

AN INVESTIGATION ON THE PROTEINS OF THE PEANUT, ARACHIS HYPOGAEA

By

MILLARD J. HORN

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GENERAL

Object of the Investigation

In previous investigations on the vegetable proteins the workers have been most interested in determining the number of different proteins the seed or material contained, and in establishing their elementary composition and amino acid content, little attention being paid to the quantitative estimation of the various proteins. Although the amino acid content of the individual proteins in a given material may be known, unless data regarding the relative proportions in which the proteins are present are available, it is obviously impossible to estimate the percentages of amino acids in the whole plant material. This is an important consideration from a practical standpoint. For instance, the dietitian, or animal husbandry man, is interested in knowing how much of the different nutritionally essential amino acids the whole wheat, or corn, contains rather than the percentages of the amino acids in gliadin, glutenin, or zein.

Earlier work on the peanut established the fact that it contains two globulins, arachin and conarachin, and possibly an albumin, although no positive evidence for the latter was obtained. Glutelins and prelamins were not looked for, nor were the exact amounts of arachin and conarachin determined. This is important as conarachin contains an unusually large amount of the nutritionally essential amino acids, and it is very desirable to know the amount

of these amino acids in the whole meal.

This investigation was undertaken to determine the relative amounts of the two globulins; to isolate, if present, albumin, glutelin, and prolamins; to ascertain the properties of all the proteins extracted; to estimate quantitatively the protein and non-protein nitrogen; to determine the precipitation limits of arachin and conarachin with ammonium sulfate; and to study the effect of the tannin in the skins on the extraction of the different proteins present.

Historical

Ritthausen (1) was the first to study the proteins of the peanut. This worker extracted oil-free peanut meal with a solution of sodium chloride and with solutions of potassium, calcium, and barium hydroxides. He obtained the globulin by saturating the diluted extract with carbon dioxide or simply by diluting the extract with a large volume of water. He apparently obtained the same globulin by acidifying the alkaline extracts with acetic or sulfuric acids. Ritthausen showed no evidence that indicated the presence of more than one globulin in the peanut.

Ritthausen dried the peanut meal over sulfuric acid in a desiccator and analyzed it for nitrogen, obtaining 10.18 per cent or 56 per cent of protein. The latter value he obtained by multiplying the percentage of nitrogen by 5.5, assuming that the protein contained 18 per cent of nitrogen. When this meal

was extracted with 10 per cent sodium chloride solution he isolated 27 per cent of globulin by diluting the extract with water and then saturating with carbon dioxide.

Johns and Jones (2), using shelled Virginia peanuts, extracted the oil-free air-dried peanut meal with 10 per cent sodium chloride. From this meal containing 42 per cent protein ($N \times 5.5$) was obtained 32 per cent of protein in the sodium chloride extract at room temperature. Raising the temperature did not increase the yield. By saturating the extract with carbon dioxide they obtained 25 grams of protein from a volume of extract calculated to contain 32 grams. This yield was based on the actual weight of pure protein after drying at $100^{\circ} C.$ and did not take in consideration the losses incurred during its isolation.

They made fractional precipitations of the protein from the extract and isolated two globulins, one of which was present only to a small extent. The globulin composing the greater part of the precipitate obtained by diluting the 10 per cent sodium chloride extract with water, they obtained by adding ammonium sulfate to 20 per cent of saturation. To this globulin they gave the name arachin. The second globulin obtained by saturating the filtrate with ammonium sulfate or by dialysis they called conarachin.

They gave the following analysis and comparison of the two proteins:

	<u>Arachin</u>	<u>Gonarashin</u>
C	52.15	51.17
H	6.93	6.87
N	18.29	18.29
S	0.40	1.09
O	22.23	22.58

They called attention here to the fact that conarashin contained three times as much sulfur as arachin.

Another striking difference was shown by their table of the nitrogen distribution.

<u>Nitrogen</u>	<u>Arachin</u>	<u>Gonarashin</u>
Amide	2.03	2.07
Humin	0.23	0.23
Basic	4.95	6.55
Non-basic	<u>11.07</u>	<u>9.40</u>
TOTAL	18.28	18.24

Gonarashin was shown to have more basic nitrogen than any other seed globulin hitherto examined. The percentage of basic nitrogen of the mixture of these globulins was also shown to be high, being 5.23 per cent. They gave a table showing the basic nitrogen in some proteins from seeds commonly used for food, taken from "The Vegetable Proteins," by Osborne, as follows:

<u>Protein</u>	<u>Source</u>	<u>Basic Nitrogen</u> <u>Per cent</u>
Zein	Maize	0.49
Gliadin	Rye	0.91
"	Wheat	1.09
Phaseolin	Kidney bean	3.62
Vicilin	Pea	4.92
Arachin	Peanut	4.96
Legumin	Pea	5.11
Excelsin	Brazil nut	5.76
Edestin	Hempseed	5.97
Globulin	Cocconut	6.06
Conarachin	Peanut	6.55

They also called attention to the fact that a trace of albumin was found in the extract which coagulated at 65-70° C., but the quantity was so small that a complete analysis could not be obtained.

Quantitative Separation of the Proteins

It was desired at first to grind the peanuts as fine as possible, but the oil rendered this impracticable, clogging up the mill. Therefore, the peanuts were ground as small as was convenient, treated with several liters of petroleum ether, dried, and then ground to a fine flour. This method saves time and a better product was obtained.

Two methods were used for the estimation of the number of proteins in the extract, coagulation by heat and fractional precipitation with ammonium sulfate, determining from the breaks in the curve the number of proteins present. Both methods showed the presence of but two proteins, arachin and conarachin.

For quantitative work it was found best to extract a 50 gram sample in a 500 c.c. centrifuge bottle by using a shaker. After

4 hours' shaking the whole was centrifuged and a fresh 400 c.c. of 10 per cent sodium chloride added to the residue and the mixture shaken again. It required about 12 hours to extract all the globulin quantitatively. The supernatant liquid was very easy to filter after centrifuging and there was but little chance for loss. By this method, 88 to 89 per cent of the total protein (based on nitrogen content) was extracted. As later tests gave no evidence for other protein nitrogen, 10 per cent sodium chloride must have extracted practically all the protein in the peanut.

For all quantitative work on the peanut extract it is necessary to use freshly prepared material. A solution over three days' old gives different results due to the decomposition of some of the protein and precipitation of some material insoluble in the sodium chloride.

At 40 per cent saturation with ammonium sulfate a clean cut separation of the two globulins was obtained, and here again it was found convenient to use centrifuge bottles for carrying out the precipitation. The arachin formed a compact mass on the bottom, and the supernatant liquid could be poured off without losing any of the precipitate. The supernatant liquid was filtered, then dialyzed for two weeks, but only a small quantity of an unpromising material separated. Conarachin is appreciably soluble in water unless all traces of salts are removed, and this method of dialysis does not remove the last traces of salts. The isolation of β -globulins is somewhat difficult due to this fact,

and they may be mistaken for albumins owing to their solubility in water containing traces of salt. We are now designing an electro-dialyzing outfit with which we hope to eliminate these last traces of salts and obtain quantitatively the β -globulins. By this method all doubts as to whether we have a trace of albumin or soluble β -globulin will be settled.

The quantitative estimation of conarachin, consequently, had to be made indirectly, but first it had to be ascertained whether all the nitrogen extracted was protein nitrogen, whether the extract contained more than two proteins, and whether there were any protein degradation products of a proteose or peptone character. By use of the ordinary method of ammonium sulfate precipitation, by use of various precipitating agents and by coagulation, it was shown that there were only two proteins present, that these were intact proteins, except for a trace, and that the extract contained no non-protein nitrogen. Therefore, advantage was taken of the wide range in the coagulation points of the two proteins. Conarachin coagulates at 80°, while arachin does not separate even at a boiling temperature. The arachin, therefore, can be estimated easily by determining the nitrogen in the filtrate from the coagulated conarachin by the Kjeldahl method. The difference between this result and the nitrogen of the original filtrate gave the arachin nitrogen. This method checked with the precipitation method and the direct method of weighing the arachin.

It was desired to find if some other salts could be found to separate arachin and conarachin. The sodium sulfate method of

Howe (18) was studied, but the higher temperature used in this method was thought undesirable. Sodium sulfate at room temperature did not effect any separation nor did lithium chloride, magnesium sulfate, sodium chloride, or potassium chloride. No clear cut separation was obtained by use of acids. Ammonium sulfate appeared to be the only salt that would effect a satisfactory separation of the two globulins.

Use of the Refractometer. The influence of dissolved proteins upon the refractive indices of various solvents has been especially studied by Reiss (3), Herlitzka (5), Schmidt (6), Robertson (4) (7-16), and Robertson and Greaves (17). Reiss used pseudo-globulins which were prepared by fractional coagulation with ammonium sulfate and purified by prolonged dialysis. These proteins were introduced in dilute salt solutions and the change in refractive indices noted. He found that the change in the refractive index of the solvent due to the introduction of these proteins was directly proportional to the quantity of protein dissolved in it, for "pseudo-globulin I" being 0.00224 and "pseudo-globulin II" 0.00230 for introduction of 1 per cent of these proteins. He found that the difference in the index between these two globulins did not constitute sufficient difference to distinguish between them and therefore could be assumed to give the same change in the refractive index.

Robertson amplified and confirmed the results of Reiss, employing a number of proteins (gliadin, casein, paramucin, serum globulin, etc.) in aqueous, alcohol water and acetone water

mixtures. He introduced the following formula showing that the change in the refractive indices of aqueous solvent due to the introduction of this protein is very accurately proportional to its concentration:

$$n - n' = ac$$

where n is the refractive index of the solution, n' , that of the solvent, c the percentage of protein in the solution, and a a constant which is characteristic of the protein. He found this formula to hold good for solutions of ovomucoid in water, paramelein in N/10 KOH, potassium serum globulinate and potassium caseinate in alcohol water mixtures and gliadin in various solvents.

Robertson came to two important conclusions: first, that the influence which a protein exerts upon the refractive index of its solution is independent of the nature or proportion of acid or base which is combined with it; second, that the refractive index of a protein solution remains unaltered by hydrolysis.

Assuming that a for arachin and conarachin are alike within the limits of experimental error, Robertson's method was used to determine the amounts of these two globulins, and the results agree well with the data obtained by other methods.

Properties of the Proteins

For studying the properties of arachin and conarachin, the proteins were precipitated from a fresh solution and used as quickly as possible in order to avoid change or decomposition. The data obtained on the coagulation point, for example, was quite different when a solution of arachin in 10 per cent sodium chloride was allowed

to stand in the ice chest for several days. Material coagulating at three different temperatures was obtained, indicating a decomposition of the protein. Another example is the precipitation point with ammonium sulfate. With an old solution there is no clear cut separation between arachin and conarachin, small amounts of material separating at 50 per cent, 60 per cent, and 70 per cent saturation with ammonium sulfate.

In working with conarachin it appeared that this protein was somewhat soluble in water. This might be due to the inability to remove the last traces of salts from the protein extract, and might account for traces of protein left in dialysate at times. Even after three weeks traces of salts are present in the dialysate.

With approximately 11 per cent protein (based on nitrogen of the meal) unaccounted for, a study of the meal left after the sodium chloride extraction was undertaken. Sixty-five per cent alcohol, and 0.2 per cent alkali removed from 2 to 8 per cent of nitrogen, but no protein was found. A carbohydrate was isolated with 65 per cent alcohol, but was not investigated further.

It is well known that tannin forms an insoluble compound with proteins and as there is tannin in the red skins of the peanut, it was thought that the tannin might affect the extraction of these proteins. Therefore, peanut meal was prepared without the red skins and extracted with 10 per cent sodium chloride solution. It was found that the tannin exerted no influence whatsoever.

Protein and Non-Protein Nitrogen

The terms protein and non-protein nitrogen are loosely used in chemical literature, and confusion exists as to just what the terms signify. As little work has been done on the quantitative estimation of the proteins in a given product and not very much is known just in what form some of the nitrogen is, the determination of nitrogen by the Kjeldahl method and multiplying by the factor 6.5, 6.0, 5.5, etc., only gives a hazy idea as to what is protein and what is non-protein. A great number of precipitating agents have been used, some claiming to precipitate only the intact protein molecule, some the intact protein together with its larger split products, and others, leaving only small peptides and amino acids in solution. In some cases it is not known whether these reagents will precipitate such non-protein nitrogenous products as purines, nucleic bases, choline, betaines, glucosides, urea, uric acid, and others, which are known to occur to some extent in many protein extracts. If we are reasonably sure that we are only precipitating protein nitrogen, how large a split product shall we leave in solution as non-protein nitrogen? On glancing over the various precipitating agents, including copper hydroxide, lead acetate, tungstic acid, picric acid, tannic acid, phosphotungstic acid, phosphomolybic acid, ethyl alcohol, colloidal iron, alumina cream, uranium acetate, sulphosalicylic acid and trichloroacetic acid, one is confronted with the problem of determining first just what

should be called non-protein nitrogen, for each one of these reagents leaves a different quantity of nitrogen in solution. After selecting the precipitating agent, it is frequently difficult to decide what to call the compounds containing the non-protein nitrogen. Are they peptones, proteoses, subpeptones, peptides?--for there is no sharp line of demarcation between these groups, nor is it understood just what is meant by these terms. Various authors differ in regard to the division of protein split products. For example, Wasteneys and Bersook (19) divide the hydrolysate into six fractions according to the nitrogen that is precipitated by various reagents, as follows:

Trichloroacetic acid	Intact protein
Sol. acid to pH 6.0	Metaprotein
Sodium sulfate	Proteose
Tannic acid	Peptone
95% alcohol + ZnCl ₂	Subpeptones
Solution	Amino acids

Other authors have divided them into coagulated proteins, meta-proteins, proteoses, peptones and peptides, and amino acids, the name protein stopping at the peptone stage. A review of the literature will show the various opinions and work done on this subject.

Non-protein nitrogen may consist of any part of these, depending on the precipitating agent used.

A. Marie (20) used ethyl acetate and showed it gave a marked precipitate with even dilute solutions of proteins and a turbidity with peptones, proteoses and albumoses, but solutions of amino acids showed no change.

Sellman (21) showed that the precipitation of proteins with tannin depended on the hydrogen ion concentration of the medium.

Marshall and Welker (22) by using an aluminum hydroxide solution precipitated the following quantitatively:

Egg albumin	Gliadin (in 70% alc.)
Edestin (in 5% NaCl)	Ovomucoid
Gelatin	Acid metaprotein (.1% HCl)
Casein (in half saturated lime water) .	Primary proteose
Glutenin (0.5% Na ₂ CO ₃) ..	Secondary proteose
Blood serum (protein) ...	Milk (including fat and protein)

Oxyhemoglobin is the only protein tested that Al(OH)₃ failed to remove.

Vinograd-Villichur and Losee (23) used colloidal ferric hydrate and claimed that it precipitated protein, some large albumins, leaving most of the intermediary products in solution.

Wolf (24) stated that Michaelis' colloidal ferric hydrate method is suitable for removing protein from blood.

Folin and Wu (25) used tungstic acid as protein precipitant for blood and found it as good as phosphotungstic acid. They called the nitrogen in the filtrate non-protein nitrogen. This reagent precipitates many of the proteoses and peptones, leaving only small peptides in solution with the amino acids.

Ritthausen (26) used copper hydroxide to precipitate proteins and this reagent has been used to precipitate gliadin and edestin quantitatively by Osborne and Leavenworth (27).

Greenwald (28) states that a precipitant for the complete removal of protein should have three properties: (1) It should be used in aqueous solution so as to make more probable the complete extraction of all non-protein water soluble substance and also to insure complete precipitation of lipoids with the proteins; (2) it should not redissolve protein when present in excess; (3) it should not interfere with subsequent digestion or distillation. He selects trichloroacetic acid as most closely approximating the ideal precipitant.

Blish (29) carried out experiments on flour extracts and found that more nitrogen was removed with copper hydroxide than any other precipitant, such as alcohol, acetic acid, trichloroacetic acid, salts of heavy metals, colloidal iron, alumina hydroxide cream, phosphotungstic acid and tannic acid. Phosphotungstic acid removed nearly as much, and tannic acid slightly less. Amino acids and peptides were probably left in the filtrate. The following is a table showing the relative value of these reagents as precipitants:

50 c.c. portions of extract of flour (Ceretana)	
Reagent	Nitrogen not precipitated Gms.
1. 5% colloidal iron precipitate at room temperature followed by 1 c.c. of concentrated Mg. SO ₄ sol.	0.00266
2. Same at boiling temperature.	0.00330
3. 10 c.c. of 10% phosphotungstic acid after making strongly acid with HCl.	0.0010
4. Tannic acid.	0.0012
5. Copper method.	0.0008

Morgulis and Jahr (30) used metaphosphoric acid as precipitant, and Folin (31) used this same reagent for blood.

Gettler and Baker (32) used acid mercuric chloride solution for determination of non-protein nitrogen in blood. They gave a table of comparison of their method with others.

<u>Non-Protein Nitrogen</u>	
	<u>Mg. per 100 c.c. of blood</u>
Gettler and Baker (32)	30-45
Folin and Denis (33)	22-37
Greenwald (28)	30
Bang (34)	19-39
Taylor and Hulton (35)	25-28
McLean and Selling (36)	23-44
Myers and Fine (37)	25-30
Hohlweg (38)	40-61

Hiller and Van Slyke (39) made a study of precipitating agents and came to the following conclusions:

Tungstic acid and picric acid precipitate protein intermediary products, leaving amino acids and small peptides. Trichloroacetic acid precipitates only whole protein.

Metaphosphoric acid, colloidal iron and mercuric chloride occupy intermediate position in regard to action on protein products. Alcohol behaves in this case like tungstic acid.

Bigelow and Cook (40) reviewed the literature for the

separation of proteoses and peptones from simpler amino bodies, referring to Stutzer's method (41) with phosphotungstic acid, Mallet's method (42) by precipitation with phosphotungstic acid in hot solutions, Allen and Searle's method (43) by precipitation with bromine, and Schjerning's method (44) by precipitation with a tannin salt solution.

Stutzer believed that his method separated peptones completely from all the simpler amino bodies, but it since has been shown that phosphotungstic acid precipitates some of the meat bases and the reagent is not suitable for the purpose first suggested.

The bromine method of Allen and Searle has been shown to be untrustworthy (45), as it decomposes both proteins and meat bases and liberates nitrogen; also that the amount precipitated under different conditions is not constant (46). Bigelow and Cook think that the tannin salt method is the best for work on meat extracts and made an extensive study of the best conditions for its use.

On working with a protein extract, therefore, the worker must have a complete knowledge of the type of nitrogen present in order to make a clear cut separation of protein, its decomposition products (peptones and proteose), and non-protein nitrogen.

EXPERIMENTAL

Preparation and Analysis of the Meal

Shelled Virginia peanuts were ground as fine as possible in an electric mill and the oil was extracted by treating the meal, first with petroleum ether and then with ethyl ether. Six extractions gave a meal free from oil. The meal was then allowed to dry in the air by spreading it out on a large sheet of paper.

Analysis of Nitrogen Content of Meal

The dry, oil-free meal was next analysed for nitrogen by the Kjeldahl method. This gave 7.36 per cent nitrogen (average). This multiplied by the factor 5.5 gives 40.48 per cent protein in the meal.

	<u>Weight of Meal</u>	<u>Cc. N/10 H₂SO₄</u>	<u>N in Meal</u> Per cent
1.	1.0688 gms.	55.9 c.c.	7.34
2.	1.0721 gms.	56.4 c.c.	7.38

Extraction of Globulins

Fifty grams of meal was treated with one liter of 10 per cent sodium chloride solution and the mixture stirred for three hours. After standing over night in an ice chest, it was filtered through finely pulped filter paper. Another liter of salt solution was used to extract from the residue the last traces of nitrogen as determined by the Kjeldahl analysis. The filtrate was clear with a phosphorescent tinge. The solution was made up to two liters, and two 10 c.c. aliquots were taken for analysis. The total nitrogen extracted was 6.41 per cent (average) or 35.27

per cent protein. This was 86 per cent of the total protein if the total nitrogen in the meal was all protein nitrogen.

<u>Nitrogen Extracted</u> Per cent	<u>Protein Extracted</u> Per cent
6.34	34.87
6.51	35.80
6.45	35.47
6.34	34.87
6.43	35.26

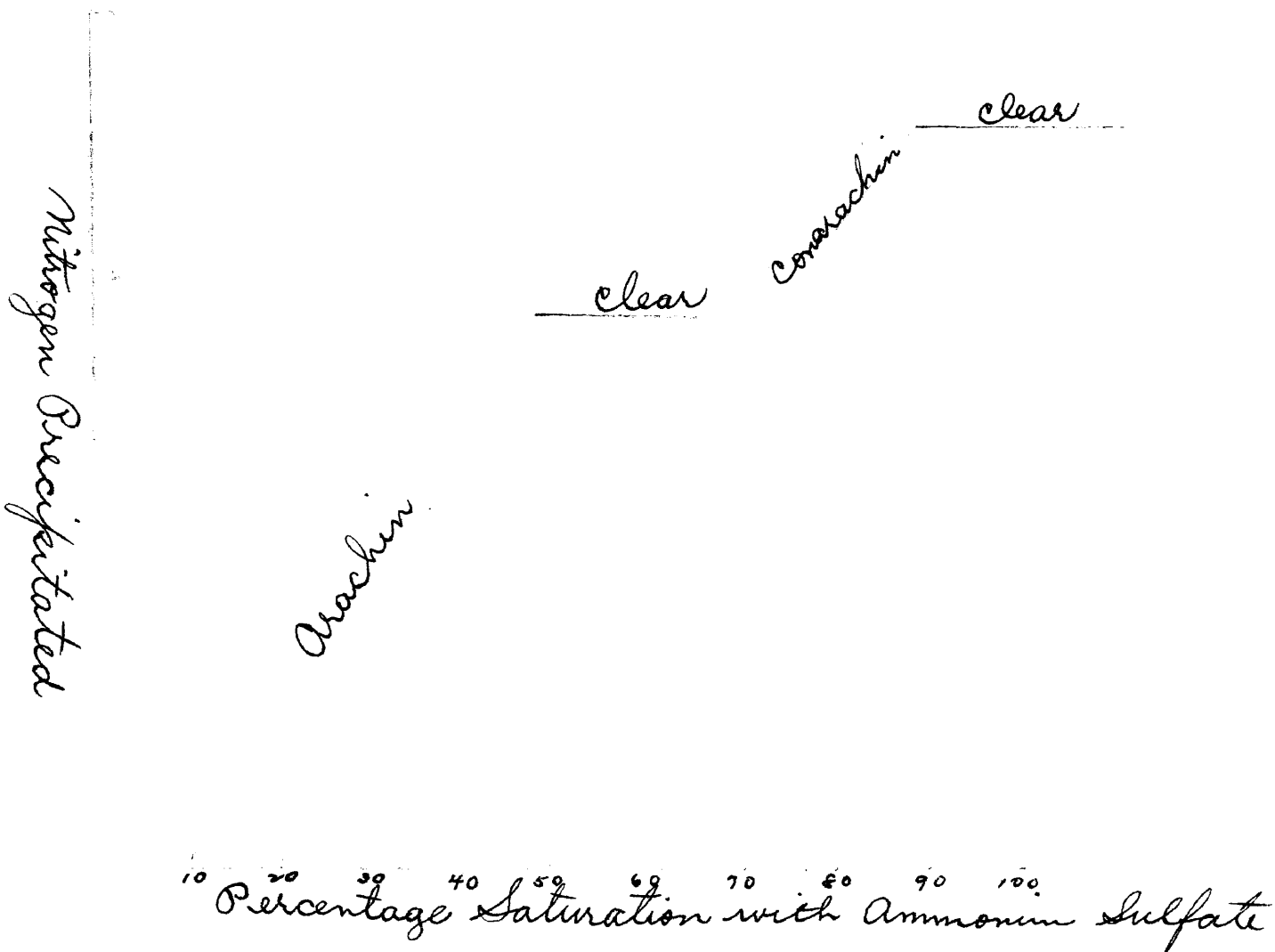
In order to ascertain whether a better extraction could be made with less danger of losses, 50 gms. of the meal was put into a 500 c.c. centrifuge bottle and shaken with a shaking machine for three hours with 250 c.c. of 10 per cent sodium chloride. The suspension was then centrifuged and the supernatant liquid poured off. This procedure was repeated until the salt extract gave no test for nitrogen. The solutions were combined and nitrogen determined on a 10 c.c. aliquot. The amount of protein extracted was practically the same as the first procedure, 35.47 per cent.

Preliminary Coagulation Tests on Extract

Four samples of 10 c.c. each were taken and heated at the rate of 4 degrees per minute. The first coagulation was at 80 degrees. This was filtered and the filtrate heated further. The solution became cloudy at 94 degrees, but there was no coagulation even when heated to boiling at 108 degrees.

Determination of Number of Proteins with Ammonium Sulfate. Solutions of ammonium sulfate were made up varying from 10 per cent to a saturated solution. Ten c.c. of the

extract was taken and enough ammonium sulfate solution added to make it saturated in steps of 10 per cent. After each step the solution was filtered and more ammonium sulfate added in the filtrate. A gradual increase in the precipitate up to 40 per cent of saturation with ammonium sulfate was obtained. Beyond that, no nitrogen was precipitated until the solution was 70 per cent saturated. Nitrogen continued to precipitate up to 80 per cent. At 90 per cent saturation there was no further precipitation, nor was there any more nitrogen precipitated at saturation with ammonium sulfate, the solution remaining clear. The following curve shows plainly that only two proteins were present:



Quantitative Estimation of Arachin

Ammonium Sulfate Method. A quantity of the clear, filtered extract (500 c.c.) was put in a centrifuge bottle and made 40 per cent saturated with ammonium sulfate (152 gms.). After standing for several hours the solution was centrifuged. In this manner the arachin was obtained in a compact mass on the bottom and the supernatant liquid could be poured off. The precipitate was redissolved in 10 per cent sodium chloride, made 40 per cent saturated and again centrifuged. The arachin was then dissolved in as little 10 per cent sodium chloride as possible and precipitated by diluting with 10 volumes of distilled water. In this way it was freed from the last traces of conarachin. The precipitate was dried by suspending in 95 per cent alcohol over night, then in absolute alcohol for 24 hours and finally in absolute ether for 24 hours. After standing in a vacuum desiccator for a day the white material was weighed.

1. Weight 2.85 gms.
2. " 2.84 "
3. " 3.00 "

The total extract from 50 grams of peanut meal was 2 liters. Therefore, the above weights multiplied by four gave the number of grams of arachin in 50 grams of the oil-free peanut meal.

1. $2.85 \times 4 = 11.40$
2. $2.84 \times 4 = 11.36$
3. $3.00 \times 4 = 12.00$

This was equivalent to 22.8 per cent, 22.7 per cent, and 25 per cent, or gave an average of 23.5 per cent. In spite of the greatest care there occurred some losses in purification and these figures may be considered low.

Coagulation of Conarachin. Ten c.c. of the sodium chloride extract was heated slowly to 85 degrees. After standing for fifteen minutes, the coagulum was filtered off on a folded filter paper. The filtration was rapid. The precipitate was washed with 10 per cent sodium chloride and nitrogen determined in the filtrate. As the total nitrogen in the extract, except for an insignificant amount, was all protein nitrogen, the nitrogen calculated was all arachin nitrogen. If

a = amount of arachin nitrogen
 c = amount of unknown nitrogen
 a + c = amount of nitrogen in filtrate

from coagulation of conarachin.

c was then determined by taking 100 c.c. of the sodium chloride extract, treating with trichloroacetic acid, filtering off the precipitate and determining the nitrogen in the filtrate. This value (c) equals .0140 gms. for the 2 liters of extract.

a + c = 2.3 gms. of nitrogen
 c = .0140 gms. of nitrogen
 a = 2.29 gms. of nitrogen
 $\frac{2.29}{18.29} = 12.5$ gms. of arachin in
 the total extract
 18.29 = per cent of nitrogen in arachin

Refractometric Method. The indices were taken with a Carl Zeiss refractometer using Palo light. The following results were obtained:

10 per cent sodium chloride	1.3510
Protein extract	1.3530

The extract was heated carefully to 85 degrees and the conarachin allowed to settle, then centrifuged and after the tube had cooled, a drop of the supernatant liquid was tested for refraction.

Reading after removal of conarachin, 1.3525

$$\begin{aligned}
 1.3530 - 1.3510 &= .0020 \text{ both arachin and conarachin} \\
 1.3525 - 1.3510 &= \frac{.0015}{.0025} \text{ arachin}
 \end{aligned}$$

$$n - n' = ac$$

n = refractive index of the solution.
 n' = refractive index of the solvent.
 c = percentage of globulins in the solution.
 a = constant (0.00236).

$$\frac{.00150}{.00236} = .635\% = 0.635 \text{ gms. arachin in 100 c.c.}$$

$$\frac{.0005}{.00236} = .2118\% = 0.2118 \text{ gms. conarachin in 100 c.c.}$$

Two liters, or the total extract from 50 gms. of meal, would therefore contain 12.70 gms. arachin, and 4.236 gms. conarachin.

Quantitative Estimation of Conarachin

Trichloroacetic Acid Method. A quantity of extract (100 c.c.) was treated with 30.4 gms. of ammonium sulfate and the precipitate filtered off. The filtrate was treated with trichloroacetic until no more conarachin was precipitated. This was removed by filtration and all ammonium salts washed out with water containing trichloroacetic acid. The precipitate and filter paper was then put in a Kjeldahl flask and the nitrogen determined.

Precipitate contained: 0.03933 gms. nitrogen. This was equivalent to 0.7665 gms. of conarachin nitrogen in the original 2 liters of extract, which is equivalent to 4.20 gms. of conarachin, or 8.4 per cent in the oil-free meal.

Indirect Determination (See Theoretical)

The method used here was an elaboration of the method for

obtaining the amount of arachin. Having (a + e) and (e) and letting (b) be equal to the amount of conarachin nitrogen, all that had to be determined was the total nitrogen in the extract (a + b + e).

This was: 3.17 gms.

$$\begin{aligned} a + b + e &= 3.17 \text{ gms. nitrogen} \\ a + e &= 2.3 \text{ gms. nitrogen} \\ b &= .87 \text{ gms. nitrogen} \end{aligned}$$

$$\frac{.87}{.1829} = 4.7 \text{ gms. in 50 gms. meal, or } 9.4\%$$

Method of generation	Arachin		Conarachin	
	Grams in 50 gms. of meal	Per cent in oil-free meal	Grams in 50 gms. of meal	Per cent in oil-free meal
	40 per cent saturation with ammonium sulfate.	11.4 11.4 12.0	22.8 22.0 24.0	
Congulation of conarachin and estimation of arachin in filtrate.	12.5	25.0	4.7	9.4
Refractometric	12.7	25.4	4.2	8.4
Precipitation with trichloroacetic acid.			4.2	8.4
Average	12.0	23.8	4.4	8.7

Due to losses in the actual isolation of arachin, the average was considered low, 25 per cent probably being the nearest to the correct amount in the oil-free meal.

Properties of Arachin

Determination of Coagulation Point. A fresh 10 per cent sodium chloride extract of the peanut meal was used. Arachin was precipitated by making the extract 40 per cent saturated with ammonium sulfate and the mixture filtered. The precipitate was redissolved in 10 per cent sodium chloride and the arachin thrown out as a white flaky mass by pouring the solution in ten times its volume of distilled water. Approximately half a gram of the moist precipitate was dissolved in 20 c.c. of 10 per cent sodium chloride. Ten c.c. portions of the solution were put into two test tubes and heated very slowly with stirring. At 90° C. the solution began to cloud, but no precipitate was obtained up to boiling.

Determination of Specific Rotation. Using a fresh extract, the arachin was purified as above and various concentrations were made up. It was found that polarimetric readings could not be made on solutions having a concentration greater than 4 per cent. The solutions were put in a decimeter tube and the reading taken with a Schmidt and Hanesch polariscope using a standard dichromate filter consisting of a 3 cm. cell containing a 3 per cent solution. The readings were taken in Ventze units and were changed to angular degrees by using the factor 0.34657. The following readings were obtained:

<u>Conc.</u> <u>Gm. per c.c.</u>	<u>Angular Degrees</u>	<u>Specific Rotation</u>
.0307	-1.2129	-39.5
.00880	-0.34657	-39.3
.04085	-1.5595	-38.1
.0205	-0.7971	-38.9
.0192	-0.7971	-41.5
	Average	-39.5

$$\alpha_D^{20} = \frac{\alpha}{l \times W}$$

- α_D = specific rotation.
- l = length of tube.
- W = weight of protein in gms. per c.c.
- α = reading in angular degrees.

Precipitation Limits with Ammonium Sulfate. A fresh 10 per cent sodium chloride solution of arachin was made up containing 2 gms. per 100 c.c. Ten c.c. of the solution was treated with trichloroacetic acid, the mixture filtered, and nitrogen determined in filtrate. No nitrogen could be detected. A series of eight 10 c.c. aliquots were now taken and various amounts of ammonium sulfate added. The precipitates were filtered off and trichloroacetic acid added to the filtrates. The amount of ammonium sulfate which had to be added to give no turbidity in the filtrate with trichloroacetic acid was taken as the point of precipitation of arachin. This was found to be at 40 per cent saturation.

<u>Degree of Saturation</u> <u>with</u> <u>Ammonium Sulfate</u> <u>Per cent</u>	<u>Filtrate treated</u> <u>with</u> <u>Trichloroacetic Acid</u>
10.0 saturated	Large ppt.
20.0 "	" "
30.0 "	" "
32.0 "	Small "
34.0 "	Cloudy
36.0 "	"
38.0 "	Slightly cloudy
40.0 "	Clear

Properties of Conarachin

Coagulation Temperature. A fresh sodium chloride extract was made 40 per cent saturated with ammonium sulfate and the arachin filtered off. The filtrate was dialyzed for twenty days in a parchment bag treated with toluene until only a trace of electrolyte was present, as shown by treating the dialysate with barium chloride plus a few drops of dilute hydrochloric acid. The clear liquid was decanted off and the conarachin scraped off. Approximately one gram was dissolved in 40 c.c. of 10 per cent sodium chloride and 10 c.c. aliquots used. The solution was heated slowly with stirring until the conarachin flocked. At 80° flocks appeared which were filtered off, and the filtrate heated further. The filtrate remained clear up to boiling.

Specific Rotation. In order to obtain a large quantity of conarachin, 500 gms. of the oil-free peanut meal was extracted with 2 liters of 10 per cent sodium chloride. By making the solution 40 per cent saturated and filtering, the arachin was removed. Enough ammonium sulfate was added to the clear filtrate to make it 85 per cent saturated and conarachin was precipitated. This was filtered and washed carefully with water. Although a large quantity of conarachin was lost in this process a few grams were obtained free from ammonium sulfate. The fresh conarachin was dissolved in 10 per cent sodium chloride and the optical rotation taken.

Conc. Gms. per c.c.	<u>Angular Degrees</u>	<u>Specific Rotation</u>
.00898	-.38122	-42.4°
.00449	-.19330	-43.0°
	Average	-42.7°

Precipitation Limits with Ammonium Sulfate. A fresh sodium chloride solution of conarachin was made up containing 2 gms. per 100 c.c. Ten c.c. of the solution was treated with trichloroacetic acid, the precipitate was filtered off, and nitrogen determined in filtrate. No nitrogen could be detected. A series of eight 10 c.c. aliquots were taken and various amounts of ammonium sulfate added. The precipitate was filtered off and trichloroacetic acid added to the filtrate. The amount of ammonium sulfate which had to be added to give no turbidity in the filtrate with trichloroacetic was taken as the point of precipitation of conarachin. This was found to be at 85 per cent saturation.

<u>Ammonium Sulfate</u> Per cent	<u>Filtrate treated with</u> <u>Trichloroacetic Acid</u>
10.0	Large ppt.
20.0	" "
30.0	" "
40.0	" "
50.0	" "
60.0	" "
70.0	" "
80.0	Cloudy
81.0	"
82.0	"
83.0	Slightly cloudy
84.0	" "
85.0	Clear

Determination of Non-Protein Nitrogen

Precipitation of Intact Protein. Freshly prepared arachin was purified several times in the manner described and approximately 3 gms. was dissolved in 10 per cent sodium chloride solution. The volume was made up to 100 c.c. in a volumetric flask. Nitrogen was determined on a 5 c.c. aliquot. Three more 5 c.c. aliquots were taken and 20 c.c. of 10 per cent sodium chloride added to each aliquot in order to facilitate filtering. One aliquot was treated with 16 per cent trichloroacetic acid until no further precipitation occurred, the second treated similarly with 10 per cent aqueous tannic acid, the third with a mixture of equal amounts of sodium tungstate and two-thirds normal sulfuric acid. The resulting precipitates were filtered off and washed with 10 per cent sodium chloride containing a few drops of the precipitating agent. Nitrogen was determined on the clear filtrates.

The following table shows that these three reagents precipitate practically all the intact protein:

Total arachin nitrogen in 5 c.c. =	.02527 gms.
Nitrogen in trichloroacetic acid filtrate =	.00028 gms.
Nitrogen in tannic acid filtrate =	.0002 gms.
Nitrogen in tungstic acid filtrate =	.0003 gms.

In order to see whether the above precipitants would also precipitate free amino acids, the following experiment was carried out:

A mixture of amino acids was prepared from 50 gms. of the α -globulin of tomato seed, treated with 100 c.c. of concentrated hydrochloric acid and 100 c.c. of water. This was heated on a

steam bath until all the solid material was in solution, then refluxed for 60 hours until it no longer gave the Biuret test. The solution was then distilled under reduced pressure until a thick syrup was obtained. The residue was redissolved in water and the solution evaporated again in order to remove as much of the hydrochloric acid as possible. The residual sirup still containing hydrochloric acid, was dissolved in a little water and neutralized with sodium hydroxide. The ammonia nitrogen was removed by making the solution slightly acid with hydrochloric acid. An equal volume of 95 per cent alcohol was added and then a 10 per cent suspension of calcium hydroxide until all the humin was thrown down and the solution remained clear. The whole solution was then distilled at 40° in vacuo until about one-half the volume had distilled over. The concentrated solution was made slightly acid and then warmed with charcoal on the steam bath for an hour. The mixture was filtered and the filtrate made up to 500 c.c. The amount of sodium chloride in the solution was determined and found to be 10 gms. per 100 c.c. On standing over night tyrosine precipitated out. This was filtered off.

Solutions were then made up of 5 c.c. of arachin solution and 5 c.c. of amine acid solution, and treated with trichloroacetic acid, tannic acid, and tungstic acid. The precipitates were filtered off, and nitrogen determined in the filtrate.

The following tables show that the intact protein was

precipitated practically complete in the presence of amino acids by these reagents:

5 c.c. of the arachin solution contained	.0171gN
5 c.c. of the amino acid solution contained	.0571gN
Nitrogen in filtrate (trichloroacetic acid)	.0542gN
Nitrogen in filtrate (tannic acid)	.0543gN
Nitrogen in filtrate (tungstic acid)	.0583gN

This experiment was repeated with 1/2 c.c. of amino acids and 5 c.c. of arachin solution.

Results

Nitrogen in 1/2 c.c. of amino acids	.0053 gms.
Nitrogen in 5 c.c. of arachin solution	.0171 gms.
Nitrogen in filtrate (trichloroacetic acid)	.0052 gms.
Nitrogen in filtrate (tannic acid)	.0057 gms.
Nitrogen in filtrate (tungstic acid)	.0054 gms.

Precipitation of Peptides and Peptones. In order to get a solution of intermediate decomposition products a bacteriologic peptone, manufactured by Parke, Davis and Company, was used. Ten grams of the peptone was dissolved in 10 per cent sodium chloride and made up to 100 c.c.

Five c.c. aliquots were taken and precipitated with tannic acid and trichloroacetic acid, filtered, and nitrogen determined in filtrate.

Nitrogen in 5 c.c. of peptone solution	.0258 gms.
Nitrogen in filtrate (tannic acid)	.0119 gms.
Nitrogen in filtrate (trichloroacetic acid)	.0258 gms.

The above results show the difference in the amount of peptone-

peptide nitrogen precipitated by tannic acid and trichloroacetic acid. Trichloroacetic acid does not precipitate any of this nitrogen.

Precipitation of a Mixture of Peptone and Protein. One-half c.c. of peptone solution and 5 c.c. of arachin were used and precipitated with trichloroacetic acid, tannic acid, and tungstic acid. The precipitate was filtered off and nitrogen determined in filtrate.

The following table shows that intact protein can be precipitated quantitatively from solution by trichloroacetic acid even when mixed with peptones, tannic acid and tungstic acid being unsatisfactory for this purpose:

Results

Nitrogen in 1/2 c.c. peptone	.00253 gms.
Nitrogen in 5 c.c. arachin	.0171 gms.
Nitrogen left in filtrate (trichloroacetic acid)	.00253 gms.
Nitrogen left in filtrate (tannic acid)	.00154 gms.
Nitrogen left in filtrate (tungstic acid)	.00140 gms.

Relative Value of Precipitating Agents with Peanut Extract. A fresh extract (25 c.c.) was treated with 2 c.c. of the sodium tungstate and sulfuric acid mixture and the precipitate filtered off. Equal amounts of the filtrate were then put in four test tubes and treated with the following reagents until precipitation was complete:

1. Tannic acid (10% aqueous solution).
2. Sulphosalicylic acid (10% aqueous solution).
3. Picric acid - saturated solution.
4. Trichloroacetic acid (16%).

Results

1. Tannic acid and tungstic acid gave a precipitate, a very copious one in the presence of sodium chloride.

2. Sulphosalicylic acid gave no precipitate.

3. Picric acid gave no precipitate.

4. Trichloroacetic acid gave no precipitate.

It was desired here to get an idea of the amount of nitrogen removed from solution by other reagents after precipitation with tungstic acid. It was found that on adding tannic acid to tungstic acid without the presence of protein a precipitate is formed which becomes larger on addition of more sodium chloride solution. Therefore this result could not be used. Sulphosalicylic acid, picric acid and trichloroacetic acid could remove no more nitrogen after precipitation with tungstic acid.

Another 25 c.c. of the peanut extracted was first treated with sulphosalicylic acid and filtered. The filtrate was put in five test tubes and treated with:

Tannic acid.
Trichloroacetic acid.
Picric acid.
Tungstic acid.
More sulphosalicylic acid.

Results

Tannic acid gave a large precipitate.

Trichloroacetic acid gave a trace of protein.

Picric acid gave a trace.

Tungstic acid gave a large precipitate.

More sulphosalicylic acid gave no further precipitate.

Here a better idea of the relative value of these precipitants is obtained. Tannic acid and tungstic acid precipitate much more nitrogen than sulphosalicylic, trichloroacetic acid and picric acid.

Quantitative Estimation of Nitrogen left in Filtrate by Various Precipitating Agents on Peanut Extract. 25 c.c. of peanut extract was treated with:

1. 10% tannic acid.
2. 16% trichloroacetic acid.
3. Saturated solution of sodium tungstate and 2/3 N sulfuric acid.
4. 10% sulphosalicylic acid.

The solutions were filtered and washed, and nitrogen determined on the clear filtrates.

Results

Total nitrogen in 25 c.c. of extract	.04115 gms.
1. Filtrate from tannic acid	.00000 gms.
2. Filtrate from trichloroacetic acid	.00940 gms.
3. Filtrate from tungstic acid	.00660 gms.
4. Filtrate from sulphosalicylic acid	.01053 gms.

As the above table did not give results consistent with the data obtained in previous experiments when precipitating with trichloroacetic acid, it was desirable to know just what caused the difference. It was found that an extract over two days' old showed a breaking down of the whole protein, even on standing in an ice chest and covered with toluene. A fresh extract of 2000 c.c. only contained .014 gms. nitrogen not precipitated by trichloroacetic acid. Tannic acid left no nitrogen in solution and tungstic acid

only a trace.

From the above results, it appears that the fresh sodium chloride extract of the oil-free peanut meal contains no non-protein nitrogen, all of the nitrogen, except for a very small amount, being in the intact protein.

Examination of Peanut Meal for other Proteins than Arachin and Conarachin

Examination for Prolamin. Fifty gms. of meal was stirred with approximately 1 liter of 65 per cent alcohol for four hours, and the mixture filtered. The clear solution was made up to 1 liter and nitrogen determined on a 100 c.c. aliquot.

Results

100 c.c. gave 0.005616 gms. nitrogen
or 1.5% of the total nitrogen in
the meal.

The alcohol solution was evaporated to dryness and a hard yellowish substance was obtained. This was dried with alcohol and ether, and analyzed for nitrogen.

0.4529 gms. sample gave 0.00238 gms.
nitrogen, or 0.5% nitrogen in the
substance.

This compound had a sweet taste and an odor like molasses, giving a strong Molisch test. It was probably a carbohydrate and was not investigated further. The nitrogen removed by the alcohol gave no indication that it was prolamin nitrogen.

Examination for Glutelin. The peanut meal after having been exhaustively extracted with 10 per cent sodium chloride was washed and 100 gms. treated with 0.2 per cent aqueous sodium hydroxide for eight hours with stirring. The mixture was centrifuged, the super-

natant liquid filtered, and made up to definite volume. Nitrogen determination made on this extract showed that 4 per cent of the total nitrogen in the original oil-free meal was extracted by the alkali. When this alkaline extract was slightly acidified with acetic acid a brown material separated. This substance when dry was found to contain 9.52 per cent nitrogen. The character and properties of this substance were not those generally characteristic of a glutelin.

By varying the time of exposure of the meal to alkali various percentages of nitrogen were extracted amounting to from 2 to 6 per cent of the total nitrogen of the original meal.

Effect of Tannin in the Red Skins
on Extractions of Proteins

Extraction with Salt Solution. The red skins were removed by slightly moistening the shelled peanuts with water and slipping off the skins. The peanuts were then ground and the oil extracted, leaving a fine white meal. This meal was dried and 50 gms. was exhaustively extracted with 10 per cent salt solution. Nitrogen was determined on the filtered extract, and the results showed quite conclusively that the tannin had no effect on the extraction of the globulins.

SUMMARY

Oil-free meal obtained by ether extraction of finely ground shelled Virginia peanuts was found to contain 7.36 per cent nitrogen, equivalent to 40.48 per cent crude protein ($N \times 5.5$).

Ten per cent sodium chloride solution extracted from the oil-free meal 6.41 per cent nitrogen, or 35.27 per cent crude protein.

Examination of the peanut meal showed the presence of only two globulins, arachin and conarachin. Quantitative estimations by three different methods of the relative amounts in which these globulins were present showed that arachin constituted 25 per cent and conarachin 8 per cent of the oil-free meal.

Arachin in 10 per cent sodium chloride does not coagulate even at boiling temperature of the saline solution; it has a specific rotation, $(\alpha)_{20}^D = -39.5$. It precipitates completely from its sodium chloride solution at 40 per cent saturation with ammonium sulfate.

Conarachin coagulates at 80° C.; has a specific rotation, $(\alpha)_{20}^D = -42.7^\circ$, and is precipitated at 85 per cent saturation with ammonium sulfate.

Practically all of the nitrogen extracted from a fresh 10 per cent sodium chloride extract of peanut meal is precipitated by trichloroacetic acid, tannic acid, and tungstic acid, showing that the extract contained little or no nitrogen other than that of arachin or conarachin.

The tannin in the red skins of the peanuts has no significant

effect on the extraction of the globulins.

An extensive study was made of different methods employed for the precipitation and differentiation of the intact protein molecule and its degradation products.

Peanuts were examined for the presence of albumins, prolamins and glutelins. No evidence was obtained to show the presence of these classes of proteins in significant amounts.

The use of the refractometer for the quantitative estimation of arachin and conarachin gave results closely agreeing with those obtained by other methods.

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