.33
6.	10.0	36.9	10.289	1.02	27.11	29.51
Shaking time 30 minutes, 40 mesh Mung bean meal.						
1.	5.0	31.6	8.813	1.76	45.22	49.25
2.	7.5	45.4	12.644	1.68	44.42	48.38
3.	10.0	59.5	16.578	1.65	43.68	47.58
4.	12.5	72.8	20.286	1.62	42.76	46.57
5.	15.0	84.8	23.615	1.57	41.48	45.18
6.	10.00	39.7	11.063	1.10	29.15	31.75
Shaking time 40 minutes, 40 mesh Mung bean meal.						
1.	5.0	31.7	8.824	1.76	46.50	50.65
2.	7.5	47.1	13.122	1.74	46.10	50.21
3.	10.0	62.0	17.254	1.72	45.64	49.52
4.	12.5	76.3	21.254	1.70	44.80	48.80
5.	15.0	90.25	25.135	1.67	44.15	48.09
6.	10.0	41.3	11.511	1.15	30.33	33.03
Shaking time 20 minutes, 20 mesh Mung bean meal.						
1.	5.0	17.5	4.873	0.97	25.68	27.97
2.	7.5	25.35	7.059	0.94	24.80	27.01
3.	10.0	32.05	8.926	0.89	23.52	25.62
4.	12.5	37.8	10.537	0.84	22.21	24.19
5.	15.0	42.3	11.785	0.78	20.70	22.54
6.	10.0	20.9	5.818	0.58	15.33	16.69
Shaking time 30 minutes, 20 mesh Mung bean meal.						
1.	5.0	17.9	4.987	0.99	26.28	28.62
2.	7.5	25.9	7.213	0.96	25.34	27.60
3.	10.0	33.35	9.294	0.92	24.49	26.67
4.	12.5	39.9	11.106	0.88	23.40	25.50
5.	15.0	45.1	12.553	0.83	22.05	24.01
6.	10.0	24.25	6.759	0.65	17.81	19.40
Shaking time 40 minutes, 20 mesh Mung bean meal.						
1.	5.0	20.65	5.753	1.15	30.32	33.02
2.	7.5	30.1	8.380	1.11	29.44	32.06
3.	10.0	38.9	10.835	1.08	28.55	31.10
4.	12.5	47.35	13.184	1.05	27.79	30.07
5.	15.0	53.4	14.876	0.99	26.13	28.46
6.	10.0	33.55	9.340	0.93	24.61	26.80

From Table XXII we can calculate the effect of particle size and time of extraction in terms of percentage (Table XXIII).

TABLE XXIII

EFFECT OF PARTICLE SIZE AND TIME OF EXTRACTION IN TERMS OF PERCENTAGE

No.	1	2	3	4	5	6
S-S-R	5:100	7.5:100	10:100	12.5:100	15:100	10:100 water
			Extraction time 20 minutes			
20 Mesh	25.6	24.8	23.5	22.2	20.7	15.3
40 Mesh	44.7	44.1	43.2	42.1	40.7	27.1
60 Mesh	69.6	67.6	65.0	61.9	57.8	31.8
			Extraction time 30 minutes			
20 Mesh	26.2	25.3	24.5	23.4	22.0	17.8
40 Mesh	45.2	44.4	43.6	42.7	41.5	29.1
60 Mesh	70.2	68.6	66.6	64.6	62.2	32.4
			Extraction time 40 minutes			
20 Mesh	30.3	29.4	28.5	27.8	26.1	24.6
40 Mesh	46.5	46.1	45.4	44.8	44.1	30.3
60 Mesh	70.3	69.3	68.1	66.8	65.1	32.7

This work involved three variables: 1) the particle size, 2) the sample-solvent ratio and 3) extraction time. It is evident that the particle size affects the degree of peptization to a greater extent than either the S-S-R or the extraction time.

The 60 mesh size gave about 20 percent greater yield of nitrogen over the 40 mesh size which in turn gave about 20 percent greater yield of nitrogen over the 20 mesh size.

When using 5:100 as the lowest S-S-R, there was a decrease in yield of nitrogen when the S-S-R was increased.

An increase in extracting time produced a slight increase in the yield of nitrogen. The results are plotted in Figure 6.

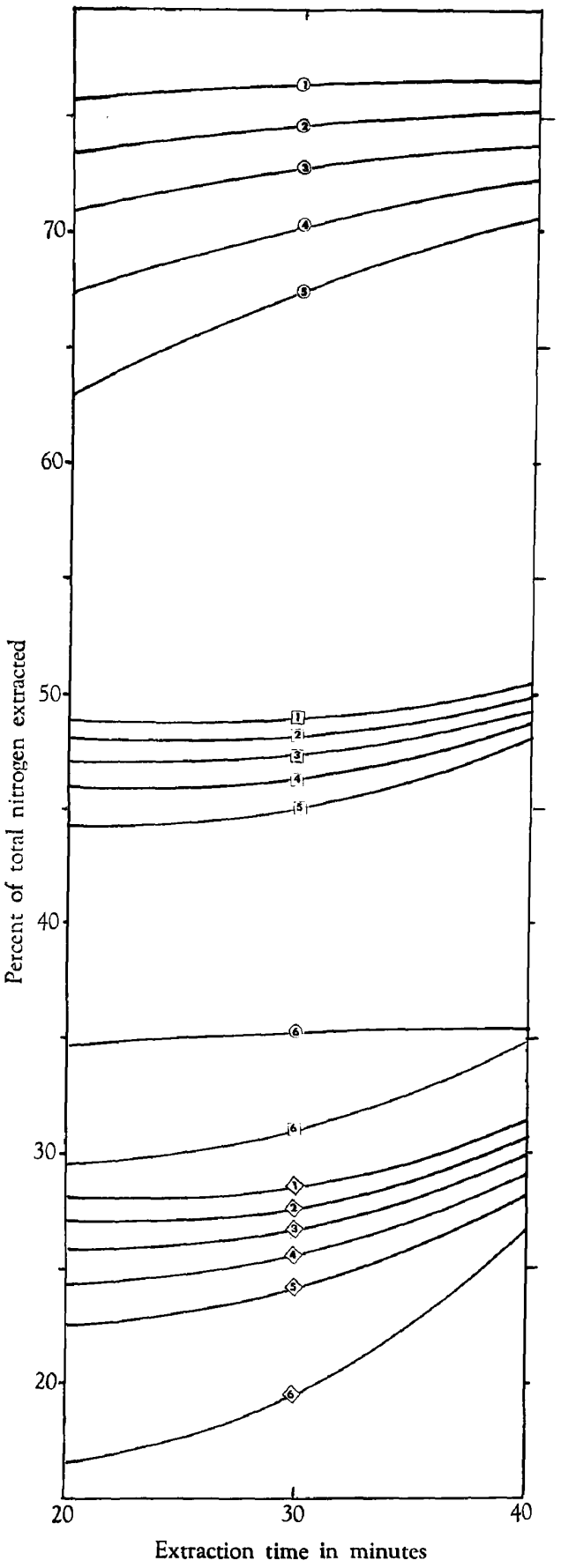


Figure 6.

This figure shows the effect of the particle size, time and sample - solvent ratio on the degree of peptization of Mung bean meal in 0.4 M NaCl. Sample sizes are indicated by:

- ○ for 60 mesh
- □ for 40 mesh
- ◇ for 20 mesh

Curves in each case numbered 1, 2, 3, 4 and 5 represent 5, 7.5, 10, 12.5, and 15 grams respectively of sample per 100 ml 0.4 M NaCl. All curves numbered 6 represent 10 grams of sample per 100 ml water.

(C) Comparative Effects of Mechanical Shaking and Hand Stirring on the Amount of Nitrogen Peptized from Mung Bean Meal.

Mung bean meal (60 mesh) was extracted with 0.4M NaCl at five sample-solvent ratios (see Table XXII). Sample No. 6 was 10 gm. of meal to 100 ml. of distilled water. Six samples were extracted at one time for 30 minutes at 25°C.

After the first extraction was made from the six samples, the clear liquids were poured into Kjeldahl flasks for nitrogen determination. The residues were saved for a second extraction. Five 100 ml. portions of 0.4M NaCl were added to the residues of Nos. 1-5, and 100 ml. of distilled water was added to the No. 6 residue for the second extraction. A third extraction was conducted in the same manner.

The nitrogen content of all extracts was determined and reported in terms of percent of nitrogen per total nitrogen on air-dried basis and shown in Table XXIV, XXV, XXVI and Figure 7.

TABLE XXIV

DETERMINATION OF NITROGEN CONTENT OF THREE SUCCESSIVE EXTRACTIONS OF MUNG BEAN MEAL SAMPLES

(A) With Mechanical Shaking for 30 minutes at 25°C.

No.	Sample 60 Mesh	Solvent 100 ml.	Kjeldahl Titration 0.1989N HCl	Mgm. N	% N per Sample	% N/T N air-dried
1st Extraction						
1.	5.0	.4M NaCl	49.1	13.672	2.73	64.54
2.	7.5	" "	70.2	19.547	2.60	61.52
3.	10.0	" "	92.25	25.688	2.56	60.62
4.	12.5	" "	116.1	32.329	2.58	61.04
5.	15.0	" "	138.0	38.427	2.56	60.45
6.	10.0	water	44.0	12.252	1.22	28.91
2nd Extraction						
1.	5.0	.4M NaCl	8.1	2.255	0.45	10.64
2.	7.5	" "	11.5	3.202	0.42	10.08
3.	10.00	" "	14.0	3.926	0.39	9.25
4.	12.5	" "	18.2	5.067	0.40	9.56
5.	15.0	" "	21.1	5.875	0.39	9.23
6.	10.0	water	30.4	8.465	0.84	19.97
3rd Extraction						
1.	5.0	.4M NaCl	2.0	0.557	0.11	2.62
2.	7.5	" "	3.5	0.974	0.12	3.04
3.	10.0	" "	4.8	1.336	0.13	3.13
4.	12.5	" "	6.0	1.670	0.13	3.13
5.	15.0	" "	7.1	1.977	0.13	3.09
6.	10.0	water	17.0	4.733	0.47	11.16
Residues						
1.	5.0		16.9	9.705	0.94	22.21
2.	7.5		28.95	8.061	1.07	25.35
3.	10.0		41.0	11.416	1.14	26.93
4.	12.5		49.9	13.895	1.11	26.22
5.	15.0		62.1	17.292	1.15	27.19
6.	10.0		60.8	16.930	1.69	39.96

TABLE XXV

DETERMINATION OF NITROGEN CONTENT OF THREE SUCCESSIVE EXTRACTIONS OF MUNG BEAN MEAL SAMPLES

(B) With Hand Stirring Occasionally for 30 minutes. Temperature 25°C.

No.	Sample 60 Mesh	Solvent 100 ml.	Kjeldahl Titration 0.1989N HCl	Mgm. N	% N per Sample	% N/T N air-dried
1st. Extraction						
1.	5.0	.4M NaCl	48.7	13.561	2.71	63.99
2.	7.5	" "	70.2	19.547	2.60	61.49
3.	10.0	" "	92.4	25.739	2.57	60.69
4.	12.5	" "	112.4	31.298	2.50	59.06
5.	15.0	" "	133.5	37.174	2.47	58.47
6.	10.0	water	43.5	12.113	1.21	28.57
2nd. Extraction						
1.	5.0	.4M NaCl	6.2	1.726	0.34	8.14
2.	7.5	" "	10.8	3.007	0.40	9.43
3.	10.0	" "	16.0	4.455	0.44	10.50
4.	12.5	" "	23.2	6.460	0.51	12.17
5.	15.0	" "	29.3	8.158	0.54	12.81
6.	10.0	water	29.0	8.075	0.80	19.04
3rd. Extraction						
1.	5.0	.4M NaCl	3.3	0.919	0.18	4.31
2.	7.5	" "	4.3	1.197	0.15	3.75
3.	10.0	" "	5.9	1.643	0.16	3.87
4.	12.5	" "	8.2	2.283	0.18	4.29
5.	15.0	" "	9.2	2.561	0.17	4.01
6.	10.0	water	14.6	4.065	0.40	9.58
Residues						
1.	5.0		17.9	4.984	0.99	23.50
2.	7.5		28.85	8.033	1.07	25.27
3.	10.0		37.9	10.553	1.05	24.89
4.	12.5		46.4	12.920	1.03	24.37
5.	15.0		56.3	15.677	1.04	24.65
6.	10.0		65.1	18.127	1.81	42.75

TABLE XXVI

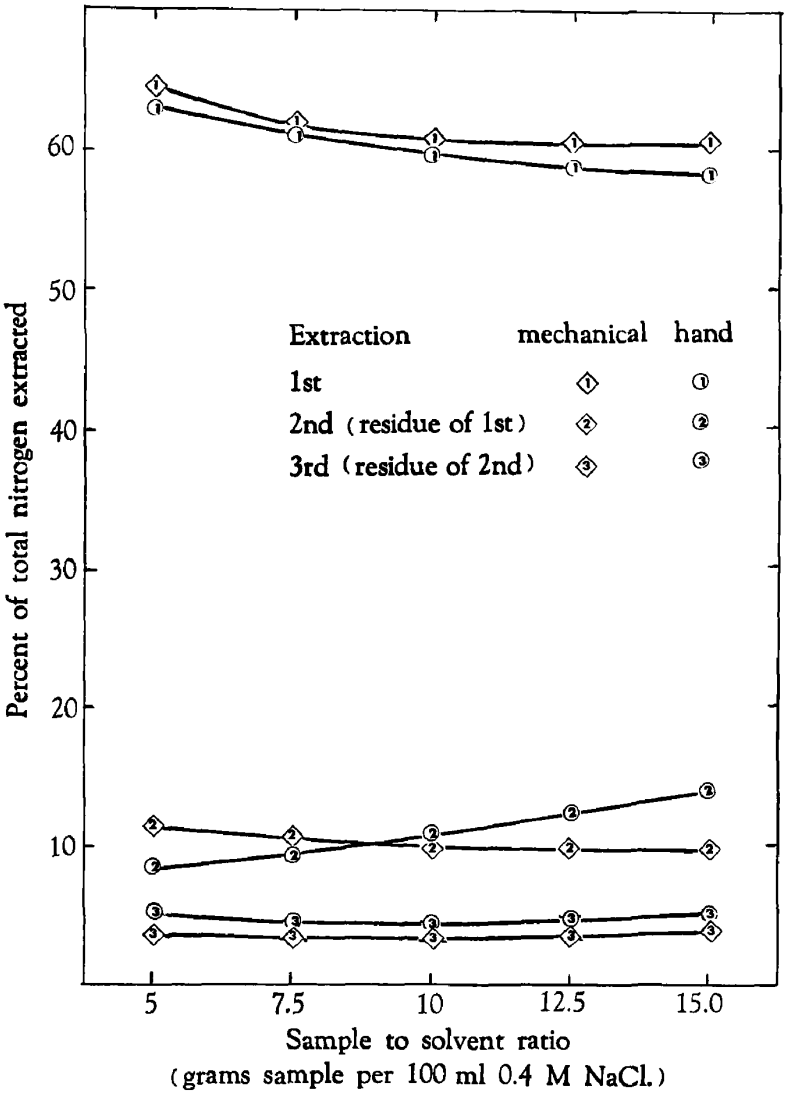
DETERMINATION OF NITROGEN CONTENT OF THREE SUCCESSIVE EXTRACTIONS OF MUNG BEAN MEAL SAMPLES

No.	S-S-R	1st Ext. % N	2nd Ext. % N	3rd Ext. % N	Residue % N	Recovery % N
A.	With Mechanical Shaking for 30 minutes at 25°C.					
1.	5:100	64.54*	10.64*	2.62*	22.21	100.01
2.	7.5:100	61.52	10.08	3.04	25.35	99.99
3.	10:100	60.62	9.25	3.13	26.93	99.93
4.	12.5:100	61.04	9.56	3.13	26.22	99.95
5.	15:100	60.45	9.23	3.09	27.19	99.86
6.	10:100	28.91	19.97	11.16	39.96	100.00
B.	With Hand Stirring Occasionally for 30 minutes at 25°C.					
1.	5:100	63.99	8.14	4.31	23.50	99.94
2.	7.5:100	61.49	9.43	3.75	25.27	99.94
3.	10:100	60.69	10.50	3.87	24.89	99.95
4.	12.5:100	59.06	12.17	4.29	24.37	99.89
5.	15:100	58.47	12.81	4.01	24.65	99.94
6.	10:100	28.57	19.04	9.58	42.72	99.91

* All N values are reported as % N of total N (air-dried samples).

Nitrogen determination was made on all residues.

Figure 7.



This figure shows the comparison of mechanical to hand stirring when 3 successive extractions were made on the same sample of Mung bean meal, using various sample to solvent ratios.

(D) The Effect of Temperature on the Degree of Peptization of "Oil Free" and "Non-Oil Free" Mung Bean Meals.

Three "oil free" and three "non-oil free" samples (60 mesh) were extracted at one time. The sample-solvent ratios (S-S-R) were 1:10, thus 100 ml. of 3 different concentrations (0.4, 0.1 and 0.05M) of NaCl solutions were added to the three 10 gram "non-oil free" samples (Nos. 1, 2, 3) and to the three 9.92 gram "oil free" samples (Nos. 4, 5, 6). The oil content of Mung bean meal (non-oil free) was 0.8 percent as previously determined.

Determinations were made in water baths at four different temperatures (25°C., 35°C., 45°C. and 55°C.). Each extraction period was 30 minutes with occasional hand stirring with a glass rod.

After centrifugation the nitrogen content of the clear extracts were determined by Kjeldahl-Gunning method. The results of the Kjeldahl determinations were calculated in terms of:

1. Mgm. N from the sample.
2. Percentage of N per total N content of air-dried sample.
3. Percentage of N per total N content on dry weight basis.

The effect of temperature upon the degree of peptization from oil free and non-oil free samples is difficult to interpret. The oil free samples appeared, generally, to have yielded higher values than the non-oil free samples except that with 0.1M NaCl solution at 55°C. and with 0.05M NaCl solution at 45°C. and 55°C. reverse results were observed.

That temperature has an influence upon peptization was shown by the fact that the degree of peptization was decreased 8 percent in oil free

samples (with 0.4M NaCl solution) when the temperature was increased from 25°C. to 55°C. Using non-oil free samples the temperature of extraction was shown to have little or no influence upon the degree of peptization below 45°C. Above 45°C. there was a decrease in the degree of peptization in both samples. When 0.1M and 0.05M NaCl solutions were used as solvents, an increase in temperature resulted in a greater degree of peptization until the maximum degree was reached (see Table XXVI and Figure 7) and then decreased with increasing temperature. When using more dilute salt solutions for extraction, it was noted that non-oil free samples yielded lower values for the amount of nitrogen peptized than oil free samples until higher temperatures were reached when oil free samples gave lower values. The general falling off of the curves in all instances above 45°C. may be due to the heat coagulation of the protein material. A greater decrease occurred when the highest salt concentration was used (0.4M NaCl). The removal of oil from samples to be used for the peptization of globulin is not a safe practice. Osborne (56) found that the proteins of ground flax seed which had been freed from oil when extracted with NaCl solutions became a water soluble product whereas natural globulin should not be soluble in water.

It is interesting to note that when a non-oil free sample was extracted with 0.4M NaCl solution albumin is apt to be coagulated to a greater degree at 55°C. than when the concentration of NaCl solution is lowered to 0.05M. It may be possible that proteins are protected from coagulation by heat (55°C.) by the presence of oil in the sample (57) (58).

TABLE XXVII

THE EFFECT OF TEMPERATURE ON THE DEGREE OF PEPTIZATION OF "OIL FREE" AND "NON-OIL FREE" MUNG BEAN MEALS

No.	Wt. Sample Grams	100 ml. NaCl	Kjeldahl Titration .1989N HCl	Mgm. N	% N/T N air-dried	% N/T N dry wt. basis
25°C.						
1.	10 non-O.F.	.4M	73.2	23.332	53.57	58.35
2.	10 non-O.F.	.1M	47.5	13.193	34.76	37.86
3.	10 non-O.F.	.05M	33.6	9.332	24.58	26.78
4.	9.92 O.F.	.4M	77.5	21.526	56.71	61.78
5.	9.92 O.F.	.1M	49.8	13.832	36.44	39.69
6.	9.92 O.F.	.05M	35.8	9.943	26.19	28.53
35°C.						
1.	10 non-O.F.	.4M	73.3	20.359	53.64	58.43
2.	10 non-O.F.	.1M	55.2	15.366	40.48	44.10
3.	10 non-O.F.	.05M	40.1	11.138	29.34	31.96
4.	9.92 O.F.	.4M	76.2	21.165	55.76	60.74
5.	9.92 O.F.	.1M	56.6	15.721	41.42	45.12
6.	9.92 O.F.	.05M	41.0	11.388	30.00	32.68
45°C.						
1.	10 non-O.F.	.4M	72.68	20.238	53.32	58.08
2.	10 non-O.F.	.1M	56.8	15.776	41.56	45.27
3.	10 non-O.F.	.05M	42.8	11.888	31.32	34.12
4.	9.92 O.F.	.4M	74.4	20.665	54.44	59.31
5.	9.92 O.F.	.1M	57.0	15.832	41.71	45.43
6.	9.92 O.F.	.05M	41.8	11.610	30.58	33.32
55°C.						
1.	10 non-O.F.	.4M	66.0	18.332	48.30	52.61
2.	10 non-O.F.	.1M	51.4	14.315	37.71	41.08
3.	10 non-O.F.	.05M	39.3	10.952	28.85	31.43
4.	9.92 O.F.	.4M	66.7	18.526	48.81	53.17
5.	9.92 O.F.	.1M	51.1	14.193	37.39	40.73
6.	9.92 O.F.	.05M	36.2	10.054	26.49	28.85

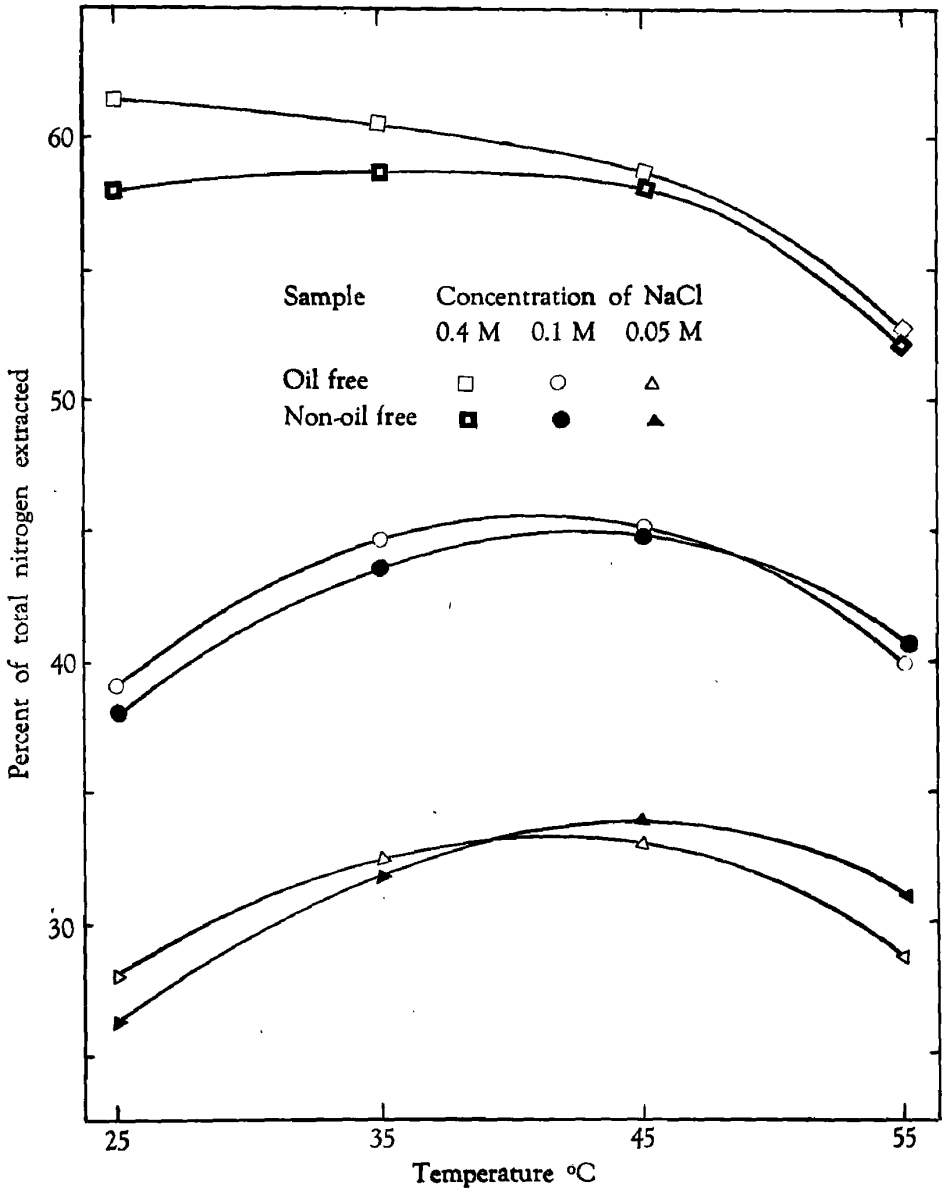
TABLE XXVIII

THE EFFECT OF TEMPERATURE AND PRESENCE OF OIL IN MUNG BEAN MEAL ON THE AMOUNT OF PROTEIN NITROGEN EXTRACTED BY THREE DIFFERENT CONCENTRATIONS OF NaCl
(Tabulated from Table XXVII)

Temp.	Material	% N per Total N of air-dried Samples		
		.4M NaCl	.1M NaCl	.05M NaCl
25°C.	Non-oil free	53.57	34.76	24.58
	Oil free	56.71	36.44	26.19
	Difference*	+3.14	+1.68	+1.61
35°C.	Non-oil free	53.64	40.48	29.34
	Oil free	55.76	41.42	30.00
	Difference*	+2.12	+ .94	+ .66
45°C.	Non-oil free	53.32	41.56	31.32
	Oil free	54.44	41.71	30.59
	Difference*	+1.15	+ .15	- .73
55°C.	Non-oil free	48.30	37.71	28.85
	Oil free	48.81	37.39	26.49
	Difference*	+ .51	- .32	-2.36

* Difference between "Oil Free" and "Non-oil Free" samples.

Figure 8.



This figure shows the effect of temperature upon the degree of peptization of oil free and non-oil free Mung bean meal.

PRECIPITATION

It was observed in the previous experiment that Mung bean protein extracted with sodium chloride or potassium chloride gave a very narrow solubility range as shown by the "steep slope" curves (Figures 2 and 3). In other words it was obvious that the protein which was highly soluble at 0.4M NaCl would be insoluble or precipitable by dilution with a small volume of water.

According to the definition globulins are insoluble in water but soluble in dilute salt solutions of the salts of strong acids and bases (59). Gortner et al reported (40) experiments in which wheat flour was extracted with 5 percent potassium sulfate solution and with 10 percent sodium chloride solution and they found that the amount and nature of the protein material dissolved by these two reagents were markedly different. According to the definition of globulin, the two solutions should have yielded identical fractions. They studied the question further and asked "of what salts and what concentrations?", but ignored the definition of globulin. Similar questions were also raised by Ferry, Cohn and Newman (60) "how soluble? or how insoluble?"

For practical purposes the "soluble-insoluble" range of Mung bean globulin must be measured and confirmed. In order to solve this problem a scheme for this determination was devised for precipitation of the globulin from a 0.4M NaCl extract by the dilution procedure. This procedure served to replace the salting out method for precipitating globulins. The determinations were carried out as follows:

A. Determination of protein nitrogen - non-protein nitrogen ratio and the albumin-globulin ratio.

B. Demonstration of the effect of dilution upon the sedimentation time of globulin fraction.

C. Protein sedimentation from 0.4M NaCl extract of Mung bean meal at various hydrogen ion concentrations.

D. Suggested procedure for the isolation of the globulin from Mung bean meal.

E. A quantitative study of the procedure.

(A) Determination of Protein Nitrogen - Non-protein Nitrogen Ratio and Albumin-Globulin Ratio.

Procedure: Twenty grams of Mung bean meal (60 mesh) were extracted with 200 ml. of 0.4M NaCl solution for 30 minutes with occasional hand stirring. The clear extract was separated from the residue by centrifugation and the extract was used for the following experiments:

1. Triplicate 10 ml. portions of the extract were pipetted into separate Kjeldahl flasks for the total nitrogen determination.

2. Ten ml. of the extract were pipetted into a 50 ml. centrifuge tube and ten ml. of 20 percent trichloroacetic acid were added. The protein matter precipitated immediately and was separated from the supernatant by centrifugation. The precipitate (with the aid of 0.4M NaCl) was transferred to a Kjeldahl flask for protein nitrogen determination.

3. The clear liquid separated by the above trichloroacetic acid precipitation was used for non-protein nitrogen determination.

4. Three blanks were also determined: to one an additional 50 ml.

of distilled water were added, to the second 100 ml. and to third 150 ml.

5. A series of ten 100 ml. graduate cylinders were arranged. The cylinders were marked as follows: the first cylinder on the left 1 - 1, the second 1 - 2, the third 1 - 3 and so on until the one at the right end was 1 - 10. To each of the ten cylinders 10 ml. of the extract was introduced by a pipette. Then to the first cylinder 10 ml. of distilled water were added to make a 1:1 dilution; to the second 20 ml. of distilled water; to the third 30 ml. and so on until the 10 cylinders were diluted with distilled water accordingly.

a. Determination of Globulin Nitrogen fraction. After standing for one hour the protein matter (globulin*) was precipitated and had settled to the bottom of the cylinders by gravitation. The precipitate was separated from the liquid by centrifugation. The liquid was poured into a clean cylinder marked to correspond to the above series. The precipitate was transferred to a Kjeldahl flask for globulin determination.

b. Determination of albumin nitrogen fraction. An equal volume of 20 percent trichloroacetic acid was added to each of the 10 cylinders which contained the clear liquid separated from the globulin protein. Precipitation occurred immediately. The precipitate was separated from the liquid by centrifugation. The nitrogen content of the liquid and

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* By the term globulin is meant the fraction which is soluble in neutral salt solution (0.4M NaCl) and is insoluble when diluted with water (59).

the precipitate were determined and recorded as non-protein nitrogen and albumin nitrogen*, respectively.

The blank determination was substracted from all the Kjeldahl titrations. Results are expressed in terms of percentage of: (1) total nitrogen (TN) as 100 percent, (2) protein nitrogen (PN), (3) globulin nitrogen (GN), (4) albumin nitrogen (AN), and (5) non-protein nitrogen (NPN) and are given in Tables XXIX and XXX and Figure 9.

(B) Demonstration of the Effect of Dilution Upon the Sedimentation Time of Globulin Fraction.

Ten ml. portions of 0.4M NaCl extract of Mung bean meal were introduced into each of ten 100 ml. graduated cylinders. Ten ml. of distilled water were added to the No. 1 cylinder making a 1:1 dilution; 20 ml. of distilled water were added to the No. 2 cylinder making a 1:2 dilution and 30 ml. were added to the No. 3 cylinder making a 1:3 dilution and so on up to the No. 10 cylinder to which 100 ml. of distilled water were added making a 1:10 dilution (Figure 10).

The photographs were taken 15, 45 and 75 minutes respectively after dilutions were made and show that the 1:4 and 1:5 dilutions were faster in precipitation and sedimentation of the total protein, whereas No. 6 through No. 10 showed slower settling than Nos. 4 and 5. This rate of settling may be due to the larger volume of dilution which apparently slows down the rate of sedimentation. With the 1:1 dilution no precipitation occurred and the protein was still in solution. With the 1:2

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* By the term albumin is meant the fraction which is soluble in water and in neutral salt solution (59).

TABLE XXIX

THE DETERMINATION OF PROTEIN-NITROGEN, NON-PROTEIN-NITROGEN, GLOBULIN-NITROGEN AND ALBUMIN-NITROGEN FROM 0.4M NaCl SOLUTION EXTRACT OF MUNG BEAN MEAL

	Determination of	Titrations ml. 0.1002N HCl	Mgm. N*	% N/T N
1.	T N	20.5	28.056	100.00
2.	T N	20.55	28.126	
3.	T N	20.45	27.958	
4.	P N	18.5	25.255	89.99
5.	P N	18.5	25.25	
6.	P N	15.55	25.320	
7.	NPN	2.5	2.805	9.97
8.	NPN	2.5	2.805	
9.	NPN	2.5	2.805	
10.	G N 1-1**	1.5	1.402	5.00
11.	G N 1-2	5.2	6.593	23.50
12.	G N 1-3	8.2	10.940	38.99
13.	G N 1-4	10.6	14.168	50.50
14.	G N 1-5	11.5	15.430	55.00
15.	G N 1-6	11.8	15.851	56.00
16.	G N 1-7	12.0	16.132	57.50
17.	G N 1-8	12.1	16.272	58.00
18.	G N 1-9	12.1	16.272	58.00
19.	G N 1-10	12.0	16.132	57.50
20.	A N 1-1	17.0	23.146	82.50
21.	A N 1-2	13.25	17.885	63.74
22.	A N 1-3	10.2	13.607	48.50
23.	A N 1-4	7.85	10.310	36.75
24.	A N 1-5	7.0	9.118	32.50
25.	A N 1-6	6.7	8.697	31.00
26.	A N 1-7	6.45	8.346	29.75
27.	A N 1-8	6.35	8.206	29.25
28.	A N 1-9	6.4	8.276	29.50
29.	A N 1-10	6.5	8.416	30.00
30.	NPN 1-1	3.0	3.507	12.50
31.	NPN 1-2	3.0	3.507	12.50
32.	NPN 1-3	3.0	3.507	12.50
33.	NPN 1-4	3.0	3.507	12.50
34.	NPN 1-5	3.0	3.507	12.50
35.	NPN 1-6	3.0	3.507	12.50
36.	NPN 1-7	3.0	3.507	12.50
37.	NPN 1-8	3.0	3.507	12.50
38.	NPN 1-9	3.0	3.507	12.50
39.	NPN 1-10	3.0	3.507	12.50

* Mgm. N values were calculated from titrations minus blanks.

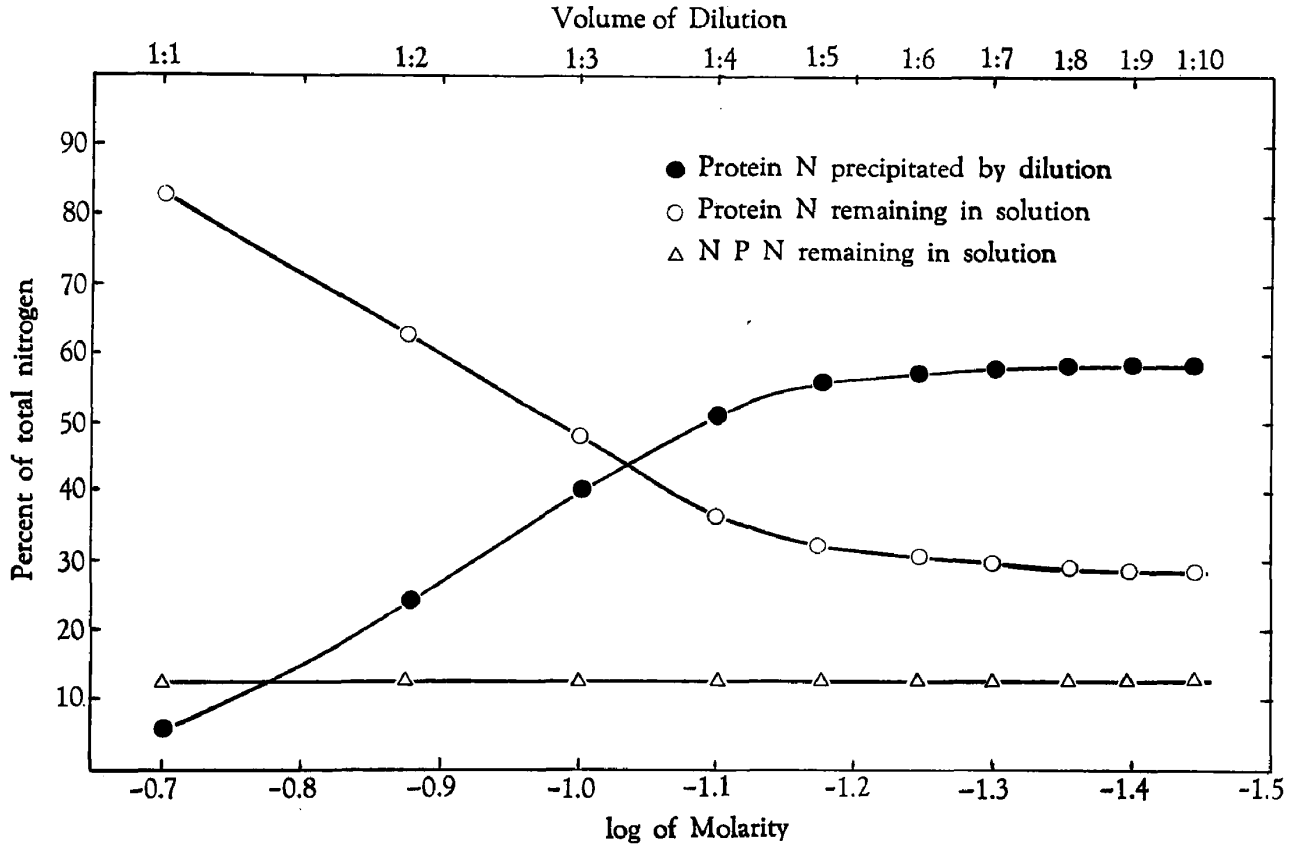
** Dilution volume, e.g., (1-2) for one volume of extract two volumes of water were added.

TABLE XXX

THE GLOBULIN -ALBUMIN RATIO G/A AND PROTEIN NITROGEN AND NON-PROTEIN NITROGEN RATIO PN/NPN OF MUNG BEAN PROTEIN

Dilution	Molarity	Log M	% GN	% AN	% NPN	Total	G/A	PN/NPN
1:1	0.2	-0.699	5.0	82.0	12.5	100.0	0.06	7.00
1:2	0.133	-0.876	23.5	63.7	12.5	99.7	0.36	6.97
1:3	0.1	-1.000	38.9	48.5	12.5	99.9	0.80	6.99
1:4	0.08	-1.097	50.5	36.7	12.5	99.7	1.37	6.97
1:5	0.066	-1.174	55.0	32.5	12.5	100.0	1.69	7.00
1:6	0.057	-1.244	56.0	31.5	12.5	100.0	1.72	6.96
1:7	0.05	-1.301	57.5	29.7	12.5	99.7	1.93	6.97
1:8	0.044	-1.358	58.0	29.2	12.5	99.7	1.98	6.97
1:9	0.04	-1.398	58.0	29.5	12.5	100.0	1.96	7.00
1:10	0.036	-1.444	57.5	30.0	12.5	100.0	1.85	6.84

Figure 9.



This figure shows the amount of protein precipitated and that which remained in solution when the salt concentration of a 0.4 M NaCl extract of Mung bean meal was diminished by dilution.

dilution a partial precipitation occurred which appeared as a white cloudiness throughout the liquid. With the 1:3 dilution there was only a small amount of protein matter flocculated and the precipitation evidently was incomplete. From cylinder No, 4 through No. 10 the precipitation with different dilutions apparently was complete and the supernatant liquids were water clear.

As a consequence of these results the 1:4 dilution was employed for the precipitation of total protein from 0.4M NaCl extract of Mung bean meal (Figure 10).

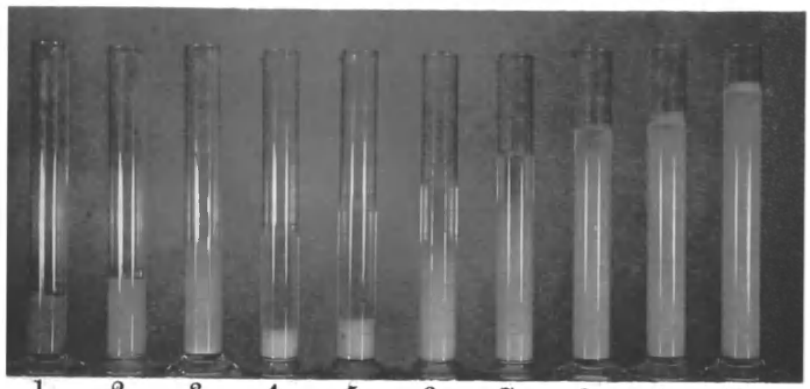
(C) Protein Sedimentation from 0.4M NaCl Extract of Mung Bean Meal at Various Hydrogen Ion Concentrations.

The purpose of this experiment was to demonstrate sedimentation of protein by variation in hydrogen ion concentration. For this work a dilution was found necessary that should not be the optimum for the precipitation of proteins as previously shown. By experiment it was found that a dilution ratio of 1:5 appeared to be less affected by simple dilution and could be used to give information regarding the influence of hydrogen ions. The procedure and results are indicated in Table XXXI.

This experiment demonstrated that in four cases, Cylinders Nos. 2, 4, 10 and 15, the precipitates appeared to be more compact as indicated by the smaller volumes (Figure 11).

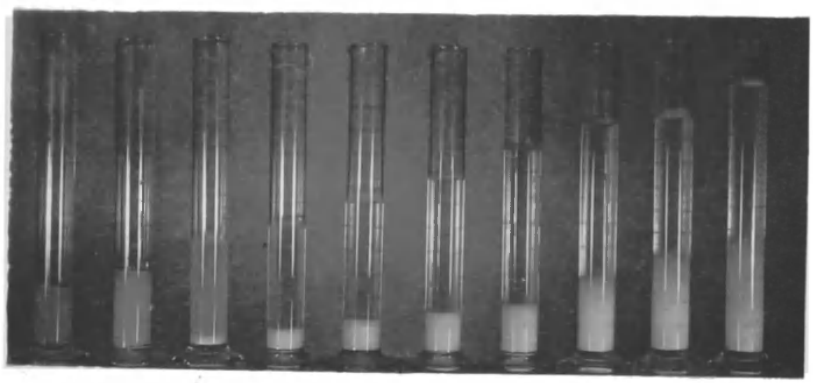
This method of precipitation was not used by this investigator for the isolation of Mung bean globulin since the addition of acid for adjusting the pH causes denaturation of the protein. When the protein is suspended in a very dilute salt solution, it is more easily denatured

Figure 10.



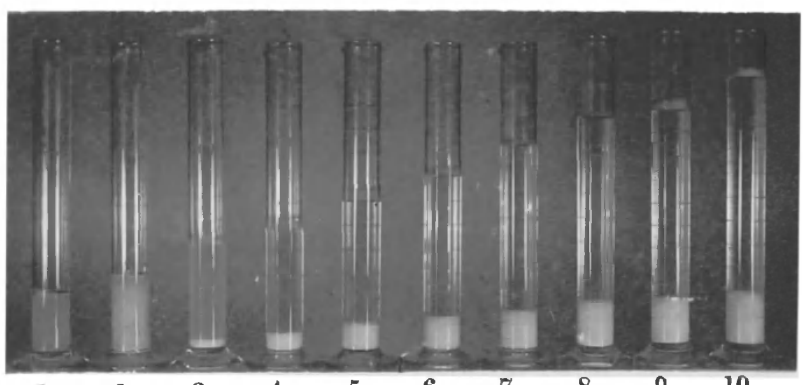
1 2 3 4 5 6 7 8 9 10

Photographed after 15 minutes



1 2 3 4 5 6 7 8 9 10

Photographed after 45 minutes

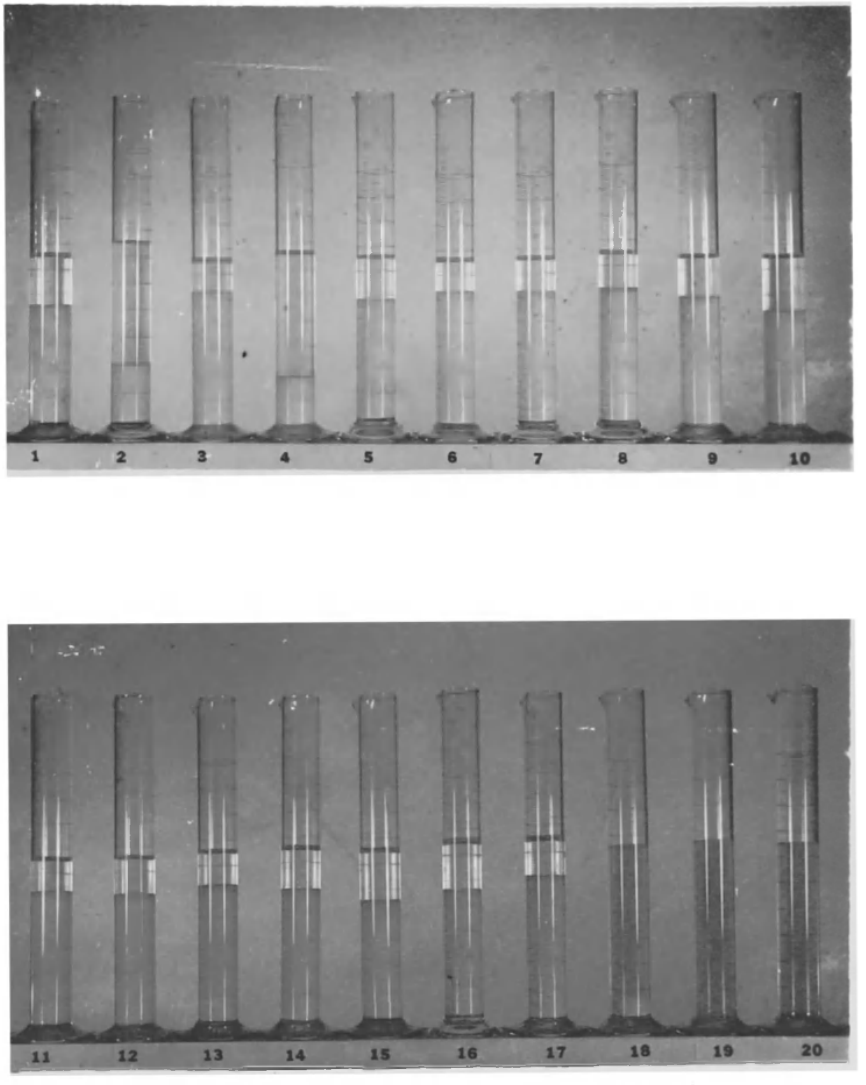


1 2 3 4 5 6 7 8 9 10

Photographed after 75 minutes

Visual observation of the gravitational sedimentation rate of Mung bean protein extract when the salt concentration was diminished by dilution. The numbers beneath the cylinders indicate the volumes with which 10 ml portions of 0.4 M NaCl extract was diluted.

Figure 11.



Visual observation of the effect of H-ion concentration (pH) on the manner of precipitation from a 0.4 M NaCl extract of Mung bean protein diluted to 0.06 M. The numbers beneath the cylinders identify the data in the preceding table.

by a very small amount of acid than when dispersed in a concentrated salt solution (61).

TABLE XXXI

SEDIMENTATION OF PROTEIN BY VARIATION OF HYDROGEN ION CONCENTARATION

Series of 100 ml. Cylinders	pH	ml. Water with pH Adjusted	ml. Extract (0.4M NaCl) added	Reading in ml. of Ppt. after 12 hrs.	Final pH
1.	6.2	55	10	46	6.17
2.	6.0	55	10	20	5.88
3.	5.8	55	10	54	5.74
4.	5.6	55	10	19	5.84
5.	5.4	55	10	49	5.75
6.	5.2	55	10	53	5.74
7.	5.0	55	10	52	5.74
8.	4.8	55	10	51	5.73
9.	4.6	55	10	50	5.74
10.	4.4	55	10	44	5.82
11.	4.2	55	10	53	5.74
12.	4.0	55	10	52	5.72
13.	3.8	55	10	52	5.72
14.	3.6	55	10	51	5.58
15.	3.4	55	10	46	5.52
16.	3.2	55	10	47	5.30
17.	3.0	55	10	52	4.82
18.	2.8	55	10	0	4.31
19.	2.6	55	10	0	3.64
20.	2.4	55	10	0	3.06

(D) Suggested Procedure for the Isolation of the Globulin from Mung Bean Meal.

From the results of the previous experiments have shown the following necessary procedure:

1. The solvent. A 0.4M NaCl solution was used.

2. Solid-solvent ratio (S-S-R) 1:10.

3. Three successive extractions were employed.

4. Extraction time. The most satisfactory results were obtained

with one hour's extraction at 25°C. with occasional hand stirring.

5. The removal of the residues. The residue was removed by centrifugation for 15 minutes at 2000 r.p.m..

6. The precipitation of protein from extract with 1:4 dilution. For each volume of extract four volumes of water were added and the total protein precipitated.

From previous analysis Mung bean protein precipitated with 1:4 dilution showed:

50.50 percent nitrogen per extractable nitrogen as globulin nitrogen

37.00 percent nitrogen per extractable nitrogen as albumin nitrogen

12.50 percent nitrogen per extractable nitrogen as N P N.

(E) A Quantative Study of the Procedure Involved.

A quantative study of the procedure just outlined was carried out.

a. Extraction. A mixture of 100 grams of Mung bean meal (60 mesh) and 1000 ml. of 0.4M NaCl solution (S-S-R 1:10) were extracted for one hour at 25°C. with occasional hand stirring. The extract was centrifuged for 15 minutes to remove the residue. The residue (I*) was re-extracted twice and all the extracts (II) were combined.

	ml. added 0.4M NaCl	ml. Centrifugate Collected
First Extraction	1000	860
Second Extraction (residue of 1st)	860	850
Third Extraction (residue of 2nd)	850	860

A volume of 860 ml. of centrifugate were collected from the first extraction so 860 ml. of 0.4M NaCl were added to the residue for the

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* See Flow Sheet - Figure 12.

second extraction, giving 850 ml. of centrifugate collected from this second extraction and 850 ml. of 0.4M NaCl were then added to the residue for the third extraction from which 860 ml. were collected. Thus the solid-solvent ratio was kept in each case at 1:10.

b. Precipitation of protein. The combined centrifugate (II) (in 0.4M NaCl) was diluted with four volumes of distilled water (1:4 ratio*). The final concentration of the diluted extract was 0.08M NaCl. The precipitation of protein occurred instantly. The precipitate was allowed to stand for a few hours, usually over night, to allow a more complete precipitation, and was then separated from the liquid by centrifugation.

c. Purification. The precipitate (III) was dissolved in 0.4M NaCl at 25°C. With gentle hand stirring, ten minutes were needed to completely dissolve the precipitate. The dispersed protein solution was then centrifuged. The residue was designated as (V) and the centrifugate as (VI). A large quantity of insoluble material separated by centrifugation, indicating a globulin-bound (Gb) (V) substance, or protein matter which is not dispersable in dilute neutral salt. The clear centrifugate (VI) was diluted with four volumes of distilled water and precipitation occurred

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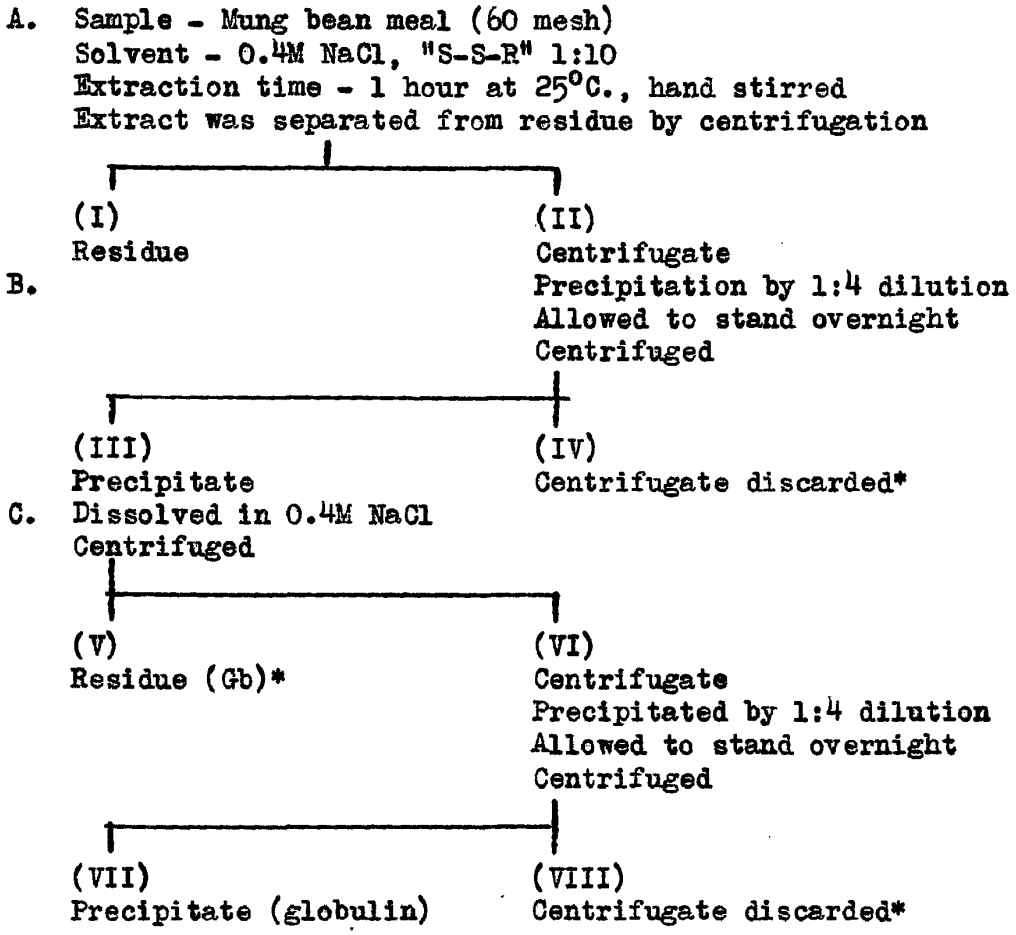
* The addition of water to the extract was conducted with a large funnel with the stem extended by glass tubing. The lower end of the tube was immersed under the surface of the extract. The time required for sedimentation of the protein was three to four hours at 6°C.

instantly. After standing for four hours, the precipitate (VII) was separated from the supernatant (VIII) by centrifugation. The process of purification (c.) was repeated until no more Gb substance could be removed.

The amount of globulin obtained from 100 grams of 60 mesh Mung bean meal by three successive extractions with 0.4M NaCl and precipitated by 1:4 dilution was then determined. The precipitate (VII) which was freed from globulin-bound substance and albumin was first dissolved in 500 ml. of 0.4M NaCl solution and two 10 ml. aliquots were taken for Kjeldahl nitrogen determination. The total globulin nitrogen from 100 gm. of Mung bean meal (air-dried) was found to be 1.4056 gm. This weight represented 37.01 percent of the total nitrogen content of 100 gm. of Mung bean meal or 49.45 percent of the total extractable nitrogen.

Figure 12

F L O W S H E E T



A - extraction, B - precipitation, C - purification.
* The centrifugates IV and VIII contain albumin fractions and were discarded. The residue V was the globulin-bound (Gb) substance insoluble in 0.4M NaCl.

FRACTIONATION AND FURTHER PURIFICATION OF GLOBULIN

In the previous experiments the purification procedure was carried out by repeated peptization in 0.4M NaCl and precipitation by direct dilution at a 1:4 ratio. The resulting product was white but amorphous in form. Since the substance obtained was not crystalline in form, other means than simple recrystallization had to be employed for purification and possible fractionation.

It was observed that when 100 ml. of protein solution (0.4M NaCl) were directly diluted with 400 ml. distilled water dropwise from a burette, then allowed to stand for 12 hours at 6°C., fine crystals formed as noted by examination under a microscope. Thus, slow dilution, as might be expected, encouraged the formation of crystals.

An effective dialyzing apparatus for precipitation and purification of protein material was constructed in this laboratory and is shown in Figure 13. A glass tank of seven liters capacity (18 in. x 6 in.) was placed in an aluminum tray which was centered and fastened by screws to a pulley six inches in diameter. The pulley was secured to the axle of the stand of a cork boring machine by two set screws. Drive for the system was obtained from a fractional horsepower (¼ H.P.) motor coupled to the axle by means of pulleys. Round leather belts (¼ in. diameter) were used to connect the pulleys. A four pulley arrangement served to reduce the motor speed from 1750 r.p.m. to 35 r.p.m. at the axle.

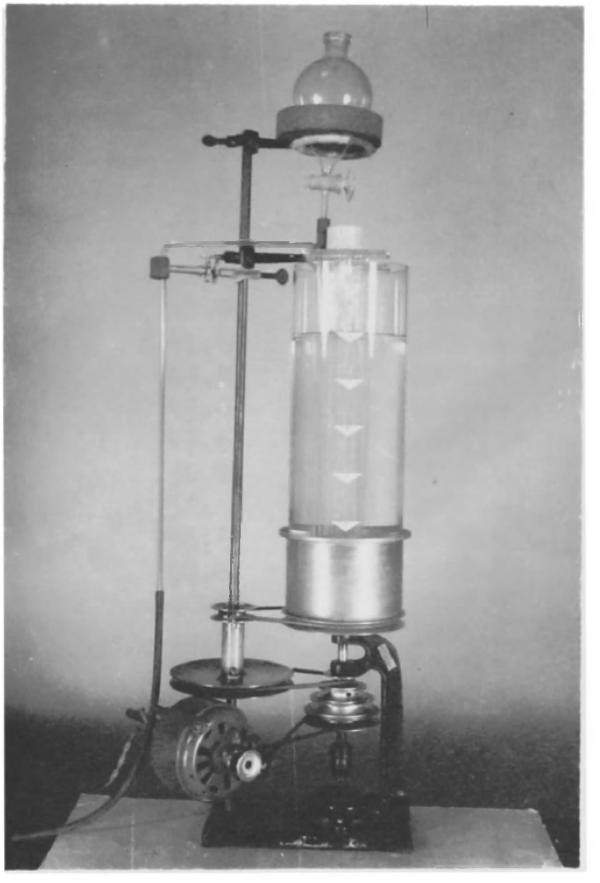
The dialyzing membrane was three inch flat width cellophane tubing 18 inches in length. The top of the tube was fastened to a ring 1 3/4 inches

in diameter. The base of the tube was secured to an open-mouth, flat-bottomed glass bottle 1 3/4 inches at top and 2 inches in depth. The top ring rested in an E-shaped metal frame which was attached to the ring stand.

The apparatus was designed for slow, step-wise, indirect dilution in order to obtain the desired precipitated and crystalline forms of the globulins. The amount of distilled water added to the tank from a reservoir placed above the tank was controlled by a stopcock. The apparatus also could be used to remove the entire salt content from a protein solution by continuous addition of water to the tank from the reservoir and removal from the tank by siphoning until the dialysing water was free of chloride ions. And further, this apparatus could be employed to reduce a solution of a known concentration to a lower desired concentration. Calculations showing the reduction of concentration are shown in Tables XXXII and XXXIII.

It is well known that protein solutions that are rapidly diluted directly by distilled water produce an amorphous form of precipitate which adsorbs many impurities. Slow direct dilution produced crystals but the process was difficult to control. The slow indirect dilution device just described overcame these difficulties. The protein solution in the membrane was suspended in the tank containing salt solution of the same concentration as the solution in the membrane. The slow addition of distilled water to the dialyzing liquid in the tank prevented direct contact of distilled water to the membrane. The membrane itself gave the proper rate of dilution. Best results were obtained when the

Figure 13.



Rotating Outside Liquid Dialyzer

TABLE XXXII

DILUTION TABLE "1000"

ml. sol.	Molarity	ml. of Water Added	Total Volume	Final Conc.
1000	0.4	142.8	1142.8	0.35
1000	0.35	29.4	1029.4	0.34
1000	0.34	30.3	1030.3	0.33
1000	0.33	31.2	1031.2	0.32
1000	0.32	32.2	1032.2	0.31
1000	0.31	33.3	1033.3	0.30
1000	0.30	34.4	1034.4	0.29
1000	0.29	35.7	1035.7	0.28
1000	0.28	37.0	1037.0	0.27
1000	0.27	38.4	1038.4	0.26
1000	0.26	40.0	1040.0	0.25
1000	0.25	41.6	1041.6	0.24
1000	0.24	43.4	1043.4	0.23
1000	0.23	45.4	1045.4	0.22
1000	0.22	47.6	1047.6	0.21
1000	0.21	50.0	1050.0	0.20
1000	0.20	52.6	1052.6	0.19
1000	0.19	55.5	1055.5	0.18
1000	0.18	58.8	1058.8	0.17
1000	0.17	62.5	1062.5	0.16
1000	0.16	66.6	1066.6	0.15
1000	0.15	71.4	1071.4	0.14
1000	0.14	76.9	1076.9	0.13
1000	0.13	83.3	1083.3	0.12
1000	0.12	90.9	1090.9	0.11
1000	0.11	100.0	1100.0	0.10
1000	0.10	111.1	1111.1	0.09
1000	0.09	125.0	1125.0	0.08
1000	0.08	142.8	1142.8	0.07
1000	0.07	166.6	1166.6	0.06
1000	0.06	200.0	1200.0	0.05
1000	0.05	250.0	1250.0	0.04
1000	0.04	333.3	1333.3	0.03
1000	0.03	500.0	1500.0	0.02
1000	0.02	1000.0	2000.0	0.01

TABLE XXXIII

DILUTION TABLE "1000x"

Ml. Sol to be Diluted	Molarity	Ml. of Water Added	Total Volume	Final Conc.
1000	0.4	142.8	1142.8	0.35
1142.8	0.35	33.6	1176.4	0.34
1176.4	0.34	35.7	1212.1	0.33
1212.1	0.33	37.8	1250.0	0.32
1250.0	0.32	40.3	1290.3	0.31
1290.3	0.31	43.0	1333.3	0.30
1333.3	0.30	46.0	1379.3	0.29
1379.3	0.29	49.2	1428.5	0.28
1428.5	0.28	52.9	1481.4	0.27
1481.4	0.27	57.0	1538.4	0.26
1538.4	0.26	61.6	1600.0	0.25
1600.0	0.25	66.6	1666.6	0.24
1666.6	0.24	72.4	1739.0	0.23
1739.0	0.23	79.0	1818.0	0.22
1818.0	0.22	86.8	1904.8	0.21
1904.8	0.21	95.2	2000.0	0.20
2000.0	0.20	105.2	2105.2	0.19
2105.2	0.19	117.0	2222.2	0.18
2222.2	0.18	130.7	2352.9	0.17
2352.9	0.17	147.1	2500.0	0.16
2500.0	0.16	166.6	2666.6	0.15
2666.6	0.15	190.5	2857.1	0.14
2857.1	0.14	219.8	3076.9	0.13
3076.9	0.13	256.4	3333.3	0.12
3333.3	0.12	303.0	3636.3	0.11
3636.3	0.11	363.7	4000.0	0.10
4000.0	0.10	444.4	4444.4	0.09
4444.4	0.09	555.6	5000.0	0.08
5000.0	0.08	714.3	5714.3	0.07
5714.3	0.07	952.4	6666.7	0.06
6666.7	0.06	1333.3	8000.0	0.05
8000.0	0.05	2000.0	10000.0	0.04

position of the membrane was eccentric, thus creating a gentle agitation of the dialyzing water when the tank was rotated. Another important point was to fill the membrane no more than three-fourths full. When immersed in the tank, the solution in the membrane rose to the level of the outside dialyzing water. This increased the interface between solution and the cellophane membrane. When the tank was rotated, both the dialyzing water and the solution were gently agitated. If the membrane was completely filled, there was little or no movement inside the membrane. Movement inside the membrane aided in establishing an equilibrium more promptly while still maintaining the proper rate of dialysis.

APPLICATION OF THE SLOW STEPWISE DILUTION PROCEDURE AND ROTATING OUTSIDE LIQUID DIALYSIS FOR THE PURIFICATION OF MUNG BEAN GLOBULIN

A 300 ml. portion of a preparation of Mung bean protein extracted with 0.4M NaCl, precipitated by 1:4 dilution and redissolved in 0.4M NaCl was placed in cellophane tubing. The dialyzer was suspended in the dialyzing tank which was also half full of 0.4M NaCl solution. The level of the solution in the tank was then adjusted to the level of the protein solution in the cellophane tube.

The indirect, slow, step-wise dilution was begun by introducing distilled water from the reservoir into the dialyzing tank by means of glass tubing. The flow was controlled by a stopcock to that of 500 ml. per hour. The water level of the tank slowly rose to the six liter mark. The dialyzing water level was maintained at the six liter mark by means of siphoning. The dialyzing water level could be adjusted to any height by regulating the length of the siphon tube.

The dialysis of the 300 ml. protein solution, covered with toluene, was allowed to proceed overnight. At that time two distinct layers of precipitate were observed in the bottom of the cellophane membrane. The lower fraction was faint yellowish in color and the upper portion was a snowy white, fluffy material. Tentatively the symbol G_3 was assigned to the material in the upper layer and G_2 to that of the lower layer.

The two portions were separated by pouring the contents of the cellophane tube into a beaker. The upper layer or G_3 poured out but the lower layer of G_2 was gelatinous material and firmly packed in the bottom of

the tube.

To further purify the G_2 fraction the gelatinous material was redissolved in 300 ml. of 0.4M NaCl solution. The solution was centrifuged to remove any insoluble material. The solution was of bluish opalescence. The clarified protein solution was placed in the dialyzing cellophane tube and dialysis proceeded as previously described. When an equal amount of water had been added to the tank, G_2 was reprecipitated. At this point the approximate NaCl concentration was 0.2M. It was observed that the dialysate appeared cloudy with white fluffy material suspended throughout.

DETERMINATION OF THE MOLAR CONCENTRATION OF SODIUM CHLORIDE AT WHICH THE G_2 FRACTION PRECIPITATES FROM THE EXTRACT

A fresh 0.4M NaCl extract was prepared according to the previously described standardized procedure. The centrifugate was diluted with four volumes of distilled water and allowed to stand four hours until the precipitation was complete. The precipitation was separated by centrifugation. The precipitate was redispersed with 0.4M NaCl, stirring until it was completely dissolved. The material was centrifuged to remove the insoluble material or globulin-bound substance (Gb). The clarified solution in 0.4M NaCl contained the total globulin and was used for the determination of the molar concentration at which G_2 precipitates.

The molar concentration of NaCl at which G_2 precipitated from the solution containing total globulins was determined by indirect, slow, step-wise dilution procedure with the dialyzing apparatus. For this work a 350 ml. portion of 0.4M NaCl solution containing the total globulins

was placed in the cellophane tube and 2650 ml. of 0.4M NaCl solution were placed in the tank. The protein solution was covered with a thin film of toluene. There was, therefore, a total of 3000 ml. of 0.4M NaCl solution if both the solution inside and that outside the cellophane tube were considered.

The indirect, slow, step-wise dilution procedure was carried out by adding to the tank from a separatory funnel a measured volume of water at a speed of 100 ml. per hour while the tank was rotating. The first measured volume of water added was 482.6 ml.. This volume was used so that the final calculated concentration of NaCl in the tank and tube would be 0.35M. After the 482.6 ml. were added, the tank and tube were rotated for a period of time to allow the material on both sides of the membrane to reach equilibrium. It was noted that there was no sign of precipitation. Therefore a second measured volume of water was added. This volume of 100.8 ml. brought the final concentration to 0.34M. Time was allowed for equilibrium to be reached. Since there was not sign of precipitation, further dilution with water was continued in measured volumes with time allowed to establish equilibrium after each addition. Rotation of the tank was continuous throughout the entire procedure. The results showing each addition, the final concentration of NaCl and visual observation made during the experiment were recorded in Table XXXIV.

It was observed that the dialysate began to cloud when the calculated concentration was 0.23M NaCl and mass precipitation occurred at .22M. Since the cloudiness of the dialysate not only persisted but increased at lower concentrations, it was somewhat confusing as to what definite

salt concentration G_2 completely precipitated. Further means were sought for determination of the precise NaCl concentration at which G_2 precipitated.

TABLE XXXIV

DETERMINATION OF THE MOLAR CONCENTRATION OF NaCl AT WHICH G_2 PRECIPITATES
(Indirect, slow, step-wise dilution)

Total Vol. of Sol. in Tank and Tube	Molarity of NaCl	ml. Water Added	Total Volume	Final Conc.	Visual Observation
3000	0.4	482.6	3428.6	0.35	no ppt.
3428.6	0.35	100.8	3529.4	0.34	no ppt.
3529.4	0.34	106.9	3636.3	0.33	no ppt.
3636.3	0.33	113.6	3750.0	0.32	no ppt.
3750.0	0.32	121.0	3871.0	0.31	no ppt.
3871.0	0.31	129.0	4000.0	0.30	no ppt.
4000.0	0.30	137.9	4137.9	0.29	no ppt.
4137.9	0.29	147.8	4285.7	0.28	no ppt.
4285.7	0.28	158.7	4444.4	0.27	no ppt.
4444.4	0.27	170.9	4615.4	0.26	no ppt.
4615.4	0.26	184.6	4800.0	0.25	no ppt.
4800.0	0.25	200.0	5000.0	0.24	no ppt.
5000.0	0.24	217.3	5217.4	0.23	slight clouding
5217.4	0.23	237.1	5454.5	0.22	ppt. occurred
5454.5	0.22	259.7	5714.3	0.21	cloudiness
5714.3	0.21	285.7	6000.0	0.20	Flocculation complete

This further study of the proper molar concentration was conducted with a new batch of globulin preparation from which the globulin-bound substance was removed. The direct, slow, step-wise dilution method was again employed. To each of the following series of nine 100 ml. graduated cylinders a 50 ml. portion of globulin solution in 0.4M NaCl was added. Varied volumes of distilled water were added to each cylinder from a burette dropwise until the desired approximate concentration was reached in each instance. The solutions were gently stirred and stored

at 6°C. for 12 hours. The results as recorded in Table XXXV were observed.

TABLE XXXV

DETERMINATION OF THE MOLAR CONCENTRATION OF NaCl AT WHICH G_2 PRECIPITATES (Direct Dilution Method)

Cylinder	Ml. Globulin in 0.4M NaCl	Ml. Water Added	Final Molarity	Approximate Amt. in Ml. after 12 hrs. at 6°C.
1.	50	12.5	0.32	no ppt.
2.	50	16.65	0.30	no ppt.
3.	50	21.43	0.28	no ppt.
4.	50	26.9	0.26	no ppt.
5.	50	33.3	0.24	incomplete ppt.
6.	50	40.9	0.22	2.8
7.	50	50.0	0.20	3.0
8.	50	61.1	0.18	4.0
9.	50	75.0	0.16	10.0

The characteristics of the system in each of the nine cylinders are described below.

Cylinders Nos. 1 through 4 were alike in having no sign of precipitation or cloudiness.

In cylinder No. 5 there was incomplete precipitation. There was no sharp dividing line or boundary between precipitation and supernatant.

In cylinder No. 6 dense precipitation occurred which appeared as a faint yellow, opalescent material. The volume of precipitate was about 2.8 ml. after standing 12 hours at 6°C.

In cylinder No. 7 the precipitation appeared somewhat whiter than in No. 6.

Cylinder No. 8 produced a precipitate which appeared whiter than No. 7 and was larger in volume. It was further noticed that there was a very small amount of white fluffy precipitation on top of the principal

precipitate. This did not occur in No. 7.

In cylinder No. 9 precipitation was complete and was uniformly whitish. It had the largest volume (10 ml.) and was whiter than No. 7. The supernatant was the clearest of any in the set.

The indications from this study of the molar concentration at which G_2 precipitates could be summarized in the following manner. The precipitate in cylinder No. 6 at the concentration of 0.22M which exhibited faint yellow colored, opalescent material was G_2 which had been isolated by slow, step-wise continuous dilution. The volume, 2.8 ml., was small but apparently dense. Cylinder No. 9 at molarity of 0.16 had the largest volume of precipitate but entirely lost the G_2 characteristic appearance. Therefore, 0.16M NaCl did not seem advisable for fractionation of G_2. In both cylinder No. 7 and No. 8 the precipitations were probably composed of G_2 fraction but they had been contaminated with other globulin fractions. From the above observations, it appeared that G_2 in pure fraction precipitated best at 0.22 molar concentration of NaCl.

FRACTIONATION OF G_3 FROM G_2-FREE DIALYSATE

The dialysate or supernatant from which G_2 was isolated by the indirect, slow, step-wise dilution procedure when tested by the use of the addition of trichloroacetic acid and by further dilution to 0.08M NaCl was found to contain a substantial quantity of insoluble protein matter. It was decided that in order to detect or isolate the further fraction or fractions, both (1) direct and (2) indirect, slow, step-wise dilution procedures should be followed.

(1) Direct, slow, step-wise dilution procedure was employed for detecting the molar concentration of NaCl at which G_3 precipitated from the supernatant. Into each of ten 100 ml. graduated cylinders was added a 50 ml. portion of the G_2-free supernatant. Various amounts of distilled water were added to each cylinder from a burette dropwise to secure a desired dilution for each. The solutions were gently stirred throughout the entire period of dilution and then were allowed to stand for 12 hours at 6°C. The results obtained are presented in Table XXXVI.

TABLE XXXVI

DETERMINATION OF THE MOLAR CONCENTRATION OF NaCl AT WHICH G_3 PRECIPITATES
(Direct, slow, step-wise dilution)

Cylinder	Dialysate 0.2M NaCl ml.	Ml. Water Added	Final Molarity	Visual Observation
1.	50	2.6	0.19	no ppt.
2.	50	5.5	0.18	no ppt.
3.	50	8.8	0.17	no ppt.
4.	50	12.5	0.16	no ppt.
5.	50	16.6	0.15	no ppt.
6.	50	21.4	0.14	no ppt.
7.	50	26.9	0.13	no ppt.
8.	50	33.3	0.12	no ppt.
9.	50	40.9	0.11	ppt. occurred
10.	50	50.0	0.10	ppt. occurred

It was observed that heavy precipitation of G_3 occurred at 0.11 and 0.10 molarity. The precipitate appeared almost white with a yellowish tinge.

(2) For further study the indirect, slow, step-wise dilution procedure was employed. A 350 ml. portion of the dialysate (0.22M NaCl) was placed in the dialyzer and 1468 ml. of 0.22M NaCl were added to the tank. The dilution procedure was the same as that for the precipitation of G_2. Two hours were allowed for establishing equilibrium after the addition of the calculated amount of water. Rotation of the tank was continuous throughout the entire procedure. The calculated volumes of water used and the final molar concentrations of NaCl in each case are recorded in Table XXXVII.

TABLE XXXVII

DETERMINATION OF THE MOLAR CONCENTRATION OF NaCl AT WHICH G_3 PRECIPITATES
(Indirect, slow, step-wise dilution)

Total Vol. of Sol. in Tank and Tube	NaCl Molarity	Water Added ml.	Total Volume ml.	Final NaCl Conc. Molarity	Visual Observations
3000.0	0.22	142.8	3142.8	0.21	no ppt.
3142.8	0.21	157.2	3300.0	0.20	no ppt.
3300.0	0.20	173.7	3473.7	0.19	no ppt.
3473.7	0.19	192.9	3666.6	0.18	no ppt.
3666.6	0.18	215.7	3882.3	0.17	no ppt.
3882.3	0.17	242.7	4125.0	0.16	no ppt.
4125.0	0.16	275.0	4400.0	0.15	no ppt.
4400.0	0.15	314.3	4714.3	0.14	no ppt.
4714.3	0.14	362.6	5076.9	0.13	no ppt.
5076.9	0.13	423.1	5500.0	0.12	no ppt.
5500.0	0.12	500.0	6000.0	0.11	precipitation occurred

The precipitation was allowed to stand in the dialyzer overnight.

At the end of this period the precipitation was complete.

The precipitate was very easy to separate from the supernatant as it was packed in the bottom of the dialyser and remained there when the supernatant was poured off. The precipitate was very soluble in 0.4M NaCl and gave a clear solution without stirring. From the above data it may be concluded that the NaCl molar concentration at which G_3 precipitates is 0.11.

INVESTIGATION OF FRACTION G_4 OF GLOBULIN IN A G_2- AND G_3-FREE DIALYSATE

With the isolation of G_2 and G_3 from the total protein mixture, the supernatant from which G_2 and G_3 were removed was still thought to contain unprecipitated protein material. Since the molar concentration of NaCl at which G_2 precipitated was 0.22 and for G_3, 0.11, it was thought that inasmuch as the solution still contained protein material, as indicated by the trichloroacetic precipitate, this protein might be precipitated as the result of a further reduction in the NaCl concentration.

The combined supernatant free of G_2 and G_3 was used for the investigation of still another fraction whose symbol has been designated as G_4. The isolation of G_4 was carried out with the indirect, slow, step-wise dilution procedure. The dilutions and observations were indicated in Table XXXVIII.

TABLE XXXVIII

DETERMINATION OF THE MOLAR CONCENTRATION OF NaCl AT WHICH G_4 PRECIPITATES
(Indirect, slow, step-wise dilution)

Total Vol. Tank and Tube	NaCl Molarity	Ml. Water Added	Ml. Total Vol.	Final Conc. Molar	Visual Observations
3000.0	0.11	300.0	3300.0	0.10	no ppt.
3300.0	0.10	366.7	3666.7	0.09	no ppt.
3666.7	0.09	458.3	4125.0	0.08	no ppt.
4125.0	0.08	589.2	4714.2	0.07	precipitation

The G_4 fraction was precipitated when the molar concentration of NaCl reached 0.07M. It was a white precipitate with a faint yellowish

tinge. It was easily separated from the supernatant by pouring off the liquid; the G_4 remaining in the tube undisturbed.

The molar concentration of NaCl at which G_4 precipitated from the supernatant was further studied by employing the direct, pour-in dilution procedure. The G_4 isolated from the above experiment was dissolved in 100 ml. of 0.4M NaCl solution. A 10 ml. portion of solution was pipetted into each of eight 100 ml. cylinders. Various ratios of dilution with water were made as follows: In cylinder No. 1, 1:1; No. 2 had 1:2 dilution and so on until with cylinder No. 8 the ratio was 1:8. The general procedure and results are tabulated in Table XXXIX.

TABLE XXXIX

THE SEPARATION OF A GLOBULIN FRACTION (G_4) BY THE FURTHER DILUTION OF THE NaCl SOLUTION

Cylinder	Globulin-0.4M NaCl Sol. ml.	Water Added ml.	Amt. Ppt. 10 min.	Amt. Ppt. 20 min.
1.	10	10	-	-
2.	10	20	-	-
3.	10	30	-	-
4.	10	40	+++	++++
5.	10	50	+++	++++
6.	10	60	++	+++
7.	10	70	+	++
8.	10	80	-	+

Mass precipitation of G_4 was observed at 1:4 and 1:5 dilution. At both dilutions the precipitate was firm and was clearly separated from the supernatant fluid in 15 - 20 minutes. With greater dilutions (1:6 and 1:7), a definite precipitate was formed after 20 minutes but its separation from the supernatant was not clear-cut in comparison with 1:4 and 1:5 dilutions. With a 1:8 dilution cloudiness occurred but no settling took place.

The variation in the character of the above precipitates indicated a difference in the insolubility of globulins at different low concentrations of NaCl solution. A 0.4M NaCl solution when diluted with four volumes of distilled water (1:4) has approximately a concentration of 0.08M and the 1:5 ratio lowers the concentration to 0.06M.

The molar concentration of NaCl at which G_4 precipitated was determined to be between 0.08 and 0.06M when the direct dilution procedure was used and 0.07M when the indirect, slow, step-wise dilution method was employed.

The supernatant from which G_4 had been removed was studied still further. Further reduction of the salt concentration of the supernatant was carried out with the indirect, slow, step-wise dilution method. The dialysate appeared cloudy but no precipitation occurred. The dialysis was further carried to the point where there were practically no chloride ions but no precipitation occurred. The dialysate was transferred to a container and stored at 6°C. There was an increase in cloudiness but no precipitation.

A QUANTITATIVE APPLICATION OF THE PROCEDURE FOR FRACTIONATION OF MUNG BEAN GLOBULINS

The procedure previously described for the separation of Mung bean globulins, G_2, G_3, G_4 was subjected to a quantitative study. Mung bean meal extracted with 0.4M NaCl solution and freed of globulin-bound substance was used for this quantitative work. A portion of this solution was analyzed for total nitrogen and found to contain 0.004671 grams of nitrogen per ml.

Separation of G_2. The 300 ml. of 0.4M NaCl solution containing total globulins and a total of 1.4014 grams of nitrogen were placed in a clean cellophane membrane and 2700 ml. of 0.4M NaCl solution were introduced into the dialyzing tank. Total volume of the solutions in the and tank were 3000 ml. of 0.4M NaCl. The indirect, slow, step-wise dilution procedure was employed for the fractionation of the globulin G_2 from the total globulin mixture. G_2 was previously determined to precipitate at 0.22M NaCl, therefore 2454 ml. of distilled water were added to the tank in order to bring the total volume to 5454 ml. and to have a final concentration of 0.22M NaCl. Distilled water was added at a slow rate, two drops per second, from a separatory funnel extended with glass tubing so that the lower end was immersed in the dialyzing water in the tank. Twelve hours were allowed for completion of precipitation and sedimentation. The G_2 fraction collected in the bottom of the bottle which was attached to the end of the cellophane membrane. The fraction, G_2, formed a semi-solid paste and was easy to separate from the supernatant by decantation of the latter. The supernatant was centrifuged and

the entire G_2 fraction was collected and dissolved in 250 ml. of 0.4M NaCl. Two 10 ml. aliquots were taken for Kjeldahl nitrogen determination. Total nitrogen content of the G_2 fraction was 0.572 grams or 40.8 percent of the total globulin of Mung bean.

Fractionation of G_3. The supernatant from which G_2 was removed was made to 300 ml. volume with 0.22M NaCl solution and placed in a clean cellophane membrane. A volume of 2,700 ml. of 0.22M NaCl dialyzing water was introduced into the tank. The volume of solutions in tube and tank was 3,000 ml. of 0.22M NaCl. A volume of 3,000 ml. of distilled water was added to the tank to bring the total volume to 6000 ml. and to a final concentration of 0.11M NaCl. Twelve hours were allowed for completion of the precipitation. The fraction, G_3, was paste-like in form and was separated by decantation. The supernatant was centrifuged. The precipitate collected was combined with the G_3 and was dissolved in 250 ml. of 0.4M NaCl solution. Two 10 ml. aliquots were taken for Kjeldahl nitrogen determination. Nitrogen content of the G_3 fraction was 0.68668 gm. or 49 percent of the total globulin of Mung bean.

Fractionation of G_4. The supernatant from which G_3 had been removed was made to 300 ml. volume with 0.11M NaCl solution and placed in a clean cellophane membrane. A volume of 2700 ml. of 0.11M NaCl solution was placed in the tank. The indirect, slow, step-wise dilution was carried out as before. A volume of 1,714 ml. of distilled water was added to the tank to bring the total volume to 4714 ml. and the final concentration to 0.07M NaCl. Twelve hours were allowed for the completion of precipitation of G_4 which was separated from the supernatant by centrifugation. The amount of G_4 obtained was small and was dissolved in 100 ml.

of 0.4M NaCl. Two 10 ml. aliquots were used for Kjeldahl nitrogen determination and the nitrogen content of the G_4 fraction was 54.65 mgm. or 3.9 percent of the total globulin of Mung bean.

The summation of the quantitative fractionation is shown in Table XXXX and the Flow Sheet in Figure 14.

TABLE XXXX

NITROGEN DISTRIBUTION IN VARIOUS FRACTIONS OF MUNG BEAN GLOBULINS

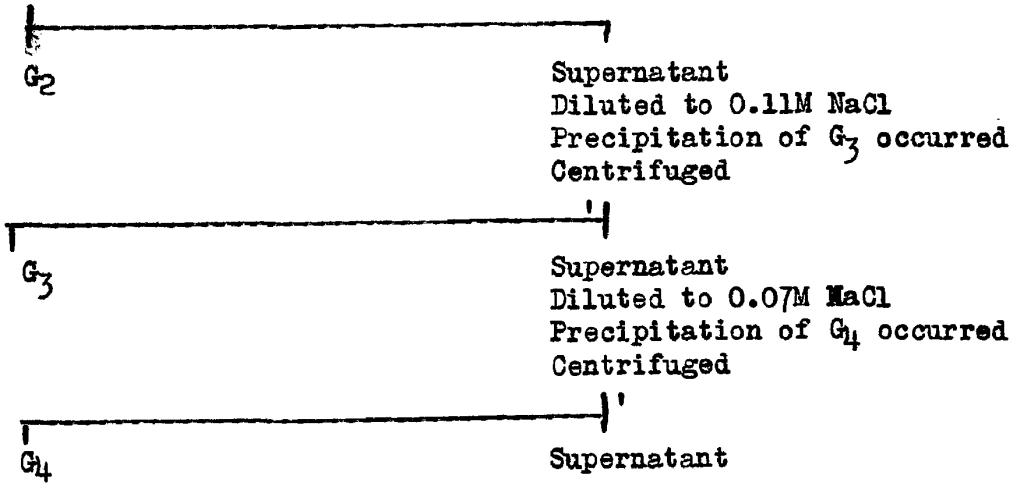
Fraction	Mgm. N	Percentage of Total
$G_2(22)$	573.39	40.95
$G_3(11)$	687.37	49.09
$G_4(07)$	54.70	3.90
Undetermined	85.94	6.06

Figure 14

F L O W S H E E T

The fractionation of Mung bean globulin was accomplished by the indirect, slow, step-wise dilution method and the use of the rotating outside liquid dialyzer.

300 ml. of total globulin in 0.4M NaCl
Diluted to 0.22M NaCl
Precipitation of G_2 occurred
Centrifuged



PURIFICATION OF FRACTIONATED MUNG BEAN GLOBULINS

There is no sharp dividing line between this procedure and that of fractionation. Since the purpose of this work was the preparation of single globulin components, it was necessary to remove small quantities of other fractions and substances which were associated with or adsorbed by these fractionated globulins.

PURIFICATION OF THE PRECIPITATE G_2. For purification, the G_2 fraction was dispersed in 0.4M NaCl. After the precipitate was completely dissolved, the solution was centrifuged to remove traces of insoluble material. For re-precipitation of the G_2 fraction, the solution was introduced into cellophane tubing and the concentration was lowered to 0.22M NaCl by membrane equilibration dilution. The precipitate formed in the tubing and was separated from the supernatant by centrifugation. The supernatant was discarded.

On repeating the purification process it was observed that there was a reduction in the quantity of the G_2 fraction. Further purification produced constantly diminishing quantity of G_2. By lowering the maximum precipitation concentration of G_2 to 0.20M and later to 0.18M NaCl, approximately the original amount of precipitate was obtained.

This change in precipitating concentration led to further investigation. A G_2 fraction was subjected to electrophoretic analysis and found to be composed of two distinct components which co-precipitated together at 0.22M NaCl, see Figure 15. Through repeated dissolution and precipitation, with removal of traces of insoluble dark material

from the mixture by centrifugation, the maximum precipitating concentration of G_2 was lowered to 0.18M NaCl. It was evident that the G_2 fraction contained certain substances other than the two globulin components and all were co-precipitated together at 0.22M NaCl. After repeating the dispersion-precipitation of G_2 and removing a small quantity of the dark substance, the maximum precipitating concentration was lowered to 0.18M NaCl. Such a complex formation may be expected to affect the solubility of the individual components. This suggested to the author that a more effective method should be sought for the removal of the dark colored substance.

RESOLUTION OF THE G_2 FRACTION. The G_2 fraction was dispersed in 0.4M NaCl and placed in cellophane tubing which was suspended in a rotating outside liquid dialyzer containing distilled water. The volume of distilled water was ten times greater than that of the protein solution in the cellophane tubing. In comparison with the previously described indirect, slow, step-wise membrane-equilibration dilution procedure, this procedure may be designated as direct, rapid membrane-equilibration. Precipitation occurred in the cellophane tubing within 15 minutes but the rotation of the tank was continued for two hours. In two hours of dialyzing equilibrium was not reached but this was to permit the sedimentation of a dark, dense precipitate while a white fluffy precipitate was separated from the dark material by decantation. The dark precipitate was subjected to dissolution for a second time and a similar phenomenon occurred. As more white fluffy material went into suspension, less of the dark material was left in the tube. The process of resolution

was repeated until little or none of the white material formed. The resolution process was then considered complete. The final residue of the G_2 fraction was dissolved in 0.4M NaCl solution and became very dark, bluish-purple in color but was not studied further.

FRACTIONATION OF THE RESOLVED PRODUCTS OF G_2. When two volumes of distilled water were added to the total collection of decantations of the resolved product of the G_2 fraction, the protein precipitated immediately. This was allowed to stand overnight at $4^{\circ}C$. The protein was separated from the water by centrifugation and the precipitate was redissolved in 0.4M NaCl. After the precipitate was completely dispersed, it was centrifuged to remove insoluble particles. The solution was ready for fractionation. The clarified protein solution was placed in a cellophane membrane which was immersed in NaCl solution of the same concentration (0.4M) in the dialyzing tank. The indirect, slow, step-wise dilution procedure was used with rotating outside liquid dialysis. The measured volume of distilled water was added to the 0.4M NaCl solution in the dialyzing tank at two drops per second. It was noted that incipient precipitation occurred at 0.18M NaCl and then the maximum precipitation occurred at 0.17M. The concentration of the solution in the tank was not diluted further. The precipitate was separated from the supernatant by centrifugation. This fraction was designated as $G_{2(17)}$.

Since the supernatant from which $G_{2(17)}$ was removed still contained an appreciable quantity of protein (trichloroacetic acid test), further fractionation for the residual protein was continued. The clear supernatant from which $G_{2(17)}$ was removed was placed in cellophane tubing.

A measured volume of distilled water was added in order to bring the concentration of the dialyser to that of 0.16M NaCl. Precipitation did not occur. The concentration of the solution was further lowered to 0.15M and later to 0.14M. The maximum precipitation occurred at 0.14M NaCl. This precipitate was recovered by centrifugation and designated as $G_{2(14)}$. The supernatant from which the $G_{2(14)}$ was removed was a clear solution and was tested with trichloroacetic acid. It contained a small amount of protein but was not studied further.

USING SPECIFIC MOLAR CONCENTRATIONS OF SODIUM CHLORIDE FOR PURIFICATION. The fractionation and purification procedure previously employed for the isolation of globulin fractions were accomplished by adjusting them to their maximum precipitating concentrations "m.p.c.". The resulting products so far as the homogenity of the protein was concerned were freed of gross contamination only. It was necessary to use both the maximum precipitating concentration "m.p.c." and the incipient precipitating concentration "i.p.c." to control the precipitation of each fraction in order to eliminate trace contamination or to secure the desired state of purity.

The purification of the $G_{2(17)}$ fraction was accomplished by subjecting it to the procedures involving definite molar concentrations of sodium chloride. The detailed account is described below.

1. The $G_{2(17)}$ fraction was dispersed in 0.4M NaCl. After complete dispersion, the solution was centrifuged and the volume measured.

2. After the cellophane tubing was rinsed with 0.4M NaCl solution, the protein solution was placed therein. The protein solution was covered

with toluene. The cellophane tubing was immersed in the dialyzing tank containing 0.4M NaCl solution. The total volume of 0.4M NaCl solution in the tubing and tank measured exactly 1000 ml.

3. The indirect, slow, step-wise membrane-equilibration dilution procedure was carried out. Following the calculations of Table XXXIII, distilled water was added to the dialyzing tank at a rate of two drops per second from the reservior to the bottom of the dialyzing tank through glass tubing. The tank was rotated throughout the dilution process.

4. After the calculated volume of water (1222 ml.) was added, the total volume was 2222 ml. and the final salt concentration was 0.18M. The incipient precipitation of fraction $G_{2(17)}$ occurred at this concentration. Rotation of the dialyzer continued for eight hours until equilibrium was reached.

5. The cloudy formation of precipitate which appeared at the incipient precipitation concentration was removed by centrifugation. The clarified centrifugate was returned to the original cellophane tube and the dilution procedure continued.

6. The calculated volume of water (130 ml.) was added slowly as before until the volume rose to 2352 ml. and the final concentration was lowered to 0.17M. Rotation of the dialyzer continued for eight hours to establish equilibrium. The precipitate which formed in the tubing was transferred to a 250 ml. centrifuge tube. After centrifuging for 15 minutes, the supernatant was decanted. This purified $G_{2(17)}$ was stored in a deep-freeze unit.

The above procedure was followed for the purification of the other

fraction, $G_{2(14)}$, $G_{3(11)}$ and $G_{4(07)}$. The incipient precipitating concentrations of the $G_{2(14)}$, $G_{3(11)}$ and $G_{4(07)}$ fractions were 0.15, 0.12 and 0.08M respectively and the maximum precipitating concentrations were 0.14, 0.11 and 0.07M. The calculated volumes of water for dilution of each salt concentration are given in Table XXXIII.

THE ELECTROPHORETIC ANALYSIS OF MUNG BEAN GLOBULINS

The knowledge that charged particles in solution migrate in an electric field has led to the development of one of the most powerful tools for characterizing proteins and numerous carbohydrates. Electrophoretic analysis has provided one of the few physico-chemical criteria of protein homogeneity. It is essential that one be familiar with the principles of the moving boundary method as used in the Tiselius electrophoresis apparatus (62,63,64 and 65) to be able to evaluate critically the data which appear in the literature.

If a protein solution buffered at any given pH is placed in a U-cell and pure buffer solution is carefully layered over it, the protein will migrate into the buffer toward one of the electrodes when direct current is passed through the solution. With a single protein, all the molecules will move at the same rate so that sharp boundaries will be maintained between the protein and the buffer. With a solution containing n-protein components, migrating at different speeds, n-boundaries will be formed soon after the current has been started. In the ascending limb of the U-cell the fastest moving protein will form a boundary against the buffer, the next fastest migrating protein will form one against the fastest protein, etc. Movement of the protein molecules may be followed by observing the boundaries.

If the protein in solution is on the alkaline side of its isoelectric point, and hence is negative in charge, the migration will be toward

the anode (+) terminal. If the protein is on the acid side of its isoelectric point, the opposite is true. If the protein is at its isoelectric point, no migration will take place.

At any specific pH, temperature and salt concentration, the distance (d) moved by a given protein boundary per unit of time (t) will depend upon the potential gradient (F). For a given potential gradient, the rate of migration d/t will be characteristic for each individual protein. The potential gradient may be calculated by the expression below:

$$F = \frac{i}{ak}$$

where F = the potential gradient, i = current (amp.), a = the cross sectional area of the U-cell and k = the conductivity of the buffer or protein solution.

The speed of migration, or electrophoretic mobility (u), may be defined as the distance moved in centimeters per second under a potential gradient of 1 volt/cm. at a certain pH, in a definite buffer of a defined

$$u\ (\text{cm/volt-sec}) = \frac{d}{tF} = \frac{dak}{it}\ \left(\frac{\text{cm}}{\text{sec/volt/cm}}\right)$$

ionic strength.

For the determinations of electrophoretic mobility, it is necessary to measure accurately 1) the distance moved by the protein boundary, 2) the time in seconds, 3) the current passing through (ampere), 4) the conductivity of the solution ($k = k_c/r$), and 5) the cross sectional area of the cell in cm^2.

APPARATUS

The electrophoretic analysis of Mung bean globulin was conducted in this laboratory with the new compact Tiselius electrophoresis apparatus*. This apparatus, based on Longsworth's scanning modification of the Toepler schlieren method, has been described by Moore and White (66). This apparatus has the valuable features of the Tiselius method yet avoids its disadvantages of large size and difficulty of operation. An electrophoresis cell of the type developed by Tiselius is used to contain the sample and in which the boundaries are formed. It has a capacity for two ml. of solution, the optic channels have dimensions of 2 mm. in width, 15 mm. along the optic path and 50 mm. in height. This cell has excellent dimensions for electrophoretic analysis because it is so narrow that a relatively large amount of heat may be generated in it by the passage of current without causing convection, permitting higher field strength and more rapid analysis.

The actual measurement of the protein distribution can best be made by Longsworth's modification of the Toepler schlieren method. The cell and its contents are illuminated with parallel light and the deviations of the rays caused by the refractive index gradients are observed. In this scanning method the cell is photographed on a moving photographic plate. Both plate and knife edges are driven simultaneously by a small motor that moves the plate at right angles to the motion of the knife edges and of the light beam, giving pictures similar to those in Figure 15.

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* Manufactured by Perkin Elmer.

BUFFER EFFECTS

Since the charge and the magnitude of charge of a protein molecule depends upon the surrounding reaction (67), it is necessary that electrophoresis experiments be carried out in suitable buffers in order to obtain comparable results. The buffer chosen as a solvent should have a high buffer capacity itself so that the protein buffer capacity is relatively reduced. This will result in fewer boundary anomalaties (68). A buffer with a low specific conductance is desirable in order to reduce the disturbances due to the heating effect of the current. The generation of heat results from friction of the ions passing through the solution and is related to the speed of migration of the ions (69). Both buffer capacity and conductance increase with the concentration of buffer salts, and it has been pointed out that because of this incompatibility a compromise must be made (67). Since buffer capacity does not depend upon ionic mobilities, buffer salts the ions of which have low mobilities should be selected.

Buffer solvents for use in the electrophoretic analysis of human plasma and serum have been studied at length by Longsworth (64). The results of these experiments show that in resolving power none of the buffers are superior to the diethylbarbiturate (veronal) solution at pH 8.6. The differences in resolving power of three different buffers at the same ionic strength and potential gradient on normal human plasma were studied by Longsworth. The patterns obtained in the barbital buffer of pH 8.6 with ionic strength of 0.1 showed the components well

separated from each other and the peaks sharp and well defined. Whereas, the same sample separated in a carbonate buffer of pH 9.9 μ 0.1, and a phosphate buffer of pH 7.7 and μ 0.1 showed that the resolving power of these two buffers was less satisfactory with human plasma. Horse plasma was also examined in the same buffers as those used in the above work. In contrast with human plasma, this material gave a more satisfactory pattern in the phosphate than in the diethyl-barbiturate buffer.

Karon (70) reported on borate and glycine buffer that were prepared according to Clark; veronal buffer according to Longsworth, and an ammonia buffer prepared by adding 0.2 molar ammonia to 0.1 molar hydrochloric acid solution. Whereas these buffers were satisfactory solvents for peanut protein, only the glycine buffer was satisfactory as a solvent for cotton seed protein. A buffer composed of 0.2 mole of ethylamine and 0.1 mole of veronal in a liter of solution having a pH of 10.7 had the most desirable characteristics for cottonseed protein. It was thereby suggested (64) that the proper solvent for the analysis of the protein of a given type varies with the species and should be determined experimentally.

THE PREPARATION OF BUFFERS OF DESIRED pH AND IONIC STRENGTH

Tables have been prepared by Cohn (71) and by Green (72) from which the molecular ratios of salt to acid can be obtained in preparing phosphate and acetate buffers of constant ionic strength and varying pH or constant pH and varying ionic strength. For all the buffer mixtures in electrophoretic work in this study, the Henderson-Hasselbach equation was used for calculation of the pK values. A list of pK values of the more common acids used for buffer mixtures is as follows:

Acid	pK value
Acetic	4.73
Barbituric	7.90
Cacodylic	6.20
Glycine	2.35, 9.77
Phosphoric	6.77 (second dissociation)

The following are examples of several types of buffer problems that arise in practice.

(A) The preparation of a sodium acetate buffer of pH 4.7 and ionic strength of 0.1. In order to secure this buffer value one must 1). calculate the pH value of acetic acid, 2). calculate the ratio of salt/acid, 3). calculate molarity of sodium acetate and molarity of acetic acid.

1). Since the ionization constant K of acetic acid equals 1.86×10^{-5}

10^{-5} . . . . . -5.0000
log of 1.86 0.2695
-4.7305 the log of ionization K

pK = the negative log of the ionization K or -(-4.73) or 4.73

2). To calculate the ratio of salt/acid, Henderson-Hasselbach equation is used.

pH = pK + log salt/acid or
4.70 = 4.73 + log salt/acid or
-0.03 = log salt/acid

positive value of -0.03 is 9.97 - 10
antilog of 9.97 is 0.933
0.933 is the ratio of salt/acid

3). To calculate M of sodium acetate and M of acetic acid. The ionic strength of univalent compounds is equal to the molar concentration. Since weak acid does not contribute appreciably to the ionic strength, the salt concentration had to be 0.1M in order to give an ionic strength of 0.1.

$$\text{ratio } 0.933 = \frac{0.933 \text{ salt}}{1.000 \text{ acid}}$$

ratio x acid = salt
0.933 x acid = salt
salt + acid = 0.1M
(0.933 acid) + acid = 0.1M
1.933 acid = 0.1M

$\frac{0.100}{1.933} = 0.0517$M acid
0.0483M salt

(B) The preparation of a sodium phosphate buffer of pH 7.0 and ionic strength of 0.1.
The pK_2 of phosphoric acid = 6.77

1). To calculate the ratio of salt/acid.

$$pH = pK + \log \text{salt/acid}$$
$$7.0 = 6.77 + \log \text{salt/acid}$$
$$0.23 = \log \text{salt/acid}$$

antilog of 0.23 is 1.7
1.7 is the ratio of salt/acid

2). To find M concentration of salt and of acid.
The sodium phosphate buffer at pH 7.0 is dissociated as follows:

$$Na_2HPO_4 \longrightarrow Na^+ + NaHPO_4^- \rightleftharpoons Na^+ + HPO_4^{--}$$

$$NaH_2PO_4 \longrightarrow Na^+ + H_2PO_4^-$$

$$\mu = 0.5 \{ CV^2$$

substitute x for acid concentration, 1.7x for salt concentration

$$0.1 = 0.5 \quad (1.7x)\ (1^2) + (1.7x)\ (1^2) + (1.7x)\ (2^2)$$
$$(x)\ (1^2) + (x)\ (1^2)$$

$$0.2 = 1.7x + 1.7x + 6.8x + x + x$$

$$0.2 = 12.2x$$

$$x = \frac{0.2}{12.2x} = 0.0164 \text{ M of } NaH_2PO_4 \text{ and}$$

1.7 x 0.0164 = 0.0278 M of Na_2HPO_4 give ionic strength of 0.1 M at pH 7.0

A list of common buffers of 0.1 ionic strength (Hardt)

pH	composition
1.78	0.02N HCl + 0.08N NaCl
3.05	0.1 N HCl + 0.5N glycine
3.62	0.2 N HAc + 0.02N NaAc + 0.08N NaCl
3.91	0.1 N HAc + 0.02N NaAc + 0.08N NaCl
4.34	0.2 N HAc + 0.1 N NaAc
4.47	0.15N HAc + 0.1 N NaAc
4.64	0.1 N HAc + 0.1 N NaAc
5.33	0.02N HAc + 0.1 N NaAc
5.42	0.1 N HCac + 0.02N NaCac + 0.08N NaCl
5.65	0.01N HAc + 0.1 N NaAc
6.12	0.02N HCac + 0.02N NaCac + 0.08N NaCl
6.79	0.004N HCac + 0.02N NaCac + 0.08 N NaCl
7.83	0.02N HV + 0.02N NaV + 0.08N NaCl
8.60	0.02N HV + 0.1 N NaV
10.28	0.02N glycine + 0.1 N NaOH
10.88	0.125N glycine + 0.1 N NaOH
11.81	0.1 N glycine + 0.1 N NaOH

Ac = acetate

Cac = cacodylate

V = diethyl-barbiturate

OPERATIONAL PROCEDURE IN A COMPLETE ELECTROPHORETIC ANALYSIS

PREPARATORY

1. Electrophoretic cells should be cleaned in Dreft and must be dry before using.
2. Preparation of sample for electrophoretic analysis: The protein is dispersed in 10 ml. of the buffer in a 50 ml. beaker. The concentration of protein should be 0.5 to 1 percent. Transfer the protein to cellophane tubing and dialyze in 1000 ml. of the same buffer for two hours with rotating outside liquid dialysis.
3. Turn on the current of power supply for electrophoresis apparatus two hours before it is to be used.

ASSEMBLY OF CELL

4. Grease the contacting cell plates with special grease. Do not grease too close to the channel. Leave a bare rectangular area extending 3 mm. around each channel. It is necessary to grease only one of the two contacting surfaces.
5. Assemble bottom and center section together with rotary motion to obtain a leak-proof seal. Assemble the top section in the same manner.

MEASURING THE RESISTANCE OF BUFFER AND PROTEIN SOLUTION

6. The conductivity cell is suspended in an ice bath in a 1000 ml. beaker. Transfer 2 ml. solution (buffer first and then protein) to conductivity cell with a hypodermic syringe. In order to prevent the formation of air bubles in the cell, the needle of the syringe must touch the bottom of the cell. Allow only enough solution to fill the cell. A constant reading may be obtained after standing for a period of time.

FILLING CELL

7. Transfer 10 ml. of dialyzed protein into a small tube and mark "Protein" and stopper.
8. Overfill bottom section of cell with protein solution.
   a. To do this use a long needle syringe.
   b. Syringe is inserted through lefthand channel.
   c. To prevent the trapping of air, tip the whole assembly to the left while letting solution flow gently into the bottom section.
9. Fill left center of cell with protein and right center with buffer.
   a. To do this the center and top sections must shift to the right to segregate channels from bottom sections.

b. Over fill the left center cell with protein solution.
c. Remove excess protein solution in the right channel by a long needle syringe and rinse 3 times with buffer.
d. Overfill the right center cell with buffer.
e. Now, keep the center section in segregated position and push top section to the left. Clamp firmly with spring clamp.
f. Remove excess protein solution from top left cell and rinse 3 times with buffer.
g. Fill both sides of top section with buffer to the level of the buffer tube arms.

10. Connect buffer tubes.
11. Insert electrodes.
12. Fill buffer tubes with buffer solution to 9/10 full leaving 1/10 space for KCl solution.
13. Inject with a syringe 10 ml. of 1/3 saturated KCl solution into each electrode capillary while the connecting gate of the top section is open to allow buffer solution to flow through freely.

PLACING THE CELL IN THE ELECTROPHORETIC APPARATUS

14. Place the entire unit of cell in the bath chamber of the apparatus and clamp it to the bottom.
15. Connect the leads to electrodes.
16. Fill bath with ice. Pour cold water over ice and fill the bath to within an inch of overflow pipe.
17. Close the bath with plate.
18. Turn on stirrer switch.
19. Twenty minutes are required for reaching uniform temperature of 0°C. bath.

ASSEMBLY OF THE COMPENSATOR

20. Fill the syringe with 7 ml. of buffer, eliminate air bubbles and mount on the compensator and turn on the motor. Make sure that the small droplets of buffer are formed from the needle point. The needle point is immersed in a beaker containing buffer.
21. Insert the needle through a hole to the lefthand buffer bottle. Do not allow needle to touch the electrode.
22. Turn on the compensator for 15 seconds and turn off.

STARTING BOUNDARY

23. Push down the center gate for the cell to segregate the channels.
24. Shift the center section of cell into aligned position by turning the right hand shifting rod.
25. Turn on compensator, "in" position, to allow a gentle flow of buffer into the lefthand buffer tube until boundaries are brought into view.
26. Photograph the starting points, if desired.

27. Turn on current, "normal", and record the time of starting..
28. Adjust the current to approximately 2 watts. (Volts E times milliamps I = watts.)
29. Time required for the development of full migration of charged particles varies from 1 - 3 hours and depends upon the different materials.

TO MAKE EXPOSURE

30. Turn off current and record time.
31. Focus the cell on screen intensely by adjusting the right hand knob.
32. Mask the image with metal plate and align the slit with the ascending cell (to the right.)
33. Turn off light.
34. Take the picture of ascending line.
    a. Expose the pattern by scan from 4 to 8.5 mark.
35. Again, focus the cell on screen intensely by adjusting the right hand knob.
36. Mask the image with metal plate and align the slit with the descending line (to the left.)
37. Turn off light.
38. Take the picture of descending line.
    a. Expose the pattern by scan from 8.5 - 14.
39. Develop the negative.
    a. Develop 4 minutes in D19 in total dark.
    b. Hypo for 10 to 15 minutes.
    c. Wash 1 hour.

ELECTROPHORETIC ANALYSIS OF MUNG BEAN GLOBULINS

TOTAL GLOBULINS. As a starting point, it was considered advisable to use the buffers that Danielson (73) used in his electrophoretic studies of pea proteins. However the acetate buffer of pH 3.72 with 0.2M NaCl would not dissolve the total globulins at 0°C. When the acetate buffer was made to pH 3.19 and 0.2M NaCl was added, the protein was soluble at 0°C. and the electrophoresis analysis was conducted. Buffers other than acetate, the phosphate buffer of pH 7.5 and the borate buffer of pH 8.30 (as prepared by Danielson), were also employed for dispersingthe total globulin. None of these buffers was a satisfactory solvent for the separation of total Mung bean globulin in electrophoresis analysis. An NH_3-HCl buffer, however, of pH 9.26, as prepared by Irving (74), was superior in resolving power in comparison with those mentioned above. The various components were well separated from each other and the peaks were sharp and well defined. The electrophoretic patterns of total Mung bean proteins with various buffers are shown in Figure 15 and 16.

A later experiment showed that veronal-citrate buffer, prepared according to Stanley (75), was not satisfactory for the Mung bean globulins even though it had proved to be a superior buffer for whey proteins.

A comparison of two types of photographic film was made. The contrast process panchromatic film which was recommended for electrophoretic recording, was compared with contrast process ortho film. It was found that the later was the more desirable film for this work since it produced much sharper and smoother lines.

THE PRECIPITATE $G_{2(22)}$. The precipitate $G_{2(22)}$ was dispersed in 10 ml. of NH_3-HCl buffer of pH 9.26 and was dialyzed in 1000 ml. of the same buffer for two hours in a rotating, outside liquid dialyzer. After equilibrium was reached, the protein was subjected to electrophoretic analysis. The duration of this experiment was 5185 seconds, at potential gradient of 5.75 volts per cm^{-1}. The photographic record showed two peaks as can be seen in Figure 15.

THE FRACTION $G_{2(17)}$. In the electrophoresis of $G_{2(17)}$ one main peak was obtained as shown in Figures 15 and 17. Seven electrophoretic analyses were conducted, four of which were made with acetate buffers of pH's 3.28, 3.90, 4.36 and 4.61 and three of which were made with phosphate buffers of pH's 7.15, 7.48, and 7.78. The $G_{2(17)}$ was homogeneous by electrophoresis. The results of these measurements are shown in Table XXXXI and Figure 21, from which a determination of the isoelectric point of $G_{2(17)}$ gave the value of pH 5.4.

THE FRACTION $G_{2(14)}$. In seven electrophoretic analyses of this fraction one main peak was obtained. Four acetate buffers of pH 3.27, 3.85, 4.44 and 4.78 and three phosphate buffers of pH 7.27, 7.52 and 7.75 were used. The $G_{2(14)}$ fraction was homogeneous by electrophoresis. The mobility measurements made with these seven pH values indicated that the isoelectric point of this fraction was 5.7. (See Figure 18 and Table XXXXI.)

THE FRACTION $G_{3(11)}$. The electrophoretic mobility determination of this fraction was made with both acetate and phosphate buffers at five pH values: 3.34, 3.80, 4.40, 6.15 and 6.63. All patterns showed one peak, therefore it was a homogeneous fraction. The pH mobility curve of $G_{3(11)}$

was a straight line from which the isoelectric point of pH 5.0 was thus determined. (See Figure 19 and Table XXXXI).

THE FRACTION $G_{4(07)}$. This fraction gave one peak in electrophoretic analyses. Mobility determination of $G_{4(07)}$ was made with acetate and phosphate buffers with pH values: 3.32, 3.90, 4.21, 4.52, 7.30, 7.50 and 7.78. The results of the measurements are shown in Table XXXXI and Figure 20 from which a determination of the isoelectric point of $G_{4(07)}$ gave the value of 5.15.

RECONSTITUTION OF MUNG BEAN GLOBULINS. An electrophoretic study of reconstitution of purified Mung bean globulin fractions was conducted. Approximately 0.4 percent of each of the $G_{2(17)}$, $G_{2(14)}$, $G_{3(11)}$ and $G_{4(07)}$ was used. The concentration of total protein of the mixture was determined to contain 1.70 percent of protein (N x 6.25). A 10 ml. portion of the protein mixture was dialyzed in 2000 ml. of NH_3-HCl buffer of pH 9.26. After two hours dialysis the final pH of the buffer was 9.0. An electrophoretic pattern showed four peaks, two of which were separate single peaks and two were peaks that overlapped as can be seen in Figure 15.

TABLE XXXXI. ELECTROPHORETIC MOBILITY CALCULATIONS

Globulin Fraction	Run No.	pH	d_1 Ascending (cm)	d_2 Descending (cm)	q cross section area of cell (cm)	k_c at 1°C.	R Resistance of protein solution in ohms
$G_2(17)$	114	3.28	2.67	2.40	0.3	.0048937	80
	115	3.90	2.07	1.93	0.3	.0048937	76
	116	4.36	2.16	2.03	0.3	.0048937	68
	120	4.61	1.14	1.09	0.3	.0048937	69
	121	7.15	0.85	0.78	0.3	.0048937	72
	119	7.48	1.65	1.59	0.3	.0048937	72.5
	122	7.78	2.62	2.94	0.3	.0048937	74.5
$G_2(14)$	124	3.27	2.19	2.02	0.3	.0048937	81
	128	3.85	2.21	2.19	0.3	.0048937	72.3
	126	4.44	1.52	1.45	0.3	.0048937	65
	127	4.78	0.92	0.95	0.3	.0048937	69.5
	136	7.27	1.96	1.71	0.3	.0048937	78
	130	7.52	2.27	2.45	0.3	.0048937	77
	129	7.75	1.69	2.14	0.3	.0048937	62.7
$G_3(11)$	97	3.34	2.91	2.60	0.3	.0048937	62.6
	96	3.80	2.80	2.73	0.3	.0048937	70
	99	4.40	1.21	1.07	0.3	.0048937	53.5
	105	6.15	1.87	2.06	0.3	.0048937	74
	104	6.63	2.77	2.85	0.3	.0048937	78
$G_4(07)$	137	3.32	2.64	2.42	0.3	.0048937	76.5
	138	3.90	1.75	1.67	0.3	.0048937	70.3
	139	4.21	1.43	1.41	0.3	.0048937	64
	140	4.52	1.14	1.32	0.3	.0048937	68.5
	152	7.30	1.65	1.67	0.3	.0048937	58.4
	142	7.50	2.27	2.29	0.3	.0048937	74.5
	153	7.78	1.91	1.82	0.3	.0048937	60.5

OF MUNG BEAN GLOBULIN FRACTIONS

$K = \frac{k_c}{R}$ Conductance of protein solution at 1°C.	$d_1 q^k$	i Current as amperes	t Time in seconds	it	$F = \frac{i}{qk}$ potential gradient volt cm^{-1}	$u = \frac{d_1}{tF} \times 10^{-5}$ potential gradient volt cm^{-1}	or $u = \frac{d_1 qk}{it} \times 10^{-5}$
.006117	.004900	.0100	7200	72.00	5.449	6.805	6.805
.006439	.003998	.0100	9000	90.00	5.176	4.443	4.442
.007196	.004663	.0140	10800	151.00	6.485	3.084	3.084
.007092	.002425	.0140	7376	103.26	6.580	2.349	2.349
.006797	.001733	.0140	3657	51.20	6.865	3.385	3.385
.006750	.003341	.0118	7226	85.26	5.827	3.918	3.918
.006568	.005163	.0140	8000	112.00	7.105	4.609	4.609
.006041	.003969	.0100	7200	72.00	5.517	5.513	5.513
.006768	.004487	.0141	7067	99.64	6.944	4.503	4.503
.007528	.003433	.0142	9007	127.90	6.287	2.684	2.684
.007041	.001943	.0158	6000	94.80	7.480	2.050	2.050
.006274	.003689	.0141	7200	101.52	7.491	3.634	3.634
.006355	.004328	.0141	7200	101.52	7.395	4.263	4.263
.007800	.003955	.0141	6000	84.60	6.025	4.674	4.674
.007817	.006824	.0160	9110	145.76	6.822	4.682	4.682
.006991	.005827	.0155	10960	169.88	7.390	3.457	3.457
.009147	.003320	.0168	10114	169.91	6.122	1.954	1.954
.006613	.003710	.0150	7289	109.47	7.560	3.393	3.393
.006274	.005213	.0150	7196	107.94	7.969	4.830	4.830
.006397	.005066	.0102	7200	73.80	5.341	6.865	6.865
.006961	.003654	.0121	7200	87.12	5.794	4.194	4.194
.007646	.003280	.0141	7200	101.52	6.147	3.231	3.231
.007149	.002443	.0152	7200	109.44	7.087	2.232	2.232
.008379	.004148	.0142	7200	101.52	5.649	4.056	4.056
.006568	.004473	.0141	7244	102.14	7.156	4.379	4.379
.008088	.004635	.0142	7200	101.52	5.852	4.533	4.533

Figure 15.

FRACTIONATION SCHEME OF TOTAL GLOBULINS EXTRACTED FROM MUNG BEAN

Purified extract in 0.4 M NaCl

1. Membrane–equilibration diluted to 0.22 M
2. Precipitate separated by centrifugation

Supernatant (0.22 M)

1. Membrane–equilibration diluted to 0.11 M
2. Precipitate separated by centrifugation

Supernatant (0.11 M)

1. Membrane–equilibration diluted to 0.07 M
2. Precipitate separated by centrifugation

Supernatant (0.07 M) (not further studied)

Fraction $G_{4\,(07)}$

Fraction $G_{3\,(11)}$

Precipitate $G_{2\,(22)}$

1. Peptized in 0.4 M NaCl
2. Centrifuged clear
3. Membrane-equilibration diluted to 0.17 M
4. Precipitate separated by centrifugation

Supernatant (0.17 M)

1. Membrane–equilibration diluted to 0.14 M
2. Precipitate separated by centrifugation

Supernatant (0.14 M) (not further studied)

Fraction $G_{2\,(14)}$

Fraction $G_{2\,(17)}$

Fractions $G_{2\,(17)}$ + $G_{2\,(14)}$ + $G_{3\,(11)}$ + $G_{4\,(07)}$ reconstituted in ca equal amounts

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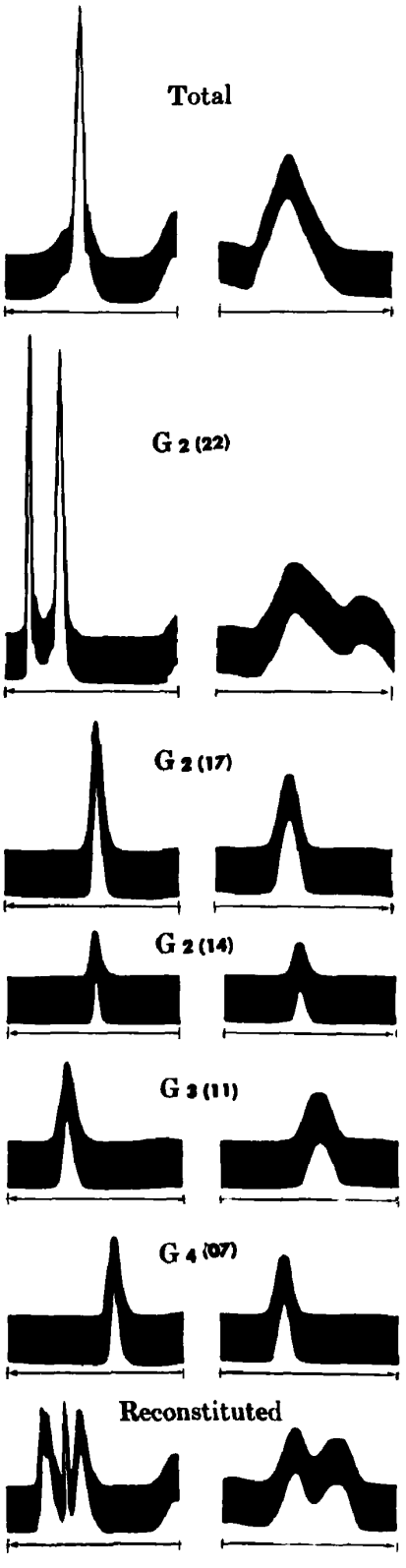


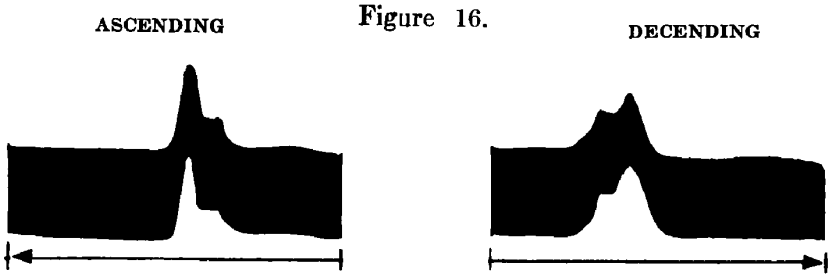
DATA ON ELECTROPHORETIC PATTERNS*

Fraction	pH	Buffer	μ (total)	Time secs.	Potential gradient v cm^{-1}	Concn percent
Total	9.10	ammonia	0.20	6500	4.95	1.55
$G_{2\,(22)}$	9.26	ammonia	0.20	5185	6 75	2.53
$G_{2\,(17)}$	3.90	acetate	0.21	9000	4.66	0.78
$G_{2\,(14)}$	3.27	acetate	0.21	7200	5.51	0.25
$G_{3\,(11)}$	3.34	acetate	0.21	9110	6.82	0.55
$G_{4\,(07)}$	3.90	acetate	0.21	7200	5.20	0.43
Recons.	9.00	ammonia	0.20	3600	7.81	1.70

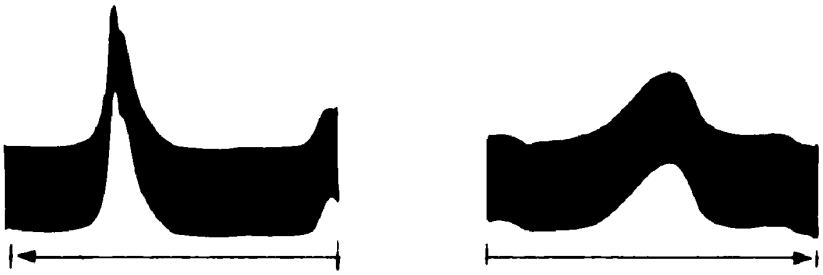
(* 0.5 magnification)

ELECTROPHORETIC PATTERNS OF TOTAL PROTEINS FROM MUNG BEAN IN THE PRECIPITATE OF A 0.4 M NaCl EXTRACT DILUTED 1 : 4

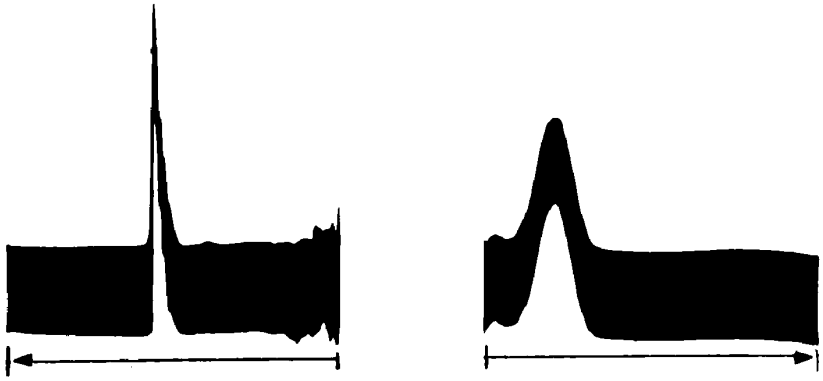
Figure 16.



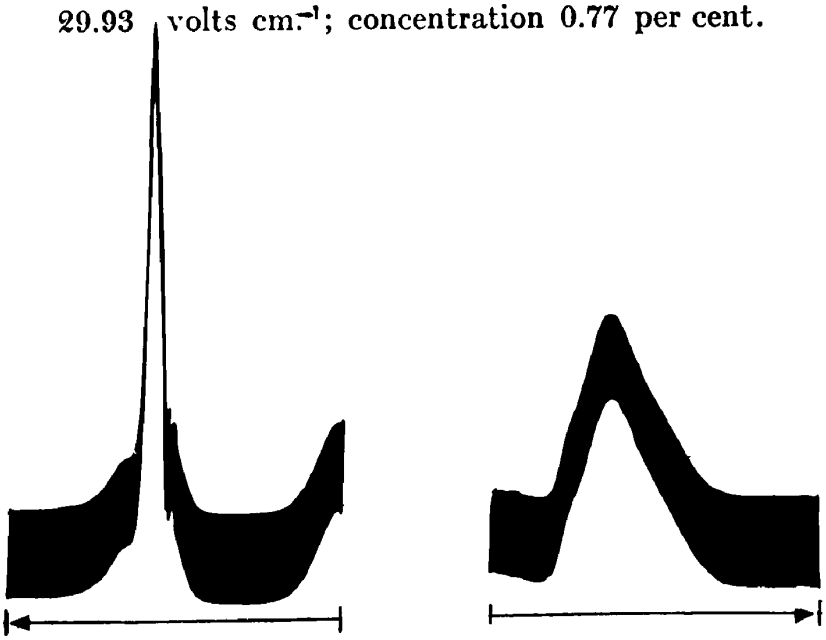
pH 3.19; acetate buffer + 0.2 M NaCl; u = 0.21; 7200 secs; 4.33 volts $cm.^{-1}$; concentration 0.77 per cent.



pH 7.50; phosphate buffer; u = 0.03; 1800 secs; 17.05 volts $cm.^{-1}$; concentration 0.75 per cent.



pH 8.30; borate buffer; u = 0.003; 334 secs; 29.93 volts $cm.^{-1}$; concentration 0.77 per cent.

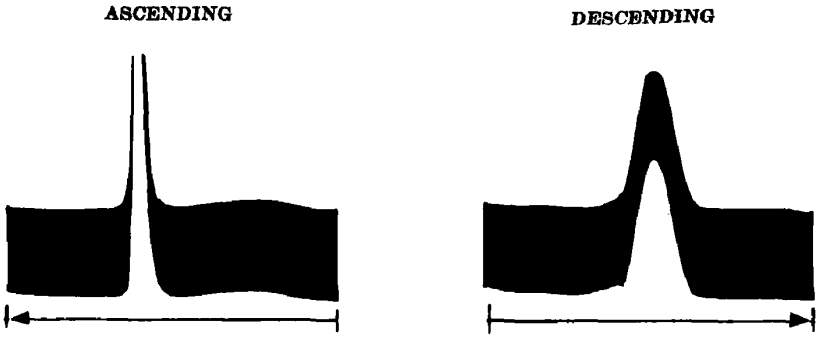


pH 9.10; ammonia buffer; u = 0.2; 6500 secs; 5.47 volts $cm.^{-1}$; concentration 1.55 per cent.

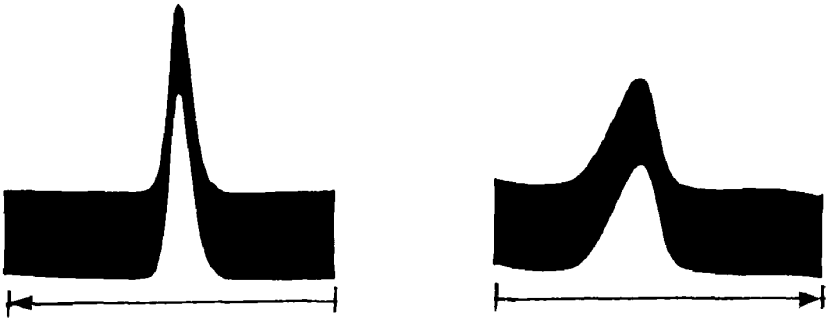
Figure 17.

ELECTROPHORETIC PATTERNS OF THE FRACTION $G_{2(17)}$

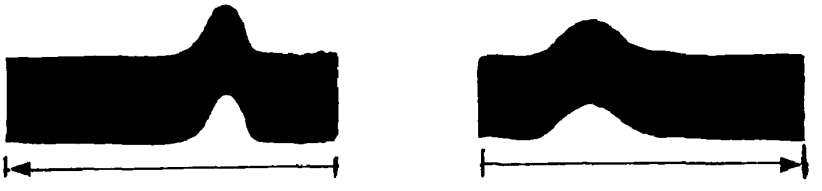
A purified fraction of the total globulins extracted with 0.4 M NaCl from Mung bean meal by: (1) membrane-equilibration dilution of the extract to 0.22 M NaCl thus precipitating a fraction called $G_{2(22)}$, and (2) repeptization of $G_{2(22)}$ in 0.4 M NaCl followed by similar dilution to 0.17 M NaCl. This yielded the precipitate designated as fraction $G_{2(17)}$.



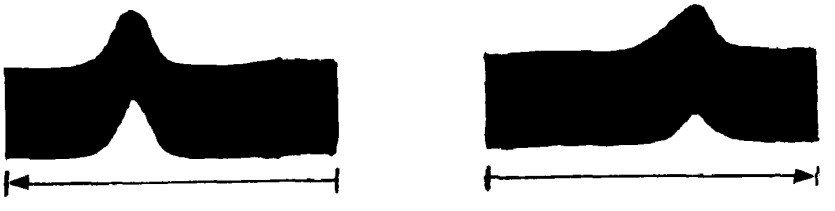
pH 3.28; acetate buffer + 0.2 M NaCl; u = 0.21;
7200 secs; 5.44 volts $cm.^{-1}$; concentration 0.78 per cent.



pH 4.36; acetate buffer + 0.2 M NaCl; u = 0.21;
10800 secs; 6.51 volts $cm.^{-1}$; concentration 0.78 per cent



pH 7.48; phosphate buffer + 0.2 M NaCl; u = 0.23;
7200 secs; 5.82 volts $cm.^{-1}$; concentration 0.68 per cent

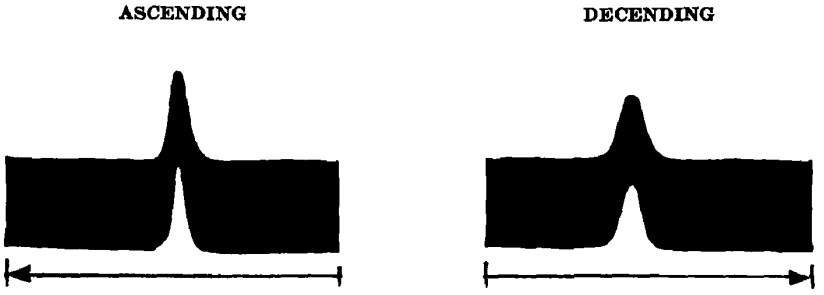


pH 7.78; phosphate buffer + 0.2 M NaCl; u = 0.23;
8000 secs; 7.10 volts $cm.^{-1}$; concentration 0.60 per cent.

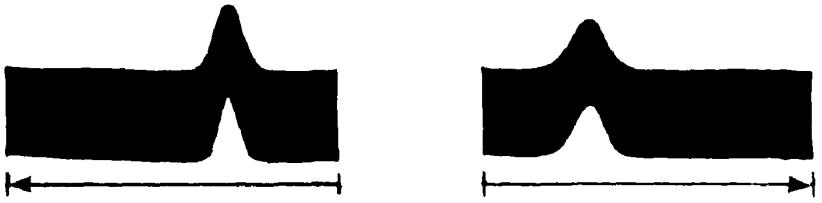
Figure 18.

ELECTROPHORETIC PATTERNS OF THE FRACTION $G_{2(14)}$

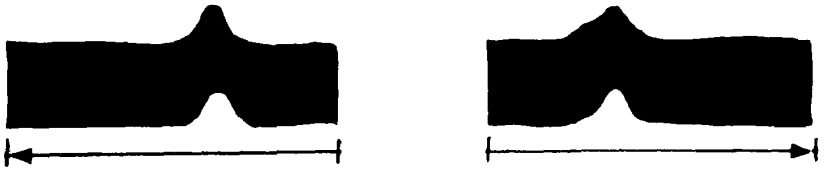
A purified fraction of the total globulins of Mung bean meal obtained by membrane-equilibration dilution of the supernatant from precipitate $G_{2(17)}$ to 0.14 M NaCl. This yielded the precipitate designated as fraction $G_{2(14)}$.



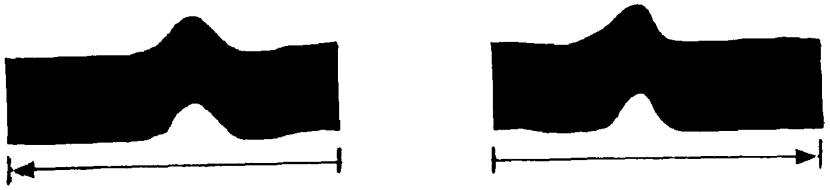
pH 3.27; acetate buffer + 0.2 M NaCl; u = 0.21;
7200 secs; 5.51 volts $cm.^{-1}$; concentration 0.25 per cent.



pH 4.44; acetate buffer + 0.2 M NaCl; u = 0.21;
9000 secs; 6.28 volts $cm.^{-1}$; concentration 0.28 per cent.



pH 7.27; phosphate buffer + 0.2 M NaCl; u = 0.23;
7200 secs; 7.49 volts $cm.^{-1}$; concentration 0.35 per cent.

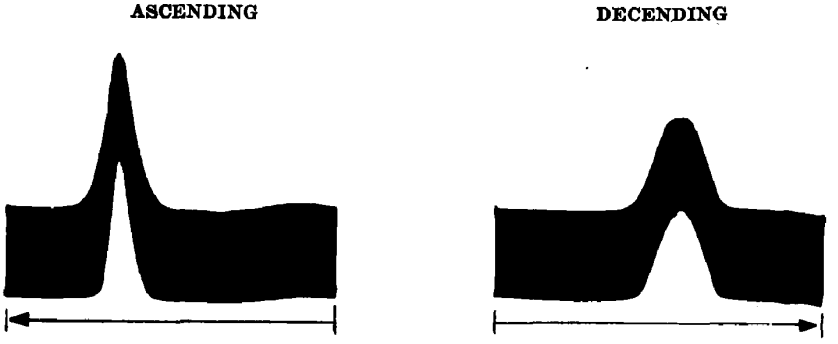


pH 7.75; phosphate buffer + 0.2 M NaCl; u = 0.23;
6000 secs; 6.02 volts $cm.^{-1}$; concentration 0.34 per cent.

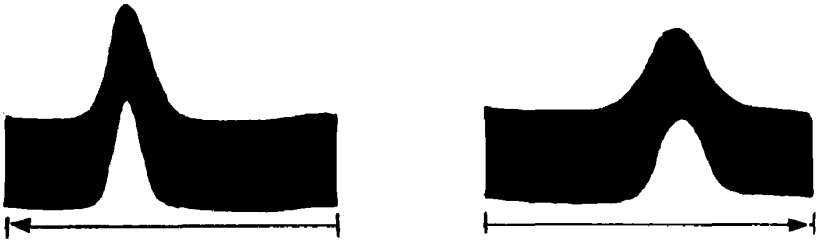
Figure 19.

ELECTROPHORETIC PATTERNS OF THE FRACTION $G_{3(11)}$

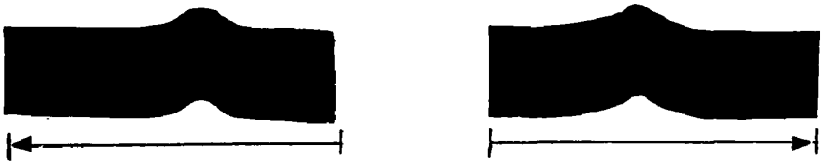
A purified fraction of the total globulins of Mung bean meal obtained by membrane-equilibration dilution of the supernatant from precipitate $G_{2(22)}$ to 0.11 M NaCl. This yielded the precipitate designated as fraction $G_{3(11)}$.



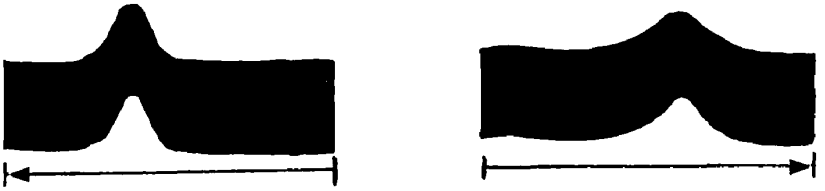
pH 3.34; acetate buffer + 0.2 M NaCl; u = 0.21;
9110 secs; 6.82 volts $cm.^{-1}$; concentration 0.55 per cent.



pH 3.80; acetate buffer + 0.2 M NaCl; u = 0.21;
10960 secs; 7.60 volts $cm.^{-1}$; concentration 0.74 per cent.



pH 6.15; phosphate buffer + 0.2 M NaCl; u = 0.23;
7296 secs; 7.56 volts $cm.^{-1}$; concentration 0.33 per cent.

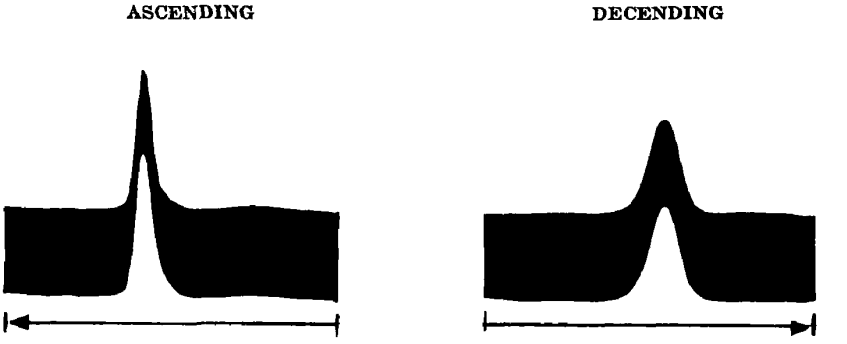


pH 6.63; phosphate buffer + 0.2 M NaCl; u = 0.23;
7196 secs; 7.96 volts $cm.^{-1}$; concentration 0.62 per cent.

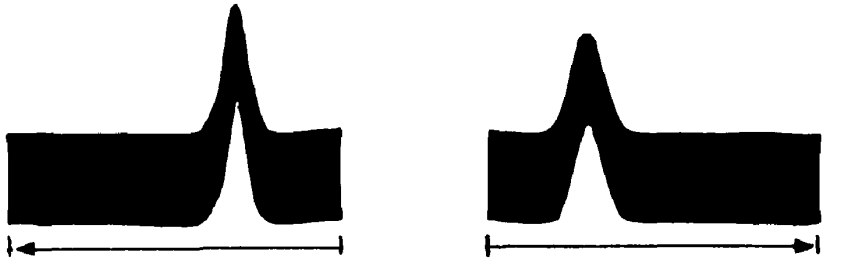
Figure 20.

ELECTROPHORETIC PATTERNS OF THE FRACTION $G_{4(07)}$

A purified fraction of the total globulins of Mung bean meal obtained by membrane-equilibration dilution of the supernatant from precipitate $G_{3(11)}$ to 0.07 M NaCl. This yielded the precipitate designated as fraction $G_{4(07)}$.

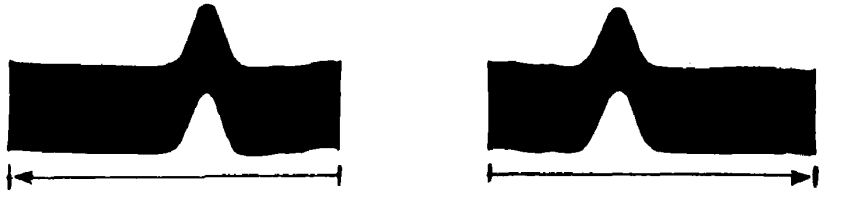


pH 3.32; acetate buffer + 0.2 M NaCl; u = 0.21;
7200 secs; 6.82 volts $cm.^{-1}$; concentration 0.43 per cent.



pH 4.21; acetate buffer + 0.2 M NaCl; u = 0.21;
7200 secs; 6.14 volts $cm.^{-1}$; concentration 0.43 per cent.

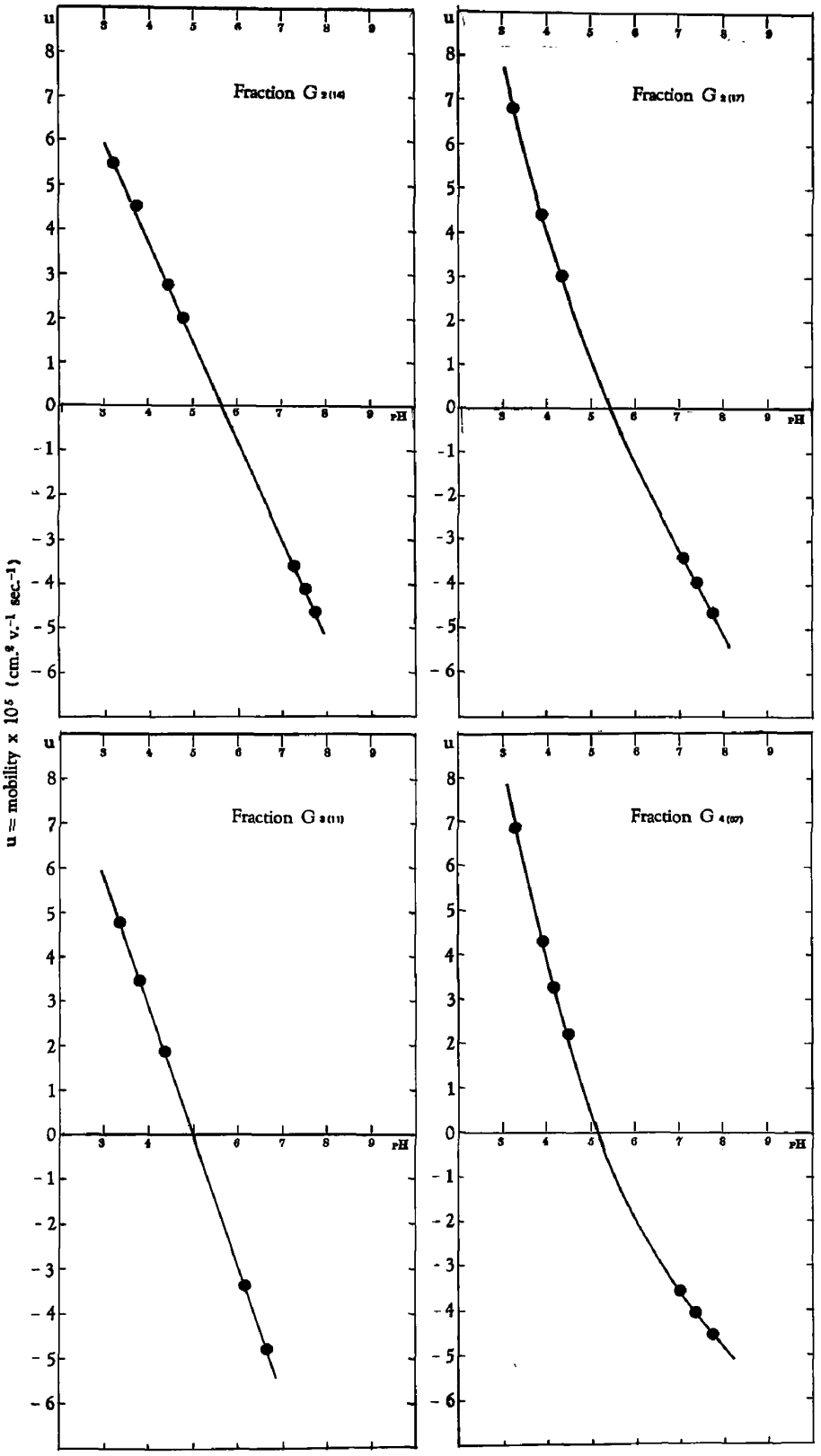
pH 7.05; phosphate buffer + 0.2 M NaCl; u = 0.23;
7200 secs; 7.71 volts $cm.^{-1}$; concentration 0.43 per cent.



pH 7.78; phosphate buffer + 0.2 M NaCl; u = 0.23;
7200 secs; 5.85 volts $cm.^{-1}$; concentration 0.36 per cent.

Figure 21.

pH — MOBILITY CURVES OF THE PURIFIED FRACTIONS OF TOTAL GLOBULINS FROM MUNG BEAN



SUMMARY AND CONCLUSION

The Relationships Between the Solubility of Mung Bean Protein and the Nature and Concentration of Solvents

The results obtained from the study are shown in Tables VI through XIV and Figure 2.

A. The salts of strong acids and bases, such as those of chloride and sulfate, showed nearly the same effectiveness in peptizing approximately equal amounts (72 percent of the total nitrogen on a dry weight basis) from Mung bean meal. The salts of phosphates, carbonate and sulfite also showed parallel effectiveness in peptizing nearly equal amounts, 83 to 85 percent of the total nitrogen from the same samples.

B. The range of ionic strength of the neutral solvents was very narrow. It was noted that the highest peptizing power of NaCl solution was very close to 0.4M. On the other hand the range of ionic strength of solutions of phosphate, carbonate and sulfite was much broader.

C. Since the definition of globulin refers to the solubility in neutral salt solution, it is obvious that the maximum amount of Mung bean globulin peptized by chlorides and sulfates was 72 percent. The question arises as to what kind of protein (beyond the 72 percent) is peptized by those alkaline solvents (phosphate, carbonate and sulfite). Since this study was devoted to globulins, other proteins were not studied in detail. However, these data support the proposition that there is a variation in the nature or kind of protein peptized by these two groups of solvents.

D. It was noted that the sodium chloride solvent yielded a curve with

a steep slope, part of which was nearly vertical. The significance of this type of curve has been pointed out previously in that it not only illustrates the solubility behavior of Mung bean protein in this solvent but it also reveals a scheme by which the protein might be isolated by lowering the concentration of the solute by dilution.

Factors that Influence the Degree of Peptization of Protein

A. Table XXI and Figure 5 demonstrate the effect of time and sample-solvent ratios and show that in all instances an increase in extracting time increased the amount of total nitrogen peptized from the samples. Of the five sample-solvent ratios employed, the one of 5:100 gave the highest value of peptization and that of 15:100 gave the lowest. When the S-S-R was small (5:100), time had less influence upon peptization. On the other hand when the S-S-R was large (15:100), time had more influence upon peptization.

B. Table XXIII and Figure 6 showed that the particle size affected the degree of peptization to a great extent. The 60 mesh size gave about 20 percent greater yield of nitrogen over the 40 mesh size which in turn gave about 20 percent greater yield of nitrogen over the 20 mesh size.

C. Table XXVI and Figure 7 show no appreciable difference between the mechanical and hand stirring. There was a great difference between the first extraction and the second and third extractions. When the S-S-R was 10:100, 60 percent of the nitrogen was peptized by the first extraction, 9 percent by the second and 3 percent by the final extraction with 27 percent of the nitrogen remaining in the residue.

D. The effect of temperature upon the degree of peptization from oil free and non-oil free samples is somewhat complex. From Table XXVIII and Figure 8 it appears that oil free samples generally yielded higher values than the non-oil free samples except that with lower concentrations and higher temperatures reverse results were observed. Using non-oil free samples the temperature of extraction was shown to have little or no influence upon the degree of peptization below 45°C. Above 45°C. there was a decrease in the degree of peptization in both the oil free and the non-oil free samples. The downward slope of the curves in all instances above 45°C. may be due to the heat coagulation of some of the protein material. When using low concentrations of salt, it seems that the presence of oil in the natural sample gave some protection against heat coagulation of protein at higher temperatures.

The Precipitation of Protein as Effected by Dilution

Figure 10 shows the sedimentation rate of Mung bean globulin when a series of ten different rates of dilution was made.

A. It was interesting to note the precipitating behavior of Mung bean globulin when one volume of 0.4M NaCl extract was diluted with four volumes of distilled water (final concentration 0.08M); the rate of sedimentation was faster and the precipitate was more compact than in any other dilutions in the series.

B. The solubility range of Mung bean globulin was thus concluded to be between 0.4M NaCl as the maximum solubility concentration and 0.08M NaCl as the precipitating concentration.

C. This leads to a consideration of the globulin behavior at dilutions greater than 1:4. In Figure 10 it was shown that as the dilution ratios were increased (to the Number 10 cylinder), the volume of the precipitates were correspondingly larger. Since the amount of protein in all cylinders was the same, why did the volume of the precipitate increase as the dilution ratio increased? It is obvious that under such conditions the hydration is greater and probably the amount of adsorption of albumin is also greater. This is supported by data in Table XXX. Therefore, a greater dilution for precipitating globulin must not be employed because it may lead to excessive occlusion of other proteins (albumin) from the solution.

D. The removal of albumin from globulin was accomplished by the dilution method. There is no existing method of separation of albumin from globulin which is superior to precipitation of the globulin by dilution, leaving the albumin in the solution.

E. From the above discussion it will be seen that the precipitation effected by the 1:4 dilution represents the globulin fraction of Mung bean. Hence Table XXX (dilution 1:4) represents the more accurate fractional values: globulin nitrogen 50.5 percent, albumin nitrogen 36.7 percent and N.P.N. 12.5 percent. The globulin/albumin ratio was 1.37 and the protein nitrogen/non-protein nitrogen ratio was 6.97. These ratio values may be used as a means of characterization of the species from which the proteins are secured.

Conditions Essential for the Isolation of and Upon Which the Characterization of Mung Bean Globulins Depend

The isolation of proteins in their natural condition is still in a state of development. The nature and the quantity of protein peptized from different sources has varied from extract to extract since much depends upon the technics employed. In order to establish a method by which the results of this study can be reproduced, the conditions essential for the preparation of Mung bean globulins must be considered.

1. Nature of solvent: A solution of neutral salt should be used.

2. Ionic strength of solvent: A solution of maximum ionic strength should be used for peptization. However, a solution of ionic strength above the maximum must be avoided since excessive concentration causes occlusion of proteins other than globulin. Contrariwise, solutions of lower than precipitation concentration should be avoided to prevent denaturation of protein.

3. Solubility range: A narrow solubility range requires less dilution to effect precipitation.

4. Particle size of sample: The smaller the particle size, the greater the yield.

5. Sample-solvent ratio: A large ratio results in an inefficient extraction. On the other hand, a small ratio gives efficient extraction but may result in a volume of extract which is difficult to handle when diluted for precipitation. Therefore, an efficient and convenient S-S-R must be chosen.

6. Extraction time: A period of time that is of long duration

must be avoided to prevent contamination, denaturation, growth of microorganisms and fermentation. A period of time that is too short results in inefficient extraction.

7. Extraction temperature: Room temperature of 20 to 25°C. is usually efficient. Higher temperatures are apt to cause denaturation. On the contrary low temperature, which might appear desirable, decrease the rate of peptization and may result in precipitation of peptized protein.

8. Agitation: Occasional hand stirring was as effective for extraction as mechanical stirring. High speed and vibrational agitation may cause denaturation.

9. Successive extraction: This is the only way to secure an exhaustive extraction of a sample.

10. Separation of residue: The ordinary standard centrifuge seems most desirable. A Sharples centrifuge should not be employed for this type of work.

11. Precipitation: When precipitation is effected by dilution, water must be added to the extract at a slow rate with continuous mixing.

12. Removal of other proteins: This can be achieved by repeated dispersal of the precipitate in salt solution followed by precipitation by dilution.

Rotating Outside Liquid Dialysis Apparatus

The development of the rotating outside liquid dialysis apparatus for rapid membrane equilibration dilution proved to be efficient for precipitation, fractionation and purification of protein. The essential characteristics of this type of dialyzer are: 1) the container which rotates

at a speed of 42 r.p.m., 2) the cellophane tubing only two thirds full with protein solution instead of completely filled to the level of the dialyzing water, 3) the cellophane tubing suspended eccentrically in the liquid in the tank in order to increase agitation of the entire system when the container is in motion. As a result of the movement, the diffusable ions adjacent to both sides of the membrane wall are constantly removed with a change of concentration and thus equilibration is effected in a shorter time.

Some of the specific applications of this type of dialyzer are:

1. Precipitation of protein by indirect dilution was accomplished when a measured volume of protein solution was placed in the cellophane tubing and immersed in a measured volume of distilled water in the dialyzing tank. The protein precipitated when the desired concentration of the solvent was reached. Indirect dilution for precipitating protein prevents denaturation of the protein by avoiding direct contact with an excess amount of water.

2. An indirect, slow stepwise membrane equilibration dilution technic with rotating outside liquid dialysis was employed for fractionation and purification of protein. This was accomplished when the protein in 0.4M NaCl solution in the cellophane tubing was suspended in the same concentration of NaCl solution in the dialyzing tank. The total volume of both were measured and then a measured volume of water was slowly added to the tank. When equilibrium was reached, a desired concentration was obtained. The Mung bean globulin was separated into its component fractions by this technic.

3. This dialyzer can also be used for continuous dialysis to remove all diffusable ions. Continuous change of dialyzing water is conducted in a very convenient way.

The Fractionation of Mung Bean Globulin and the Isolation of G_2, G_3 and G_4

Using the membrane equilibration dilution procedure for lowering the salt ion concentration of the protein solution, the first fractional precipitation of Mung bean globulin occurred at 0.22M NaCl. This precipitate was designated as fraction precipitate $G_{2(22)}$. The centrifugate from the precipitate $G_{2(22)}$ was diluted similarly to 0.11M NaCl and yielded a precipitate which was designated as fraction $G_{3(11)}$. The centrifugate of the precipitate $G_{3(11)}$ was further diluted to 0.07M NaCl and a third precipitate was obtained. This fraction was designated as $G_{4(07)}$. The nitrogen distribution in terms of percentage of total globulin in these fractions are 40.95, 49.09 and 3.90 for $G_{2(22)}$, $G_{3(11)}$ and $G_{4(07)}$ respectively. The electrophoretic patterns of these fractions showed that the $G_{2(22)}$ contained two components whereas the others were single components.

Development of a Dissolution Process for Resolving the G_2 Complex

The precipitate $G_{2(22)}$ was subjected to further fractionation by the dissolution technic. It was first peptized in a small volume of 0.4M NaCl solution in order to keep both the protein concentration and the dielectric constant of the medium high. This technic decreased the interaction of components of different species and other charged substances and the decomposition of any complex was possible under such an environment.

It was found that the fractional precipitate, $G_{2(22)}$, was a complex of two globulin components and some pigmentary material which were bound together and co-precipitated at the concentration of 0.22M NaCl. Such complex formation may be expected to affect the solubility of the individual components.

The dissolution procedure for the decomposition of such a complex was simple to perform but difficult to perfect. Since the isolation of each of the single components from a protein mixture was the primary object of this study, considerable time was devoted to this problem.

The fractional precipitate $G_{2(22)}$ which was peptized in 0.4M NaCl was introduced into the cellophane tubing which was then suspended in distilled water in the container. The volume of distilled water was ten times greater than that of the protein solution in the cellophane tubing. Precipitation occurred in the cellophane tubing within 15 minutes, but a period of two hours was allowed for sedimentation of the fast precipitating components. The fast precipitating components, pigmentary substance combined with protein, were co-precipitated and appeared as dark, dense material in the bottom of the tubing. The true globulin appeared as white fluffy precipitate (amorphous in form) which was suspended throughout the supernatant and was separated from the dark precipitate by decantation. The dissolution procedure was repeated with the dark precipitate until all the globulin was released from the pigmentary material.

Further fractionation of the dissolution product resulted in one component precipitating at 0.17M NaCl and designated as $G_{2(17)}$, and the

other precipitating at 0.14M NaCl and called $G_{2(14)}$. Electrophoretic patterns showed that the $G_{2(17)}$ and the $G_{2(14)}$ were single components.

Use of Maximum Precipitation Concentration and Incipient Precipitation Concentration to Control the Purification of the Globulin Fractions

The fractionation and purification procedure previously employed for the isolation of globulin fractions were accomplished by adjusting them to their maximum precipitation concentration "m.p.c.". The resulting products, so far as the homogenity of the protein was concerned, were freed of gross contamination only. It was necessary to use specific molar concentrations of the solvent, both the maximum precipitation concentration and the incipient precipitation concentration "i.p.c.", to control the precipitation of each fraction and eliminate trace contamination and secure the desired state of purity.

The maximum precipitation concentrations of the Mung bean globulin fractions $G_{2(17)}$, $G_{2(14)}$, $G_{3(11)}$ and $G_{4(07)}$ were carefully determined to be 0.17, 0.14, 0.11 and 0.07M NaCl respectively. Their incipient precipitation concentrations were accordingly 0.18, 0.15, 0.12 and 0.08M NaCl.

By means of electrophoretic analysis, the patterns show that four individual single globulin components have been isolated in supposedly undenatured state from Mung bean meal. The problems of globulin individuality, globulin definition and globulin isolation have been studied critically and the author has attempted to follow the advice of Osborne in keeping himself from all preconceived notions as to what constitutes orthodox or non-orthodox technics.

Osborne stated years ago "we can, for the present, treat as individual proteins only those products whose extensive fractionation has given no evidence that a mixture is being dealt with, and we must await new methods of study before anyone of these proteins can be accepted as true chemical entities." The Mung bean globulins herein described have shown such constancy of composition and properties that we feel justified in considering them as substances of reasonably definite character.

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ADDENDA

A NEW APPARATUS FOR FREEZING-DEHYDRATION

AND

TECHNIQUE FOR HIGH VACUUM MANIPULATION

This apparatus is designed and constructed for drying proteins and biological materials.

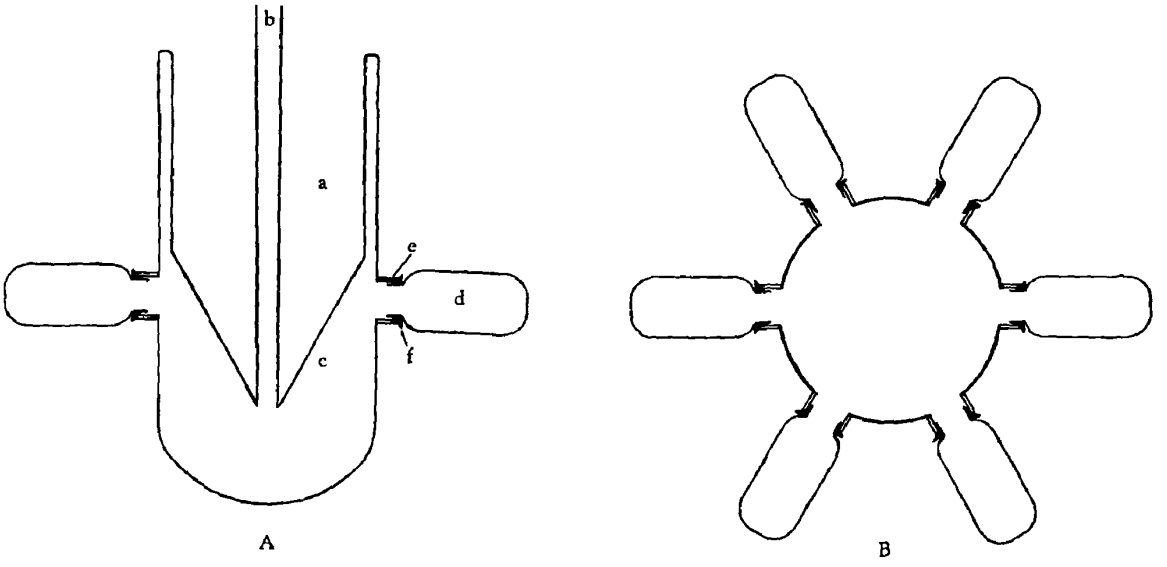

Figure 1. Freezing-dehydration tank A. Side section view B. Top section view
a. freezing chamber, b. center pipe, c. freezing cone, d. 250 ml centrifuge bottle, e. adapter, f. rubber ring

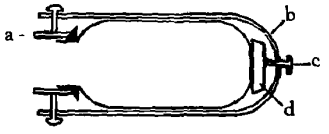

Figure 2. a adapter
b U clamp
c setting screw
d wooden block

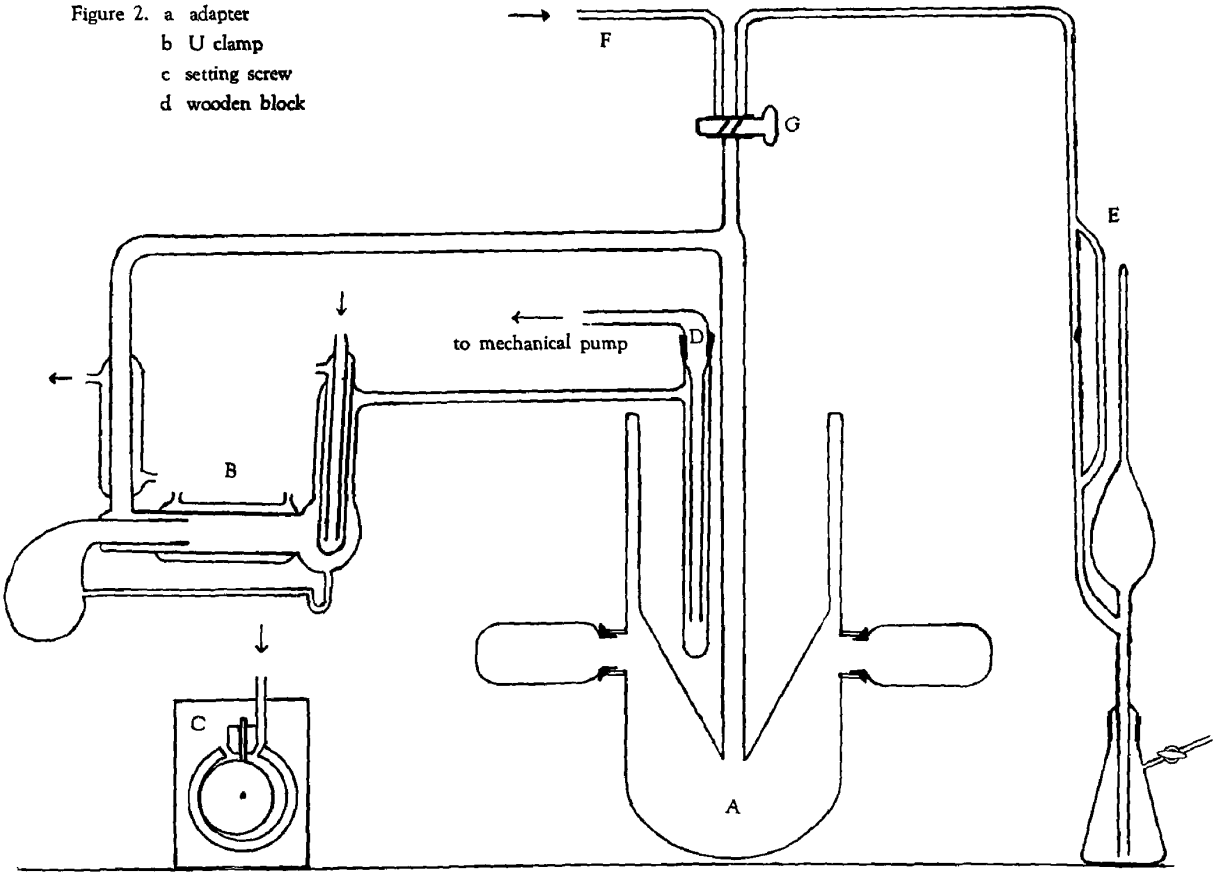

Figure 3. Assembly of apparatus A. Freezing-dehydration tank B. mercury condensation pump
C. mechanical pump D. cold trap E. McLeod gauge F. dry air inlet G. two way stopcock

A NEW APPARATUS FOR FREEZING-DEHYDRATION AND TECHNIQUE FOR HIGH VACUUM MANIPULATION

By rapid freezing and dehydration from frozen state under low pressure biological materials can be dried to a very low moisture content without shrinkage in volume (1). This technique has been utilized in the fixation of tissue in morphological studies and in histo-chemistry (2,3). In a recent publication (4) the use of freezing-dehydration method for the determination of moisture content of dehydrated vegetables was described. It involved the addition of a large amount of water to a weighed sample of dried vegetable, freezing and drying from the frozen state and completion of the drying in a vacuum oven or vacuum desiccator in the presence of an efficient water absorbent. It was observed that there was a marked increase in the drying rate of samples of dehydrated vegetables when first saturated with water, then frozen and subsequently dried in vacuo from the frozen state to a low moisture content. The increase in the drying rate may be attributed to two factors. One is an increase in volume of the frozen tissue and the other is the increase in porosity. It also was found that the completion of the drying in a vacuum oven at suitable temperatures produced the same results as drying in an evacuated desiccator at room temperature.

To meet the requirements of this technique a new apparatus for freezing-dehydration has been designed and constructed in accordance with the principles here described. The design of this freezing-dehydration vacuum

tank resembles a Dewar flask (double-walled structure) (Figure 1A). The open chamber (a) contains the freezing mixture, dry ice suspended in 95 percent ethyl alcohol, which maintains a low temperature of -72°C. The chamber serves as a cold bath for the quick freezing of samples. This chamber will hold six 250 ml. centrifuge bottles. For the drying procedure these bottles are attached to the tank by six adapters which are distributed around the sides of the outer tank (Figure 1B). When the air is evacuated, the tank and the six bottles become one unit under the same low pressure.

In the drying operation water vaporizes from the samples in these bottles under the low pressure and is diffused out of them and condenses upon the inner cold wall (Figure 1Ac) as long as the freezing mixture in the chamber remains at a low temperature. The vaporization continues as long as there is a greater concentration of water vapor in the bottles than in the tank. This results in a difference in diffusion pressures between the bottles and the tank. Since the greater concentration of water molecules is in the bottles, this determines the direction of diffusion; i.e. from the bottles to the tank (5). The tank is so constructed that when it is in operation it maintains the diffusion pressure at practically zero in the tank because of the rapid condensation of water vapor to solid ice upon the cone of the lower part of the inner tank (Figure 1Ac). The volume of space in the vacuum tank is four times the volume of the bottles which also contributes to the movement of water vapor from the bottles into the tank. Hence, the direction of the diffusion of water vapor from the bottles to the tank continues constantly until the water

vapor is exhausted in the bottles and the samples are dried.

One principle of an efficient freezing-dehydration apparatus is the establishment of a steep concentration gradient in the system. However, the rate of diffusion is also influenced by the distance through which the diffusing molecules must travel. These two factors are components of what may be called the concentration gradient. This principle has guided the construction of this freezing-dehydration vacuum tank and has met the requirement of effective extraction of water vapor from the samples in the bottles.

CONSTRUCTION OF TANK.-- The freezing-dehydration vacuum tank is made from the lower part (18 inches) of a steel gas cylinder (8 3/4 inches diameter). The thickness of the outer wall is 3/16 inch while the inner tank is made of sheet steel 1/8 inch thick. The upper part of the inner tank is rolled into a cylinder, 8 inches in height, 7 1/2 inches in diameter; the lower part is rolled into a cone, 6 inches in height and these are welded together. A 3/4 inch pipe 20 inches in length is welded through the tip of the cone. The tank is evacuated through this pipe (Figure 1A,b). The two tanks are welded together across the top and the unit is thus similar to a double-walled Dewar flask. Six openings are equally distributed around the outer tank 10 inches from the top edge. These openings provide for the connection of six 250 ml. centrifuge bottles (Figure 1B). Adapters for the bottles are welded at these openings. They are made of 1 1/2 inch pipe and cut 1 1/4 inches in length. Two holes are threaded into each piece of pipe on opposite sides at the center for fitting 1/2 inch long, 1/4 inch ball head bolts (Figure 2a). The

bolts are brazed into place on the inside of the adapters. U clamps of strip iron (Figure 2b) which hook over the two bolts on the adapter are adjusted by a set screw at the other end of the U clamp to hold the centrifuge bottles stationary (Figure 2c). A wooden block acts as a cushion between the bottom of the bottle and the set screw. The adapters are beveled at a 90° angle on the inside to accomodate a rubber ring* which fits over the neck of the bottle (Figure 1A,f). This provides an air-tight connection between the bottles and the tank.

Before welding, the inside and outside of the two tanks were painted with red oxide primer. Two coats of red oxide primer were applied after the units were welded together and the tank was evacuated while the paint was wet.

When in operation, the tank is wrapped with an inch thick wool blanket for insulation.

ASSEMBLY OF APPARATUS AND LOW PRESSURE PRODUCTION.— The entire apparatus for freezing-dehydration (Figure 3) consists of the freezing-dehydration tank, A, the mercury condensation pump B, the mechanical pump C, and the cold trap D which is connected between the mercury pump and the mechanical pump to trap water vapor and mercury vapor. These compose the active parts of the apparatus. The McLeod guage E and the dry air inlet F are used infrequently and can be isolated from the more active parts by the stopcock at G. The entire assembly occupies a horizontal space on a bench or on a movable table of two by four feet.

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* Cenco Frizzell holder No. 18107.

This enables the unit to be placed on a convenient working level. The simplicity of this unit eliminates operational difficulties. It is easy to evacuate this simple system and to keep it evacuated.

The capacity of the mechanical pump should be high. Capacity is measured by the time required to evacuate a given volume to a desired pressure. This time is in proportion to the volume of the system. A comparison of the capacity of two mechanical pumps will illustrate these points (Table I). The data in Table I indicates that a larger volume requires more time proportionally for proper evacuation. It also furnishes information that a proper choice of the mechanical pump is important in obtaining the best production of low pressure.

With the help of a mercury diffusion pump a low pressure of 10^{-5} is possible when a fast mechanical pump is used (Table II). Table II illustrates that the efficiency of a mercury pump is dependent upon a "backing pressure" produced by a mechanical pump.

The data in Table II indicate that a mercury diffusion pump will produce a higher vacuum when matched with an efficient mechanical pump. This illustrates that the use of mercury and mechanical pumps together are necessary to produce the desired low pressure and overcome the defects of the system.

Although mercury diffusion pumps backed by fast mechanical pumps are preferred for high vacuum work and have been recommended by many investigators (7), they do have one disadvantage. The vapor pressure due to the mercury is so high it tends to destroy the vacuum in the system. If a pressure of the order of 10^{-3} micron is to be maintained in

TABLE I

COMPARISON OF THE CAPACITY OF TWO VACUUM PUMPS (6)

Duo-seal pumps	No. 1405 1/3 h.p.	No. 1400 1/4 h.p.
Low pres. obtainable	0.05 micron Hg	0.1 micron Hg
Free air capacity	33.4 liters per min.	21 liters per min

Evacuated Volume in Liters and Time in Minutes

Pressure mm. Hg	5 l. min.	10 l. min.	20 l. min.	30 l. min.	50 l. min.	5 l. min.	10 l. min.	20 l. min.
10^{1}	.65	1.5	3.0	4.3	7.0	1.4	2.8	5.8
10^{0}	1.2	2.3	4.6	7.0	11.5	2.3	4.5	9.0
10^{-1}	1.7	3.5	7.0	10.8	17.2	3.0	6.5	13.0
10^{-2}	2.5	5.0	9.6	14.8	24.0	4.7	9.5	19.0
10^{-3}	3.5	6.8	13.7	20.3	33.5	7.5	15.3	31.0
10^{-4}	5.1	10.6	21.0	31.5	54.0	20.0	40.0	

TABLE II

PERFORMANCE OF MERCURY PUMP BACKED BY MECHANICAL PUMP

Pressure produced by mechanical pump	Low pressure produced by mercury pump (Todd)
0.1 mm.	0.0003 mm.
1.0 mm.	0.0003 mm.
5.0 mm.	0.0003 mm.
10.0 mm.	0.007 mm.
20.0 mm.	0.14 mm.
30.0 mm.	0.3 mm.

a system, mercury, which has a vapor pressure of 0.185 micron at 0°C. must be prevented from diffusing into the system. Since the vapor pressure of mercury at -78°C. is but 3×10^{-6} micron, the concentration of mercury may be greatly reduced in the system by the use of a cold trap (8). Thus, a trap must be used but attention should be given to the design of the trap suitable for high vacuum work such that it offers only low resistance to the flow of gas. The trap used for this apparatus has an outside tube measuring 32 mm. in diameter and an inside tube measuring 18 mm. in diameter. It has a universal joint of ground glass so that one of its arms is adjustable. Since the mercury vapor is extremely poisonous, it is especially dangerous for use in a poorly ventilated room. Both mercury vapor and water vapor endanger the efficiency of a high vacuum mechanical pump. Thus this second condensation trap is a safety device.

The important factors affecting the operation of a pumping system are: 1. leakages in the system, 2. The resistance of the pumping line, trap constrictions, bends, etc. These offer high resistance to the flow of gas. The capacity of the vacuum lines is determined by: 1. the shortest possible distance between the pump and the vacuum apparatus; 2. the largest diameter possible of the connecting tubes; 3. the use of large curves in tubing rather than sharp angles, and 4. absorption of gas on the walls of the entire system. Both glass and metal adsorb an appreciable amount of carbon dioxide, air and moisture upon their surfaces. Metal units are more porous than glass, hence they have a higher capacity for adsorbing gases than glass. In order to liberate the adsorbed gases

at a rapid rate glass must be heated up to 300°C. while pumping. New apparatus releases gases very slowly at room temperature. However, metal units are not outgased by any procedure other than that of continuous pumping. In a system for high vacuum it is imperative to remember that the pumps are an integral part of the whole system, and as such, their functioning is dependent on the design of the system. Even for a high speed pump the resistance of the pumping lines must be kept to a minimum. Short lengths of small diameter tubing must be avoided. If the resistance of the vacuum system is high, the system will be a slow system even if the pump used has great speed, because the pump speed is then limited by the system.

OPERATIONAL PROCEDURE.— The reservoir or freezing tank (Figure 1A,a) is filled with four liters of 95 percent ethanol. Small pieces of dry ice (2 or 3 ounces) are added. Vigorous CO_2 gas evolves. Addition of dry ice is continued every 5 to 10 seconds until the vigorous evolution of CO_2 gas decreases. This requires about two pounds of dry ice. Then four pieces of dry ice a half pound in size are added to assure the temperature of the alcohol at -72°C.

Six 250 ml. centrifuge bottles containing samples are suspended half-way in the freezing tank for 20 to 30 minutes. The bottles are placed in an upright position. After 30 minutes in the freezing tank, the bottles are removed and connected to the adapters and fastened with the screws at the ends of the U clamps.

After the sample bottles are removed, six pounds of dry ice are put into the freezing tank. The temperature of -72°C. will remain constant

for a period of 7 to 8 hours. Additional dry ice must be added if the operation is to proceed for a longer period. The mechanical pump is then turned on for 3 to 5 minutes. When most of the air is evacuated by the mechanical pump, then heat is applied to the mercury pump. The pressure of the system is measured by a McLeod gauge.

When this vacuum-dehydration process is completed, the apparatus should be turned off in the following manner: 1. the heater for the mercury pump is first turned off; 2. air is slowly allowed to go into the system through the drying tube by turning the two way stopcock at G (Figure 3); 3. the mechanical pump is immediately turned off; 4. remove the bottles containing dried samples, these are stored in a desiccator containing P_2O_5 or $Mg(ClO_4)_2$ and subsequently evacuated.

EXPERIMENTAL RESULTS.— A. Drying meats and vegetables. Six centrifuge bottles containing six different weighed samples were frozen in the freezing chamber of the apparatus for 30 minutes then connected to the vacuum tank for 4 hours dehydration. Pressure measured 22 micron of mercury after 1 hour. Temperature of the freezing tank was -72°C. After 4 hours dehydration the samples were reweighed.

Sample	Wt. Fresh	Wt. Dried	% Water Loss per Total Wt.
1. Lean beef	58.80 gm.	37.15 gm.	36.8
2. Kidney	38.86 gm.	22.20 gm.	43.0
3. Beef liver	31.45 gm.	18.10 gm.	42.8
4. Apple	54.65 gm.	30.09 gm.	44.9
5. Carrot	38.20 gm.	24.72 gm.	35.3
6. Cabbage	34.75 gm.	10.04 gm.	71.1

Observations made from results of this experiment:

1. When a sample was cut into large chunks, the center portion was not dried in 4 hours and remained frozen.
2. All dried samples retained their original form and size.
3. Incompletely dried samples absorbed moisture from atmospheric air. This observation was made from apple tissue which became sticky a few minutes after exposure to air.
4. Four hours dehydration was not sufficient for drying these tissues.

B. Drying meats and vegetables. Six centrifuge bottles containing six different weighed samples were frozen in the freezing chamber of the apparatus for 30 minutes then connected to the vacuum tank for eight hours dehydration. Pressure measured 20 microns after 1 hour. After 8 hours dehydration the samples were weighed and stored in a vacuum desiccator with P_2O_5 as water absorbent. The following results were obtained. Percentages of water loss per total weight were calculated after 8 hours of vacuum dehydration and after completion of drying in a vacuum desiccator for 7 days.

Sample	Wt. Fresh Sample	Wt. Sample after 8 hr. Dehydration	% Water Loss per Total Weight	% Water Loss after 7 Days in Desiccator
1. Banana	32.65 gm.	8.48 gm.	74.0	74.6
2. Green bean	24.60 gm.	3.00 gm.	87.8	84.3
3. Sweet potato	45.79 gm.	20.07 gm.	56.17	66.39
4. Carrot	32.33 gm.	8.53 gm.	73.63	86.48
5. Beef	46.10 gm.	14.20 gm.	69.19	71.14
6. Beef liver	42.00 gm.	16.30 gm.	61.19	67.07

C. Dehydration of five whole rat liver samples. Samples were frozen for 30 minutes in the freezing tank which was filled with dry ice and ethanol. The dehydration period employed was 8 hours. A low pressure of 5 micron of mercury was maintained during the last 4 hours. The samples were weighed immediately following dehydration and daily thereafter for 6 days with continuous storage over P_2O_5 in a vacuum desiccator. No further loss in weight occurred after 5 days storage. The results secured are presented in Table V.

TABLE Va

EFFICIENCY OF DEHYDRATION BY FREEZING-HIGH VACUUM TECHNIQUE

	Fresh Weight in Grams	After 8 hrs. Freezing-Dehydration	Gms. Water Loss per Total Wt.	% Water Loss per Total Wt.	% Water Loss After 5 Days in Vacuum Desiccator
1.	6.97	2.64	4.33	62.12	62.26
2.	7.59	2.82	4.77	62.84	62.97
3.	7.89	2.59	5.30	67.17	67.93
4.	6.87	2.02	4.85	70.59	70.74
5.	8.87	3.19	5.68	64.03	70.10

Using the percentage of water loss after 5 days in the vacuum desiccator (column 5) as representing 100% water loss from all samples, the data in columns 1 and 2 of Table Vb were calculated and show the difference in percent water loss by 8 hours freezing-dehydration and 5 days in a vacuum desiccator.

Samples 2 and 4 were more completely dried by the freezing-dehydration method probably because the samples in these two cases were so placed that a considerably greater surface area was exposed.

TABLE Vb

Sample	% of Total Water Loss by 5 Days in Vacuum Desiccator (P_2O_5)	% of Total Water Loss by Freezing-dehydration
1.	0.92	99.08
2.	0.21	99.79
3.	1.12	98.88
4.	0.21	99.79
5.	8.66	91.34

SUMMARY.— This apparatus for freezing-dehydration has been constructed as described herein and has yielded satisfactory results in our laboratory. Some of the features of this apparatus are discussed.

The tank itself is a device for extracting and trapping water which has vaporized in centrifuge bottles from frozen samples. The apparatus is capable of establishing a steep concentration gradient because of two contributing factors: 1. the efficient water vapor condensing mechanism and 2. the short distance between the openings of the bottles and the freezing cone (the lower part of the inner tank). The freezing chamber serves also as a cold bath for freezing samples and as the second cooling trap for mercury vapor. The double walled structure of the tank offers the advantage that the temperature of the freezing chamber remains low for a longer period of time.

The horizontal position and the large opening (25mm) of the centrifuge bottles facilitate a more rapid diffusion of vaporized water molecules. The tank has a large capacity for drying samples: six 250 ml. centrifuge bottles can be used at one time. The adapters of the tank

allow bottles to be used interchangeably which is an improvement over other types of laboratory vacuum-dehydration units. Further advantages are offered by the use of centrifuge bottles because substances which must be separated from solution by centrifugation can be dried in the same bottle without transfer which prevents possible loss of sample, limits contamination and saves time. The dried samples may be preserved in the same bottles.

The simplicity of design of the entire unit contributes to ease of construction in an ordinary machine shop.

It has been found that 8 hours operation is sufficient to dry samples to near constant weight. The apparatus is easy to operate and does not require special attention during the period of operation.

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A HIGH TEMPERATURE BATH MADE FROM ALUMINUM SHAVINGS

A HIGH TEMPERATURE BATH MADE FROM ALUMINUM SHAVINGS

The need for a high temperature bath for hydrolysis of proteins in aqueous solutions led to the work presented as follows. It was necessary to find a material for a bath in which constant boiling temperature of an aqueous solution could be obtained. Since aluminum shavings have a high conductivity of heat, it was found that a high temperature bath could be constructed from them.

An aluminum-shaving bath is constructed of a copper box which contains aluminum turnings about the size of rice granules. The box is placed on top of a copper plate a quarter of an inch thick and is fastened by screws. The copper plate is heated by three electric elements and the heat is regulated and controlled by a thermoregulator which is located in the center of the bath. (Figure 1).

Because of the high conductivity of copper a uniform temperature bath can be constructed. The copper plate must be thick enough in order that sufficient heat may be diffused to the entire unit. The entire bath is inclosed in a box of transite so that the uniform temperature of the bath can be preserved. The space between the copper box and the transite wall measures one inch and is filled with rock wool as insulator. The bath is covered with individual transite covers with notches to accommodate the necks of the flasks.

The dimensions of the inside of the bath are as follows: 40 inches in length, 6 inches in width and 4 1/2 inches in depth. Eight 300 ml. round bottom flasks can be set in a row. The flasks are conveniently

placed in metal cups made from 8 ounce aluminum measuring cups with handles removed. In case of breakage of flasks, the solution is trapped in the cups. (Figure 1). The flasks are cushioned in the cups with "Chore-girl" copper ribbon material which retains its form. Aluminum shavings were not satisfactory because they did not retain a definite form to accommodate the shape of the flasks. However, the bath can be operated without the use of metal cups.

In packing the bath the 8 metal cups with their contents are placed on the bottom of the box. (Figures 1 and 2). Then the aluminum shavings are poured in to a depth of 0.75 inch, evenly distributed but not pressed or tamped. Two aluminum bars are laid on top of the aluminum shavings in front and in back of the metal cups but not touching the cups or the side walls of the bath. After all these are in position, the bath is filled with aluminum shavings up to one inch from the top. The two aluminum bars, 39 inches in length by 1/2 inch in diameter, are imbedded in the aluminum shavings 3/4 inch above the bottom of the bath, are used as heat conductors. (Figures 2 and 3).

The bath was tested for 50 hours of continuous operation at 200°C. Recordings were made of the temperature variations at five different positions in the bath. The fluctuation of temperature of all the positions of the bath was found to be the same, ±1.5°C. (See table). It was demonstrated that the temperature of the bath was even and constant at various positions in the bath when the thermoregulator was set at 200°C.

The thermoregulator was set at temperatures ranging from 30°C. to 250°C. for 12 hour periods. The bath held the given temperature throughout

the period tested. Detailed data on specific temperatures other than 200°C. were not compiled. Temperatures higher than 250°C. could be secured by adding more heating elements.

The aluminum-shaving bath so constructed has many advantages for use in a chemical laboratory. It is especially useful at or above 100°C. for hydrolyzing or refluxing samples for long periods. Partially hydrolyzed samples can be taken from each flask for analysis of the degree of hydrolysis without disturbing the main procedure when the two-neck-flasks are used. Aluminum shavings are non-corrosive and always appear clean. Above 100°C. the shaving-bath has several advantages over a liquid bath: no liquid escapes by evaporation; no unpleasant fumes as with acid or organic liquids.

The cost of the material, excepting the thermoregulator, for building the bath is between $12.00 and $15.00. The thermoregulator is manufactured by Fenwal, Inc., Ashland, Massachusetts. An aluminum plate 3/8 inch thick, 40 inches by 6 inches, weighing 10 pounds may be used instead of copper (cost $3.00). Aluminum shavings are obtainable at machine shops at $.10 per pound.

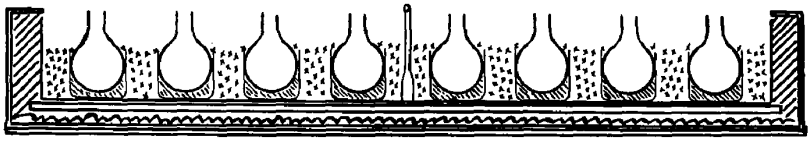

Figure 1. Front section view

Aluminum shavings

Rock wool insulation

Copper plate

Heating element

300 ml. flask placed in metal cup, cushioned with chore-girl copper ribbon

Position of thermo-regulator

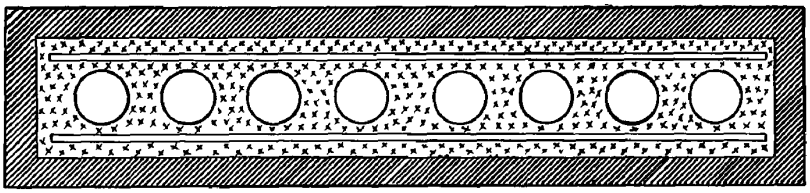

Figure 2. Top section view

Flask

Aluminum shavings

Aluminum bar

Rock wool insulation

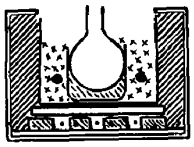

Figure 3. End section view

• • Aluminum bars · · · Heating elements

Copper plate

TEMPERATURE VARIATIONS AT FIVE POSITIONS IN THE BATH

Time in Minutes	Thermo-regulator Pilot Light	1 °C	2 °C	3 °C	4 °C	5 °C
1	off	201	203	201	202	201
2	off	201	203	201	202.5	201
3	Off	201	203	201	203	201.5
4	off	201	203	201	203	201
5	off	201	202	200	202	200
6	on	201	202	200	202	200
7	on	200	201	199	201	200
8	on	200	201	199	200	200
9	on	199	200	198	200	199
10	on	199	200	198	200	199
11	on	198.5	200	198	200	199
12	on	198	200	198	200	199
13	on	198.5	200	198	200	199
14	on	199	200.5	198	200	199.5
15	on	199	200.5	198	200	200
16	on	199.5	201	198	200	200
17	on	200	201	198.5	200.	200
18	on	200	201	199	200.5	200.5
19	off	200	201	200	200.5	200.5
20	off	200	201	200	201	201
21	off	200	201.5	200	201	201
22	off	200	202	200	201	201
23	off	200	202.5	200.5	201.5	201.5
24	off	200.5	203	200.5	202	201.5
25	off	200.5	203	200.5	202	201
26	off	200.5	203	200.5	202	200
27	off	200.5	203	201	202	200
28	off	200.5	202	200.5	202	200
29	off	200	202	200.5	202	199.5
30	off	200	202	200.5	201	199
31	off	200	202	200.5	200.5	199
32	off	200	202	200	200.5	199
33	on	200	201.5	200	200.5	199
34	on	200	201	199.5	200	199
35	on	199.5	200.5	199	200	199
36	on	199	200	198.5	200	199.5
37	on	198.5	200	198.5	200	199.5
38	on	198.5	200	198.5	200	199
39	on	198	200	198	200	199
40	on	198.5	200	198	200	199

TABLE Cont.

Time in Minutes	Thermo-regulator Pilot Light	1 °C	2 °C	3 °C	4 °C	5 °C
41	on	198.5	200	198	200	199
42	on	198.5	200	198	200	199
43	on	199	200.5	198.5	200	199.5
44	off	199	201	199	200	200
45	off	199	201	199	200	200
46	off	199	201	199.5	200	200
47	off	199.5	201.5	200	200.5	200
48	off	200	202	200	201	200.5
49	off	200	202	200	201	200.5
50	off	200	202	200	201	201
51	off	200	202	200	201	201
52	off	200	202	200	201	200.5
53	off	200	202	200	201	200.5
54	off	200	201.5	200	200.5	200.5
55	off	200	201.5	200	200.5	200
56	off	199.5	201	199.5	200	200
57	off	199	200.5	199	200	200
58	on	198.5	200	199	200	199.5
59	on	198	200	198.5	199.5	199
60	on	198	200	198.5	199.5	199
Highest reading		201	203	201	203	201
Lowest reading		198	200	198	200	199
Range or variation		3	3	3	3	2
Fluctuation		± 1.5	± 1.5	± 1.5	± 1.5	± 1