STUDIES OF THE PROTEINS OF THE TUNG NUT (*A. FORDII*)

by Hui-Lan Yeh

Two protein fractions, globulin and albumin, were isolated from defatted tung nut meal (*Aleurites fordii*, grown in Louisiana). "Globulin" is defined as that fraction of the sodium chloride extract (10 per cent sodium chloride) which was precipitated on removal of the salt by dialysis. "Albumin" is defined as that fraction which was coagulated by heat at approximately neutral reaction after the removal of the globulin.

The average nitrogen content of the globulin and albumin fractions were 17.5 per cent and 14.3 per cent, and the sulfur contents were 1.75 per cent and 0.79 per cent, respectively.

Nine amino acids (threonine, serine, cystine, methionine, arginine, tryptophane, tyrosine, histidine, phenylalanine) were determined in the acid hydrolysates of these two protein fractions. The nitrogen present in these acids totaled 44 and 35 per cent of the total nitrogen of the globulin and albumin, respectively. The methionine and cystine sulfur accounted for 97 per cent of the total sulfur of the globulin and for 101 per cent in the case of the albumin. The percentage of arginine in the globulin hydrolysate was high (approximately 25 per cent of the total nitrogen), a value similar to that previously observed in arachin, the globulin of the peanut. The analyses of the albumin fraction were distinctly different from those of the globulin.

(Yeh--thesis abstract for microfilming--contd):

The digestibilities of these proteins *in vitro* by the gastro-intestinal enzymes were slightly lower than those of many of the seed proteins, i.e., globulin 82.4 per cent and albumin 73.4 per cent.

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INTRODUCTION

The tung nut is commonly known in the United States as a natural source of oil for the manufacturing of varnishes, paints, soaps, linoleum, oil cloth, etc. In China it is extensively used to water-proof paper, cloth, and masonry, as well as for varnishes. It is the oil seed of the tropical tree called tung, a romanized Chinese name. The species introduced into the southern states of this country by the United States government from the Orient is scientifically denoted as Aleurites fordii, which belongs to the natural order Euphorbiaceae. The tung trees are usually grown on dry, thin soil not suited to general farming. They rarely exceed 25 feet in height. Each tree yields about 100 pounds of fruits, each of which contains three to four seeds. The nuts studied in this laboratory were supplied by the Department of Agriculture of the United States.

These nuts are not only rich in oil but also have a very high content of protein as do most nuts. However, because of their toxic nature, these nuts have not been used for any other purpose except as a source of oil. Usually, after the oil is pressed out from the nuts, the press-cake is either used as fertilizer or is burned. It

seems to be rather a great waste. If only more were known about the chemical nature of the nuts, a useful outlet for the residual material could certainly be found.

The present investigation is undertaken mainly to study some of the chemical properties of the principal proteins isolated from the nuts, to see how they compare with other known proteins as to:

(1) content of some amino acids

(2) digestibility by proteolytic enzymes *in vitro*.

The toxicity of the defatted nut meal was confirmed in this study, but no attempt was made to characterize the specific toxic principle. Many plant seeds are known to be toxic, and the toxic principles of many of them are known to belong to a group of substances called toxalbumins. Ricin from *Ricinus communis*, curcin from *Jatropha curcas*, and crotin from *Croton tiglium*, are some examples. Upon heating to a temperature of 60° or above, the toxicity of most of the toxalbumins is destroyed. Tung nuts are also found to be rendered non-toxic by heating. Whether the toxicity has any relationship with the water-soluble, and heat-coagulable proteins or not is a question worthy of investigation.

HISTORICAL

The toxic nature of tung nuts has been known for a long time. It probably has been known to the natives of the tung tree growing countries long before there is any record in the literature. In 1907, Holmes (1) reported that Kaloo nut (A. fordii) was poisonous having produced toxic symptoms in five children. Palmer (2) in 1933 reported many cases found in Florida. He recorded that the nuts when eaten produced a feeling of discomfort, warmth, and nausea followed by vomiting, violent cramps, pains, etc., and under some conditions might prove fatal. He suspected that the toxin might be in the oil. However, he recorded that Dr. Rivers had suggested that the toxin might be something similar to the ricin of the castor bean.

Erickson and Brown (3) reviewed Carratala's description of the symptoms of five workers who had eaten the nuts. In the same article, they reported a case of a student in the Louisiana State University who mistook a tung nut for a Brazil nut. They gave the following description:

> An hour later he felt dizzy and weak and had severe pains in the stomach. Violent vomiting, accompanied by diarrhea, set in immediately, and recurred at short intervals. Attempts to satisfy an unusual thirst seemed to aggravate this condition, which lasted for 6 or 7 hours, after which he was very weak. On the following day he had

a headache throughout the morning, but there was no pain. By the third day he had completely recovered.

There are few experimental studies of this toxic property. In an attempt to find some use for the press-cake obtained when most of the oil has been pressed out from the nuts, Godden (4) found that the nuts are unpalatable, and they contain some irritant material which has a harmful effect on the mucous membrane of the intestine. Rats, birds, dairy cattle and pigs, all refused to eat the rations containing the tung nut meal. Pigs were finally made to eat a ration containing 2.5 per cent nut meal, and some pigs were put on rations containing 5 and 10 per cent nut meal. All pigs had diarrhea, and became weak. Histological examination of livers, kidneys and intestines of the pigs fed on the ration containing 2.5 per cent nut meal showed some evidence of damage to the parenchyma of both liver and kidney. The mesenteric glands were slightly congested. The pathological findings were more marked in the animals receiving the diets containing larger percentages of the nut meal, and in addition there was some evidence of gastritis.

Gardner (5) reported that the toxic principle of tung nuts was not associated with the oil. His investigation was not extended to other parts of the tung nut. Erickson and Brown (3) showed definitely that the toxic principle was not in the oil but in the tung nut meal which had been

extracted with petroleum ether. The toxic principle was destroyed by heat, either hot air or steam. However, steam heat was more effective in this respect. Heating with steam at 230° F. for 2 hours rendered the oil-free meal non-toxic to rats; but when the heating was at 212° F. in an oven, a period of 15 hours was necessary to give the same effect. Alcohol could not extract the toxic principle. The press-cake obtained from the tung oil industry was found to be non-toxic. The toxic principle must have been destroyed as the press-cake was obtained from ground kernels which had been steamed before pressing.

The nature of the toxic principle is still unknown. Erickson and Brown (3) attempted to isolate the toxic material but were unsuccessful. Many toxalbumins have been isolated from plant seeds. Many of the toxins in plants are also heat labile such as gossypol in cotton seeds, curcin in Jatropha curcas and so forth.

Much work has been done on the tung oil, but little is known about the rest of the nut. Godden (4) reported some analytical results of the chemical nature of the extracted nuts and also on the extracted press-cake. Like most nuts, tung nuts have a very high protein content. The results obtained by Godden are presented in Table I.

Table I

Chemical Analyses Taken from Godden (4)

Sample A is the material obtained by direct extraction with benzine of the nuts and Sample B resulted from the benzine extraction of the press-cake.

	Sample A	Sample B
	per cent	per cent
Moisture	9.53	10.47
Crude protein	28.12	32.19
Ether extract	1.58	0.57
Crude fibre	19.93	22.04
Total ash	5.07	5.73
Soluble carbohydrate (by difference)	35.77	29.00
Acid insoluble ash	0.23	0.27
Lime (CaO)	0.70	0.83
Soda (Na_2O)	0.066	0.066
Potash (K_2O)	1.27	1.50
Phosphoric acid (P_2O_5)	1.51	1.82
Chlorine	0.029	0.035
Iron	0.019	0.016
Iron containing nitrogen	4.5	5.15

EXPERIMENTAL

Toxicity of the Extracted Nut Meal

In order to confirm the earlier findings on the toxicity of the nuts (3) (4), a feeding experiment was carried out. Four male rats were put on a diet containing 20 per cent oil-free nut meal. The composition of the diet is given in the appendix. In addition, all rats were given half of a yeast tablet daily. A daily food consumption record was kept. Rats were weighed once a week. The record is summarized in Table II.

Table II

Rat number	Initial weight	Final weight	Weight lost during the period	Average daily food consumption	Number of days of survival
	gm.	gm.	gm.	gm.	days
1	80.5	41.5	39.0	3.7	20
2	59.0	38.7	20.7	2.8	15
3	80.0	48.0	32.0	2.7	13
4	68.0	40.0	28.0	2.7	14

On the second day of the experiment, a slight purgative effect was noted on two of the rats. After two days all rats seemed to be sluggish and weak. At the end of one week all rats showed definite signs of a purgative effect. The diarrhea in one of the rats was so bad that the hair around the anus was completely removed. Godden (4) considered that the diarrhea was caused by some irritant material in the meal. Erickson and Brown (3) reported definite purgative effect of the nut kernel on rats.

The rats in this experiment survived from 13 to 20 days. They refused to eat and scattered food. The average initial weight of the rats was 72 gm. and they lost on the average 30 gm. in 15 days. The average total food intake in the entire period was 45 gm. The rats in Erickson and Brown's experiment survived only three days. Each of their rats was given 15 gm. of the ration containing 19.5 per cent of the oil-free nut meal. At the end of 62 hours, 2 out of 5 of their rats were found dead. Since their rats ate more of the tung nut meal per day, the toxic effect was shown more strikingly in their experiment.

On autopsy, these rats showed no peculiarity except that the intestines were enlarged and full of gas. Godden (4) found that pigs fed on a ration containing more than 2.5 per cent tung nut meal showed, on post-morten examination, some evidence of gastritis with small bloody spots in both stomach and small intestine; but pigs which received a

ration containing 2.5 per cent nut meal did not show any abnormality in their gastro-intestinal tract. The rats in this experiment probably did not have enough tung nut meal to cause such changes.

Livers and kidneys were removed immediately after the rats were found dead, and preserved in a 3 per cent formaldehyde solution. These were sent to the Department of Pathology* for histological examination. The following results were reported:

Rat #1 5020-LAV. Liver: Acute passive congestion. Slight cloudy swelling. No lipoidosis. Kidneys: Acute passive congestion. Cloudy swelling of slight degree. No lipoidosis.

Rat #3 4846-LAV. Liver: Acute passive congestion. Cloudy swelling. Slight degenerative fatty infiltration confirmed by fat stain on frozen section. Kidneys: Postmortem changes. No lipoidosis. Adrenal: Postmortem change.

Rat #4 4847-LAV. Liver: Acute passive congestion. Multiple small foci of necrosis with early inflammatory reaction. (These are not unlike the primary focal necrosis of tuberculosis.) In the frozen section these foci show a fine

* The histological examinations were made by Dr. Carl V. Weller of the Department of Pathology, to whom we wish to express our appreciation.

stippling of deposited lipids. No other lipoidosis. Kidneys: Acute passive congestion. No lipoidosis.

Godden (4) found that pigs fed on a ration containing 2.5 per cent nut meal showed damage in the parenchyma of both liver and kidney, and that these pathological findings were more marked in the animals receiving the larger percentages of the meal.

In conclusion, we may say that the oil-free nut meal is unpalatable and has a purgative effect on rats. No definite conclusion can be made on the post-mortem findings, and the histological observations.

Isolation of the Main Protein Fractions from the Tung Nuts

Proteins were prepared by the extraction of the defatted tung nut meal with 10 per cent sodium chloride solution, and by further fractionation of the extract in the usual manner.

Preparation of Defatted Tung Nut Meal

The nuts as received were in the form of dried fruits. The outer coverings (pericarp) of the dry fruits were fibrous but rather hard shells. Each fruit had from 3 to 5 seeds (nuts). The seeds looked more or less like Brazil nuts. They were somewhat triangular in outline, with

rounded edges, averaging 1 inch long, 3/4 inch broad, and 1/2 inch in thickness, slightly convex on one side, and more or less sloping on the other to a central ridge. The outer seed coat (testa), about 1/24 of an inch in thickness, was hard. The inner seed coat (tegmen) was membranous and had a slightly tan color. The kernel was creamy white. It had a slightly acrid taste.

Soon after the nuts arrived, they were hulled. The nut meat was ground into small pieces through a coarse meat chopper, and then extracted with gasoline according to the following procedure.

(1) Extracted with gasoline twice, in a percolator, assembled as shown in Figure 1.

(2) The nut meal was ground again through a finer grinder. It was put back into the same percolator but with a fresh filter paper and cotton, and extracted three more times with gasoline.

(3) The partly defatted nut meat was ground once more through a still finer grinder, and the extraction was repeated twice.

(4) The resultant nut meal was air dried by spreading it out on a piece of paper, and sieved through a 20 mesh sieve.

This nut meal was not absolutely free of oil, and had a slightly tan color.

One year later another lot of tung nuts was obtained.

Apparatus for Percolation of Tung Nut Meal with Gasoline

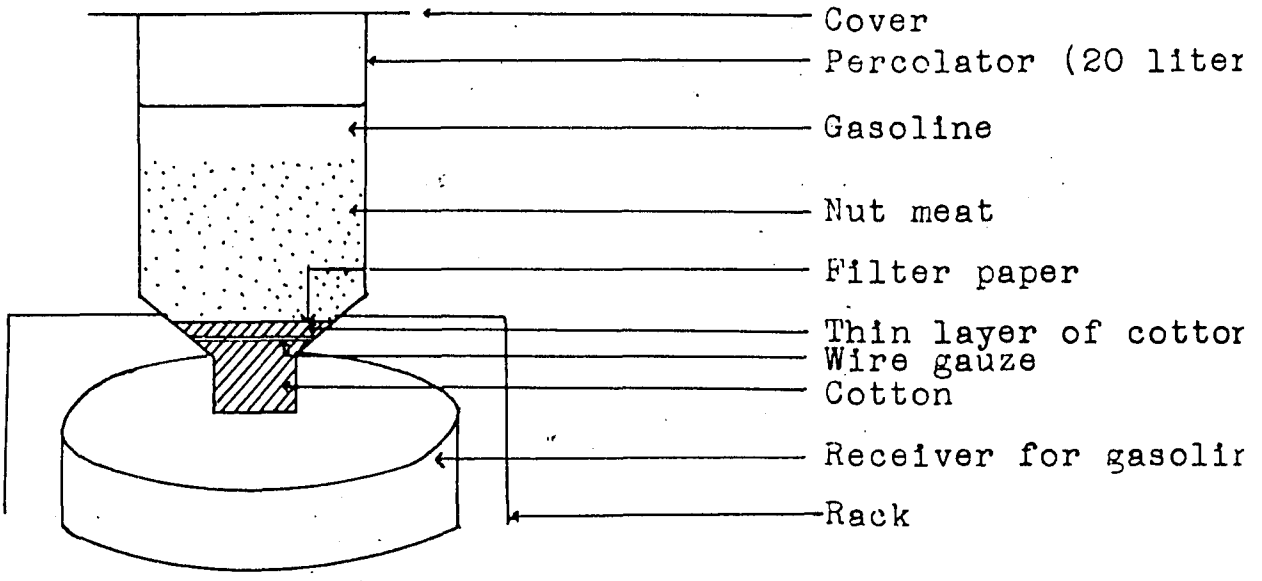


Figure 1

They were treated in exactly the same way. The meals from these two lots of nuts were analyzed for moisture, ash, nitrogen, and sulfur. Moisture was determined by heating the nut meal at 102° to a constant weight. The dried sample was then ashed in a muffle furnace at 550°. Nitrogen was determined by the macro Kjeldahl method using boric acid to receive the ammonia. Sulfur was determined by the Parr bomb method. The results are given in Table III.

Table III

These defatted nut meals are referred to as nut meal in later discussions.

Defatted tung nut meal	Moisture	Ash	Nitrogen		Sulfur	
			Water-free basis	Water-ash-free basis	Water-free basis	Water-ash-free basis
	per cent	per cent	per cent	per cent	per cent	per cent
First lot	9.08	5.86	5.88	6.25	0.55	0.58
Second lot	8.37	7.52	6.48	6.98	0.57	0.62

If the factor 6.25 be used for the calculation of protein content the nitrogen analyses would indicate a content of approximately 39.1 and 43.6 per cent of protein in extracted tung nut meal (calculated on the ash-free and moisture-free basis). The nitrogen to sulfur ratios similarly calculated are 10.8 and 11.3 respectively.

Extraction of Proteins

1. Total Nitrogenous Material Extractable by 10 Per Cent Sodium Chloride Solution.

In order to find a salt solution suitable to extract the nitrogenous material from the nut meal under optimum conditions, the following solutions, 0.5 per cent sodium carbonate, 1.0 per cent sodium carbonate, 10 per cent sodium chloride, and 0.5 N sodium benzoate, were tried. The procedure used was as follows:

Duplicates were run at the same time. An aliquot of 100 ml. of the solution to be studied was measured out to be used for the extraction. Exactly 3 gm. of nut meal were introduced into a 50 ml. centrifuge tube. About 20 to 30 ml. of the solution from the aliquot mentioned above were added and stirred well until a thin paste was formed. The tubes were incubated at 30°, with occasional stirring, for an hour. The extract was separated from the meal by centrifugation, and decanted into a 100 ml. beaker. This process was repeated but the extraction continued for only half of an hour. This extract was added to the solution obtained from the first extraction. Again the extraction was repeated, but for only a quarter of an hour. Finally the residual meal was washed with the rest of the solution. The washing was also added to the other three extracts. The combined extracts were filtered through a 1.5 inch

layer of practically dried paper pulp previously washed with the solution being studied. The filtrate was measured and aliquots were taken for nitrogen determination. The amount of extractable nitrogenous material as determined here was not quantitative, but it served at least the purpose of comparing the relative efficiency of different solvents. The result indicated that 1.0 per cent sodium carbonate extracted only slightly larger amount of nitrogenous material than 0.5 per cent sodium carbonate, 10 per cent sodium chloride, or 0.5 N sodium benzoate solution. Furthermore, since sodium carbonate is strongly alkaline, it was preferable to use 10 per cent sodium chloride or 0.5 N sodium benzoate solution to extract proteins from the nut meal.

The absolute quantity that could be extracted by 10 per cent sodium chloride or 0.5 N sodium benzoate was studied. The nut meal used here was 80 mesh instead of 20 mesh. In order to get around the difficulty encountered in using paper pulp for filtering the extract, a means of flocculating the non-protein material suspended in the extract was necessary.

It was noted that when the extract was made alkaline to a pH of about 10 with a few drops of 50 per cent potassium hydroxide solution, the suspended particles flocculated out and could be separated easily by centrifugation.

Three gm. of 80 mesh nut meal were extracted with

100 ml. of 10 per cent sodium chloride solution, in the same way as mentioned in the previous experiment. The procedure used in the experiment is also given in the form of a flow sheet in the appendix. To the resultant extract, 50 per cent potassium hydroxide was added dropwise, until maximum flocculation was observed. The point of maximum flocculation had a pH of about 10. The precipitate was removed by centrifugation, and washed once with 10 per cent sodium chloride solution.* The resultant solution was filtered through a Whatman #41 filter paper and the paper was washed three times with 10 per cent sodium chloride solution.* The filtrate was diluted to 100 ml., and 10 ml. of it were taken for nitrogen determination. The precipitate obtained by the addition of potassium hydroxide to the extract of 3 gm. of nut meal contained, on the average, only 0.00375 gm. nitrogen. When calculated on the basis of 100 gm. nut meal, the amount of nitrogen carried down in the precipitate by adding potassium hydroxide would be 0.125 gm. or less than 5 per cent of the amount of nitrogen present in the clarified extract. When this quantity was compared with the amount extracted by sodium chloride solution (2.8 gm.) and that originally present in the nut meal (5.88 gm.), it was reasonable to consider that the precipitate was not pure

*Previously adjusted to a pH of about 10.

protein but residual nut meal, which had been extracted rather thoroughly. The nitrogen in the filtrate was considered to signify the amount of nitrogen extractable by this solution with the adopted procedure.

Solution of 0.5 N sodium benzoate was also studied in the same manner. The results of these two extraction experiments are summarized in Table IV.

Table IV

Solutions	Nitrogen extractable per 100 gm. of nut meal
	gm.
10 per cent sodium chloride	2.89
	2.82
	2.68
	2.71
	2.93
	2.91
0.5 N sodium benzoate	2.76
	3.08

According to the results given above, 10 per cent sodium chloride and 0.5 N sodium benzoate were about equally effective in extracting nitrogenous materials from the nut meal. Therefore 10 per cent sodium chloride solution was chosen for the extraction of proteins from the nuts.

The extractions at 30° and 40° C. were compared. The procedure used was exactly the same as that given on the flow sheet. All the previously mentioned extraction

experiments were carried out at 30° C. Results given in Table V indicate that extraction carried out at 40° was not any better than that at 30°.

Table V

Temperature	Nitrogen extracted per 100 gm. nut meal
	gm.
30°	2.83
	2.87
40°	2.89
	2.89

For the purpose of testing whether C.P. sodium chloride would extract more of the nitrogenous material from the nut meal, a 10 per cent solution of C.P. sodium chloride was compared with a 10 per cent solution of ordinary table salt.* The pH of the C.P. sodium chloride solution was found to be 6.7, and that of the common salt solution was 7.0. Due to the high concentration of salt, these pH values were determined colorimetrically. Since both solutions were of practically the same pH, a difference in the reaction of these solutions could not be the factor for any difference in the efficiency of extraction.

*112 gm. "Diamond Crystal Salt" were dissolved in 1000 ml. boiling water. The solution was cooled and filtered through an ordinary qualitative filter paper.

The procedure used was exactly the same as described before. The result given in Table VI seems to indicate that the 10 per cent solution of common salt was essentially as effective in extracting the nitrogenous material as was the 10 per cent solution of C.P. sodium chloride.

Table VI

Solutions	Nitrogen extracted per 100 gm. nut meal
	gm.
10 per cent common salt (average of 6 determinations)	2.82
10 per cent sodium chloride C.P. (average of 2 determinations)	2.85

With the procedure described as summarized on the flow sheet in the appendix, i.e., extraction at 30° with 10 per cent solution of sodium chloride (common salt), 2.82 gm. nitrogen could be extracted from 100 gm. nut meal (80 mesh). The resultant extract was clear and the material in solution showed a point of maximum insolubility at pH 3.92. The maximum insolubility was determined by adding different amounts of hydrochloric acid to 10 ml. of the extract to obtain different degrees of turbidity. The resultant suspensions were allowed to stand over night, and then filtered. The pH values of the filtrates were determined

with a glass electrode. The pH of the solution which would not give any more precipitate or turbidity upon further acidification was taken as the point of maximum insolubility.

2. Procedure for the Extraction of Proteins.

In order to adapt the above findings to the extraction on a larger scale, the following procedure was developed.

150 gm. of nut meal (20 mesh) were placed in a 2000 ml. beaker. 750 ml. of 10 per cent solution of sodium chloride were stirred in until all of the nut meal was wetted well by the solution. This thin paste was incubated at 35°-40°C. with frequent stirring for 1 hour. The extract was separated from the meal by straining the thin paste through muslin. Only a fraction of the paste was strained at a time, for the separation in this way was better than that obtained by straining the whole quantity at once. The extract was covered with a thin layer of toluene. The residual meal was again similarly extracted with 300 ml. of the 10 per cent sodium chloride solution for a half of an hour. The extract was removed in the same way as described in the first extraction. The process was repeated once more with 250 ml. salt solution, and for a quarter of an hour. Finally the residual meal was extracted with 200 ml. of salt solution for 5 minutes. All four extracts

were combined. The combined extracts were centrifuged for about an hour to remove the large particles. The supernatent liquid was then filtered through a filter paper pulp mat, one inch in thickness, which had been saturated with 10 per cent sodium chloride solution. Since the filtration usually took about one day, toluene was added to both the funnel and the receiver, and the filtration was carried out at a temperature of about 5-10°C. Near the end of the filtration it was completed by applying slight suction. The filtered extract obtained by the procedure outlined was usually clear but yellowish. If the filtered extract was not clear, the filtration was repeated.

Separation of the Globulin and Albumin Fractions

"Globulin" is defined as that fraction of the sodium chloride extract which was precipitated on removal of the salt by dialysis. "Albumin" is defined as that fraction remaining after the removal of the globulin which could be coagulated by heat at approximately neutral reaction.

1. The Globulin Fraction.

The globulin fraction was obtained by dialyzing the clear 10 per cent sodium chloride extract of the nut meal. Toluene was used to keep the solution sterile. The dialyzing bags were made by tying two ends of a piece (1 foot) of commercial dialyzing tubing having a diameter of about 3 cm.

(36/32" visking tubing). The bags were floated in 4-liter beakers or reagent bottles full of distilled water. These were cooled in a cold room at about 10-15° C. or by immersing the bottles in running water at 8-10°, when weather permitted. On the first day of dialysis, the water (dialyzate) was changed every 2 hours. Later, the water was changed 4 times a day. The dialysis was expedited by stirring the contents of the bags mechanically and continued for 6 days. Usually the dialyzate was free from chloride at the end of 4 days. The extra period of dialysis was allowed to make sure that the protein was free from salt.

At the end of the dialysis, the bags were opened, and the contents were poured into a beaker. The precipitate was allowed to settle and most of the supernatant liquid was decanted off. The final separation was made by centrifugation. The precipitate was washed with distilled water three or four times. The resultant globulin was redissolved in a 10 per cent solution of sodium chloride. The solution obtained at this stage was usually grayish brown in color. There was usually some insoluble material. This material was removed by centrifugation and the precipitate was washed twice with a small amount of 10 per cent sodium chloride solution. The washings were added to the main solution. The clear solution was then redialyzed in the same way as described above. The precipitate obtained after the second dialysis, when viewed under a microscope, did

not show a definite crystalline form although such a form was suggested.

The precipitate obtained was washed successively, once with water, once with 50 per cent alcohol, once with 70 per cent alcohol, twice with 95 per cent alcohol, once with absolute alcohol, once with a mixture of equal parts of absolute alcohol and ether, and then finally with ether. The washings were removed by centrifugation. The product was air dried and kept at room temperature. The dry product was powdery and practically white.

2. The Albumin Fraction.

After the globulin had been removed from the salt-free extract, the remaining solution contained mainly the water-soluble, heat-coagulable protein, albumin. This solution was filtered. When the filtrate was heated to a temperature of about 50° C., it became slightly turbid, and definitely turbid at 55° C. When it was heated to boiling, the precipitate flocculated, and settled easily, leaving a very clear solution. The precipitate was removed by filtration through a hardened filter paper. Although the filtrate still gave a positive biuret test, no precipitate was formed upon further boiling. The precipitate was washed by boiling with water for a half of an hour and the water was removed by filtration. The washing was repeated twice. Finally the precipitate was dried with

alcohol and ether as described in the preparation of globulin. The dried albumin had a more or less straw color.

Many samples of globulin and albumin were prepared by these procedures. Each of them was analyzed for the content of water, ash, nitrogen, and sulfur by methods mentioned previously. The results are shown in Table VIII given in the "Discussion."

Attempts to Prepare Crystalline Globulin

In general, protein will crystallize more easily from a more concentrated solution or during a slow and undisturbed dialysis. The Sørensen procedure (6) seems to meet these requirements. In this method, the extract is dialyzed and concentrated by reduced pressure at the same time. With the hope of getting some crystalline protein and a greater yield, this more elaborate procedure was carried out.

600 gm. of nut meal were extracted with 10 per cent sodium chloride solution as described in the previous section. For easier manipulation, 600 gm. were divided into 4 lots for extraction. The final extracts were combined. One half of the extract was dialyzed in the usual way, and the other half dialyzed by the Sørensen procedure under reduced pressure. The compositions of the proteins isolated by this procedure are also given in Table VIII.

For dialysis under reduced pressure, the setup shown in Figure 2 was used. This is a modification of Sørensen's original setup, as suggested by Dr. Lila Miller of this laboratory.

Two dialyzing cells (C), each of which was put in a dialyzing bottle containing distilled water, and one reservoir (B) were grouped into one unit. They were placed in a large dish pan packed with ice. Each dialyzing bottle (B) was connected to a water receiver (A). Three such units were employed. All water receivers (A) were connected to a safety trap, manometer, and pressure regulator as shown in Figure 2.

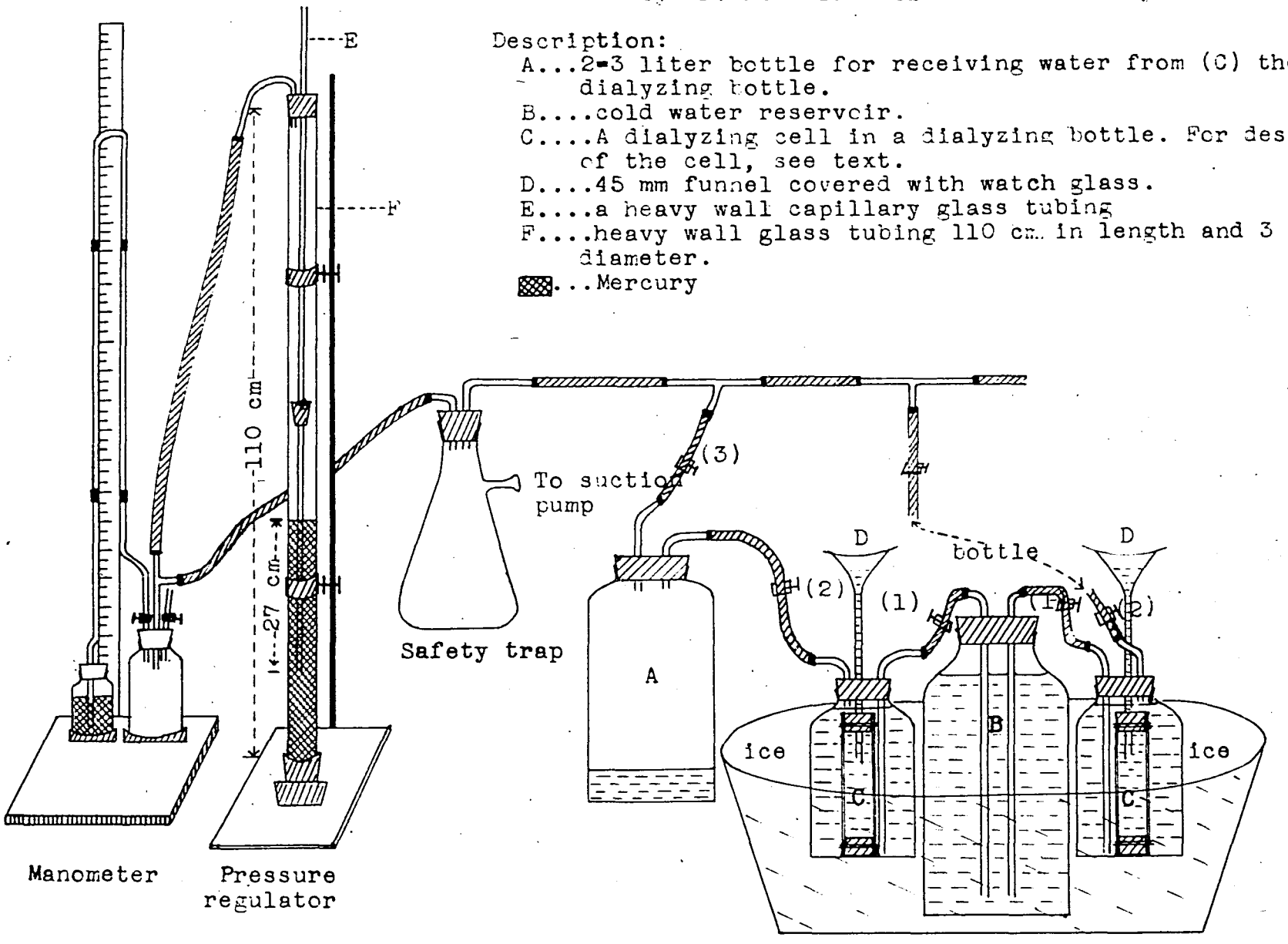
The dialyzing cell was made from the cellophane tubing already described, fitted with #6 rubber stoppers, the lower end with a solid rubber stopper and the upper end with a one-hole rubber stopper. The tubing was fastened onto the stopper with rubber bands. It was found that if two rubber bands were used at each end, each band being stretched to go around the stopper at least five times, a joint tight enough to withstand the pressure used was obtained. The cellophane tubing alone was found by Dr. Miller not to be strong enough to withstand the pressure, so she suggested that a cloth tubing should be fitted over the outside of the cellophane tubing. This worked very nicely. After the cloth tubing was fitted over the cellophane tubing, two more rubber bands were fastened on

APPARATUS FOR DIALYSIS UNDER REDUCED PRESSURE

(from Sørensen's Laboratory and modified by Dr. L. Miller of this Laboratory)

Description:

A...2-3 liter bottle for receiving water from (C) the dialyzing bottle.
B....cold water reservoir.
C....A dialyzing cell in a dialyzing bottle. For description of the cell, see text.
D....45 mm funnel covered with watch glass.
E....a heavy wall capillary glass tubing
F....heavy wall glass tubing 110 cm. in length and 3 cm. in diameter.
...Mercury



each end. The tubing was now tested for leakage by filling it with water and suspending it in the air. If the cell was satisfactory, a funnel (D) already fitted in the rubber stopper of a dialyzing bottle was inserted into the dialyzing cell. The dialyzing cell must be adjusted so that when the stopper of the dialyzing bottle was in place, the solid stopper attached to the cellophane tubing would rest right on the bottom of the dialyzing bottle.

The stopper of a dialyzing bottle had three holes. To one of them, a funnel (D) was inserted. This funnel was also inserted into the rubber stopper of the dialyzing cell as described previously. The dialyzing cell was filled through this funnel. Into the other two holes, two right-angle glass tubings were inserted. One carried water from the reservoir to the dialyzing bottle. This glass tubing should be extended to the bottom of the dialyzing bottle. The other carried dialyzate from the dialyzing bottle to the water receiver (A).

During dialysis, connections between reservoirs and dialyzing bottles were cut off by screw clamps (1), while the others (2) and (3) were opened. The water in the dialyzing bottles was changed at least four times a day by opening these screw clamps (1). When enough water had been drawn through, these clamps (1) were closed again. The water receivers had to be emptied once a day. When this was done all connections were closed. A few crystals

of thymol were put into water receivers so as to prevent putrefaction.

The dialyzing cells (C) were completely filled, and the funnels (D) were partially filled with protein solution, and the solution was covered with toluene. The funnels were refilled from time to time. In order to keep the temperature of dialysis below 10°C., the dish pans were refilled with ice three times a day.

The pressure was kept at about 26-28 cm. Hg. by a water suction pump and a pressure regulator. In general, the pressure appeared to be rather constant at 26 cm.

With this apparatus, it required 25 days to concentrate 2250 ml. nut meal extract down to less than 200 ml. and to dialyze away all of the salts. When the dialyzate in the water receiver gave a negative chloride test, the dialyzing cells were detached from the funnels. The pasty and gummy precipitates from all dialyzing cells were transferred to a small beaker. The cellophane tubings were washed many times with water. The resultant suspension was centrifuged and then washed twice with water by centrifugation. The precipitate represented the globulin fraction and the combined centrifugate and washings theoretically contained all of the nitrogenous materials of the nut meal extract with the exception of the globulin. However, this solution was usually contaminated with globulin. The last trace of globulin in this suspension probably could

be removed by adjusting the solution to the isoelectric point of the globulin, followed by filtration.

A pH of 4.8 was found to be the probable isoelectric point of the globulin, a conclusion drawn from the following experiment. A small amount of the above mentioned centrifugate, which was believed to be contaminated with globulin, was put into a test tube. An approximately 0.1 N hydrochloric acid was added to it dropwise until maximum precipitation took place. This point was found to have a pH of 4.03. The precipitate was centrifuged off. The solution was made alkaline again with 0.1 N sodium hydroxide. Acid was again added dropwise, and at pH 4.88 turbidity was first observed. This pH was taken as the isoelectric point of the globulin.

In order to remove as much globulin from the above mentioned centrifugate as possible, the whole solution was therefore adjusted to pH 4.8, and placed in a refrigerator over night. The precipitate formed was filtered off through an ordinary filter paper. The filtrate was clear though yellowish. The solution was again dialyzed and concentrated by the Sørensen procedure. The albumin fraction in this concentrated solution was precipitated by saturating the solution with ammonium sulfate, and the precipitate was dialyzed against distilled water in the ordinary manner. The salt-free, neutral dialyzed solution was finally coagulated by heating to its boiling point. This

precipitate was dried with alcohol and ether in the usual manner.

The globulin fraction, i.e. the precipitate obtained by dialyzing the extract under reduced pressure, was redissolved in 10 per cent sodium chloride solution. The resultant solution looked not only turbid, but also muddy and grayish in color. Unsuccessful attempts were made to remove the pigmented substances. The solution was finally centrifuged to remove any insoluble material. The supernatant liquid was again dialyzed under reduced pressure. The precipitate obtained, on completion of the dialysis, was still brownish yellow. This was centrifuged off. The precipitate did not show any crystalline property.

Since some of the pigmented impurities was removed from the protein solution in the second dialysis, it may be considered possible that crystalline material might be obtained, if the precipitate were dissolved in a minimum amount of 10 per cent sodium chloride at a slightly elevated temperature, and then cooled slowly. Therefore one-tenth of the precipitate was dissolved in a minimum amount of 3 per cent sodium chloride solution at 30° C. This solution was cooled to room temperature, and then put in refrigerator over night. Some precipitate formed, which was not crystalline. Even when the above process was repeated with a further purified globulin solution, no crystalline material could be detected.

It has been considered that large crystals may be obtained if the dialysis is carried out in such a manner that the concentration of salt in the dialyzing bag changes slowly and gradually (7). Another attempt was therefore made to prepare some crystalline globulin.

A 10 per cent sodium chloride solution of globulin was dialyzed against sodium chloride solution of decreasing concentration. The setup used is shown in Figure 3.

The dialyzing bags were immersed in the dialyzing bottle (B). The dialyzing bottle was placed in a cooling vessel in the sink. Distilled water was transferred from a water reservoir (A) to the dialyzing bottle. The flow was maintained by means of a screw clamp at a rate of a drop per second. The excess water in (B) overflowed into (C), from which the excess of water would overflow into the sink. The vessel (C) was kept at about 8° C. by running water from (D).

At the beginning of the dialysis, 9 per cent sodium chloride solution was placed in the dialyzing bottle (B). Salt was dialyzed out from the dialyzing bag slowly as the difference in salt concentration was only 1 per cent. As dialysis proceeded, the concentration of salt in (B) decreased, due to the incoming water from (A). In this way, the salt solution in (B) was kept at a lower concentration than that in the dialyzing cell. This process was continued without disturbance until (B) was found to be free of

Apparatus Used for the Dialysis Against Salt Solution of Decreasing Concentration

A. Distilled water reservoir
B. Dialyzing bottle, and the excess of dialyzate overflows into C
C. Cooling vessel which sits in a sink
D. Running water from a tap, and the excess of water from C overflows into the sink
E. Coil for cooling water before it enters B

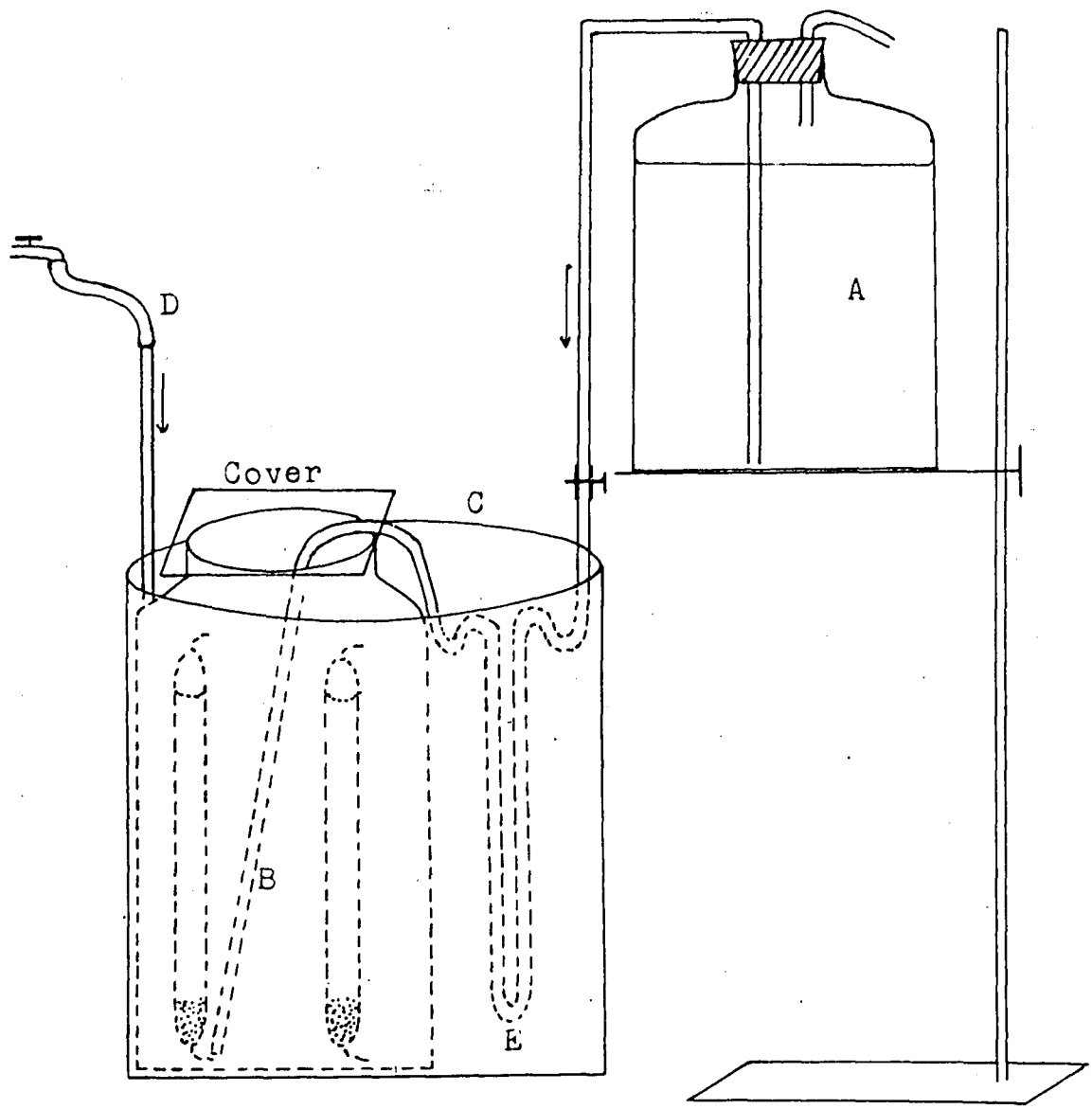


Figure 3

chloride. However, the precipitate obtained was again not crystalline.

There was an accidental observation which may be worthwhile to be recorded. About 0.1 gm. of globulin precipitate, suspended in water, had been kept in the refrigerator for more than half of a year. Before it was thrown away, the precipitate was examined under the microscope. Curiously, a few large crystals of tetragonal prism type were noted. The small particles which did not show their shapes distinctly, might be the so-called microcrystals obtained usually by rapid dialysis (7).

In order to prove that the crystals were not impurities, several tests were made. The solution gave negative tests for chloride, and sulfate. The precipitate dissolved in 10 per cent sodium chloride solution readily.

Although there were many definite large crystals, some smaller, single or in group, there were not enough for a definite identification. Furthermore, this type of crystals has not been observed in any other preparations of the globulin obtained in this study.

Determination of Amino Acid Content of The Protein Fractions

Preparation of Material for Analysis

In order to eliminate small differences in each sample, globulin samples Nos. 25, 27 and 31 were combined (Globulin C) for analysis and albumin Nos. 24, 26 and 30 were similarly combined (Albumin B). These two materials were dried in vacuo at 40° C. for two days. Then, they were spread out and exposed to air for a day. The resultant powders were stored in tightly stoppered bottles. Analyses were made only on these two mixed samples. Moisture, ash, nitrogen, and sulfur were determined on each at the beginning of the analysis. In the final check, the moisture and ash contents were again determined at the time samples were taken for hydrolysis. The results of these analyses will be given in Table VIII.

General Remark on Methods

1. Hydrolysis of Protein.

For the determination of tyrosine, tryptophane, and phenylalanine, alkaline hydrolysis was employed. Alkaline hydrolysis of protein is recommended by Lugg (8), Brand and Kassell (9), and Block et al. (10), for the determination of the aromatic amino acids. Lugg (8) reported no loss of tyrosine on hydrolysis with 5 N sodium hydroxide in a sealed

tube with plenty of air enclosed, or in an open vessel, while about 6 per cent of the tryptophane was lost by the same procedure. Brand and Kassell (9), however, found that when proteins were hydrolyzed with 5 N sodium hydroxide in a sealed tube,* there was no destruction of tryptophane, but a loss of tyrosine of approximately 4.2 per cent. In this study, the conditions employed for hydrolysis were more similar to those described by Lugg. Furthermore, it was found that a 6 per cent correction for the loss of tryptophane in hydrolysis would give a tryptophane value of casein close to that obtained by other investigators, especially that recently reported by Brown (11) on casein (Labco). Therefore all the values obtained in this study were corrected thus. For the same reason, no correction was made in the tyrosine values.

For the determination of phenylalanine, Block et al. (10) recommended hydrolysis of protein with 5 N sodium hydroxide, since they found that the destruction of phenylalanine could be reduced to a minimum by this procedure. However, it was found in this investigation that sulfuric acid hydrolysis gave comparable results.

Acid hydrolysis of proteins is usually recommended for the determination of amino acids other than the three discussed above, all of which contain a benzene ring.

*They did not mention the amount of air enclosed in the tube.

Sulfuric or hydrochloric acid may be used, but the latter is more commonly employed. In this study hydrochloric acid (1:1) was used.

2. Amino Acid Determination.

Nine amino acids (threonine, serine, cystine, methionine, tryptophane, tyrosine, histidine, phenylalanine, and arginine) were determined. For each amino acid, duplicate or in some cases triplicate determinations were made on duplicate hydrolyzates. As a final check of the values obtained on separate hydrolyzates, threonine, serine, arginine, cystine, histidine were determined on a single acid hydrolyzate (1 gm. protein), and phenylalanine, tryptophane, and tyrosine on a 0.2 gm. alkaline hydrolyzate. For methionine, a separate acid hydrolyzate was prepared, since the value obtained with the 1 gm. hydrolyzate differed greatly from that obtained previously and this was thought to be due to the difference in the method of preparation of the hydrolyzate. Moisture and ash of the protein fractions were again determined at the time samples were weighed for these hydrolyses. These figures were used in the final check for calculating the amino acids values obtained on a moisture-free and ash-free basis.

The method used for the determination of each of the above 9 amino acids was checked with pure amino acid, casein (Labco), and/or casein (Labco) hydrolyzate to which

the pure amino acid was added before it was applied to the protein fractions under investigation. Many of the methods for the determination of amino acids have an error of more than ± 5 per cent. In this group are the methods for the estimation of methionine and phenylalanine. The methionine value obtained by McCarthy and Sullivan's (12) method, and the phenylalanine value by the Kappeller-Adler method as modified by Block and Bolling (13), are affected greatly by slight variations in the conditions. A more thorough study of the exact factors that control the reactions will certainly increase the accuracy of these methods. Two new modifications of the Kappeller-Adler method have been reported recently, the one by Brown (14), and the other by Albanese (15). Each modification offers some improvement of the method.

In order to test the accuracy of the various procedures for the individual amino acids, control analyses of casein (Labco) were carried out. This seemed appropriate since casein has been analyzed extensively by many investigators and a considerable amount of data as to its amino acid composition is available. The values found in these check experiments with casein (Labco) are given in Table VII with the figures obtained by other authors for comparison. The nitrogen content of the casein on a water-ash free basis was found to 15.37 per cent and the sulfur content was 0.85 per cent, as compared with 15.40 per cent and 0.86

Table VII

Amino Acid Content of Casein

All results are calculated on a moisture-ash free basis except where noted.* Casein analyzed by different authors are not the same. Brown (14) employed casein (Labco).

	Block and Bolling (13)	Schmidt** (16)	This study	Other sources
	per cent	per cent	per cent	per cent
Phenylalanine	5.8	3.9	5.67	4.52-5.33 (17) 3.99 (14) 5.71 (18)
Serine	---	0.5	5.23 (N) 5.35 (L)	5.2 (19) 5.2 (20)
Threonine	4.6	---	3.69 (N) 4.16 (W)	3.43-3.68 (21) 4.23-4.44 (22)
Tyrosine	5.2	6.6	5.69	5.7 (11)
Cystine	0.3	0.3	0.33 (F-M) 0.27 (R) (Lu)	0.3 (23)
Methionine	3.0	3.4	3.56	2.99* (12)
Histidine	1.9	2.5	3.52	3.6 (24) 3.61 (25)
Arginine	5.3	3.8	3.54	3.37-4.12 (26)
Tryptophane	1.2	2.2	0.97	1.02-1.03 (11)

*The value has not been corrected for moisture and ash.

**These figures represent the values compiled by Schmidt.

(N) Nicolet and Shinn's method.
(L) Logan's method.
(W) Winnick's method.
(F-M) Folin-Marenzi's method.
(Lu) Lugg's modification of the Folin-Marenzi method as adapted by Block and Bolling.
(R) Rossouw and Wilken-Jorden's precipitation followed by Folin-Marenzi's method.

per cent given on the label (vitamin free casein, Labco brand).

Kappeller-Adler (17) found 4.52-5.33 per cent phenylalanine in different samples of casein. With the Block and Bolling (13) modification of this method, 5.67 per cent phenylalanine was found in this study. Both figures are considerably higher than that shown in the compilation of Schmidt (16). Recently two modifications of these methods have been proposed. Brown (14) with his modification reported 3.99 per cent on a water-ash free basis, and Albanese (15) with his modification of the same method, reported 4.77 per cent on an air dried basis. With the proper correction for moisture and ash, the latter value should easily be above 5 per cent. Hess and Sullivan (20) with their method reported 5.71 per cent. The present investigation adapted Block and Bolling's method with some slight modifications for the determination of phenylalanine.

The serine values obtained for casein (Labco) in the present study with Nicolet and Shinn's (20) method and with Logan's (19), were very similar to those previously reported. However, from a private communication, we have learned that Dr. Nicolet is studying further the behavior of serine in casein. It has been observed that nearly one-half of the serine when combined with phosphate as in casein is destroyed in acid hydrolysis. This means that the content of serine in casein may have to be revised again.

In order to conserve material, Logan's method which requires only 0.2-0.5 gm. of protein was used in this study.

The percentage of threonine in casein as determined by Shinn and Nicolet's (21) method was lower than that by Winnick's (22) method; however, the values obtained by each method check very well with those reported by the originators of the methods. The figure given by Block and Bolling (13) is even higher than that reported by Winnick (22). In this investigation, threonine was estimated by Winnick's method.

The tyrosine value found with Lugg's (27) method lies between that reported by Block and Bolling (13) and that reported in the compilation by Schmidt (16). The figure of 5.69 per cent checks exactly with the value 5.70 per cent reported by Brown for casein (Labco). The tryptophane content of casein by Brown (11) with entirely different methods is only slightly higher than that found in this study with Lugg's (27) method, 1.02-1.03 per cent as compared with 0.97 per cent. Lugg's procedure was chosen for the determination of tyrosine and tryptophane in the present study.

It is known that Folin-Marenzi's method (28) for cystine determination usually yields higher results than other methods. Here, in this study, the same phenomenon was noted. With the unmodified Folin-Marenzi method the cystine content of casein was found to be 0.33 per cent,

as compared with 0.27 per cent obtained by the Lugg (29) modification of the same method as adapted by Block and Bolling (13), or by the copper precipitation method of Rossouw and Wilken-Jorden (30), followed by Folin-Marenzi's method. However, recovery experiments on pure cystine added to protein hydrolyzate seemed to favor the use of the unmodified Folin-Marenzi method, and therefore it was employed in this study.

In the present study methionine was determined by the McCarthy and Sullivan (12) method. According to the authors their method has an accuracy of $\pm$ 4 per cent. They reported 2.99 per cent methionine for casein (not corrected for moisture and ash). This value is significantly lower than the value found with their method in this study. The value compiled by Schmidt (16) is 3.4 per cent and that given in Harrow's text book (31) is 3.6 per cent.

The histidine value obtained in this study by the use of the Koessler and Hanke (32) reaction as modified by Lang (33), is higher than any so far reported in the literature. Recently at the New York meeting of the American Chemical Society (September 1944), Saidel and Brand (34) reported that with the use of their new method, a modification of the diazotization method, all values obtained except for lactoglobulin, are higher than those obtained by the isolation procedure. The Lang (33) modification was employed in this study.

In the present investigation the Sagakuchi (35) reaction, adapted by Thomas, Ingalls and Luck (26) was used for the determination of arginine. The arginine value obtained for casein is within the range of the values reported by these authors. However, the value given by Block and Bolling (13) is much higher than that found here.

In general, the amino acids values of casein, with the exception of histidine, found in this study by the methods mentioned above fall within the ranges of those reported in the literature.

The details of all the methods used are given in the appendix.

Study of the Digestibility of the Proteins of Tung Nuts in vitro

Material

Globulin C and albumin B were used in studies of digestibility. The nitrogen, sulfur, phosphorus, ash, and water contents of these preparations are given in Table VIII. Unfortunately, due to the lack of availability of albumin B, albumin A had to be used for the estimation of the total nitrogen in peptide linkages hydrolyzed by hydrochloric acid (1:1). As shown in Table VIII, albumin A has practically the same percentage of nitrogen on water and

ash-free basis as albumin B; but the former has a slightly higher sulfur content, 0.92 per cent as compared with 0.79 per cent, and its ash content is almost four times as much as that of albumin B. With the assumption that the higher ash content was merely due to the presence of impurities and did not affect the nature of the protein molecule, the total nitrogen in peptide linkages of albumin A hydrolyzed by hydrochloric acid (1:1) was taken as a basis for the calculation of the percentage of the nitrogen in peptide linkages in albumin B that could be split by enzymes of the intestinal tract.

The pepsin used was Difco 1:20,000 and the enzymes of the pancreas and the intestine were obtained by the extraction of the respective organs of pig with glycerol in the usual manner. The details of the preparation are given in the appendix.

Procedure

1. Peptic Digestion.

The optimum acidity for peptic activity ranges from pH 1.5 to 2.2. However, in order to get the protein into solution, or fine suspension, the proteins were first dissolved in alkali, and the resultant solutions were then brought to a pH of about 1.5 by adding the calculated amount of acid with vigorous shaking. The details of this procedure are given in the appendix. The relative amounts

of pepsin and protein present in all the digests were roughly 1 and 5, that is 1 ml. of digest contained 3.75 mg. of pepsin and about 17.0 mg. of protein.

Protein might undergo a slight hydrolysis independent of the enzyme, and the enzyme might be autohydrolyzed or hydrolyzed by the presence of other enzymes, therefore a protein control and an enzyme control were run at the same time. The pH of the solutions, the concentration of protein in the protein control and the concentration of enzyme in the enzyme control were the same as those in the digest. There was usually no hydrolysis of significance in the protein control. Slight hydrolysis as indicated by increases in acidity and alkalinity was always observed in the enzyme control. The methods of preparing the digest and the controls are given in the appendix.

Toluene and thymol were used as preservatives in all the experiments. There was no evidence of contamination as measured by marked changes of pH at any time. All the experiments were conducted at 30 $\pm$ 1° C. in a constant temperature water bath. This temperature was chosen because it is the one usually specified in the measurement of the activities of the enzymes used.

The degree of hydrolysis was estimated by measuring the increase of acidity in the Willstätter titration and the increase of basicity in the Linderstrøm-Lang titration. The procedures of these two titrations are given in the

appendix. The values obtained with samples taken immediately after the introduction of the enzyme solution were taken as a zero base line. The hydrolyses of the digest were corrected for the enzyme controls. The method of calculation is given in the appendix.

Duplicate samples of 1 ml. were taken for both the Willstätter and Linderstrøm-Lang titrations. The solutions were shaken well for about 15 seconds, and allowed to settle for 30 seconds and then samples were pipetted into 50 ml. Erlenmeyer flasks containing 3 ml. acetone plus 1 ml. of water, (Linderstrøm-Lang) or 2 ml. alcohol (Willstätter) plus 1 ml. of water. The acetone or alcohol in the flasks stopped the action of pepsin as soon as the solution came into contact with it. The pH of all the solutions were determined with a glass electrode immediately after the first set of samples were taken and near the end of the digestion. Due to the rapid rate of hydrolysis at the beginning of the digestion, samples were taken at the end of 1, 2, and 4 hours, and thereafter daily. When the rate of digestion became very slow, samples were taken only every other day. The digestion was followed until no further changes in the acidity and basicity of the digest were observed. This usually occurred after about 100 to 250 hours, depending on the nature of the protein and its physical state. The pH of the digest usually increased slightly, and that of the controls remained constant. The

remaining solutions were used for the subsequent study of pancreatic digestion. During the time which was required for the preparation and estimation of the activity of the pancreatic extract, the peptic digest and its controls were kept in a refrigerator.

The hydrolysis of peptide linkages liberates free amino and free carboxyl groups. The increase in amino groups may be expressed in terms of nitrogen, as the percentage of the total nitrogen. Since one carboxyl group is liberated for every amino group in the hydrolysis, the increase of carboxyl groups may be expressed also as nitrogen. Hence, the increases of acidity and alkalinity throughout the study were expressed in terms of nitrogen as percentages of the total nitrogen. The total nitrogen of the digest and controls was determined by the micro Kjeldahl method as described in the appendix.

2. Pancreatic Digestion.

The activity of the diluted and activated pancreatic extract was determined just before it was used, for the diluted glycerol extract lost its activity rapidly upon standing. The method of preparing the enzyme extract and that for the determination of the activity of the extract are given in the appendix.

40 ml. aliquots of the peptic digest and its enzyme control were adjusted to a pH of about 7.9-8.0 with measured

amounts of sodium hydroxide. The solutions were diluted to 45 ml. with water, and 55 ml. of the borate buffer-enzyme mixture* were added to each of the solutions. Samples were taken immediately after the introduction of the enzyme-buffer mixture. 55 ml. of borate buffer were added to a 40 ml. alequot of the protein control and the pH was adjusted to 8.0-8.1. The resultant solution was diluted to 100 ml. with water. From now on the general procedure was the same as described for the peptic digestion.

This order of mixing the enzyme with the buffer before adding to the digest was necessary because it was observed that when pancreatic extract (pH 8) which contained glycerol was mixed with borate buffer (pH 8.2), the pH of the resulting mixture decreased to about 6.

In the digestion of globulin following peptic digestion, and in the first of the two similar experiments of albumin, borate buffer and peptic digest (already adjusted to pH 8) were mixed and the pH of the mixture was adjusted before the enzyme was added to it. Upon the addition of borate buffer, the pH of the mixture of peptic digest and enzyme decreased to 7.3-7.5. Therefore pH adjustment had to be

*30 ml. of borate buffer (0.2M) were mixed with 90 ml. of diluted and activated pancreatic extract, and the pH of the mixture was adjusted to 8 with sodium hydroxide. The enzyme activity of this mixture was again determined. The final concentration of borate buffer in the digest was 0.0275 M. Borate at this concentration had no effect on the proteolytic activity of the enzymes as shown by the experimental data given in the appendix (Table XXXIV).

made after the first set of samples was taken. The amount of sodium hydroxide necessary to bring the digest to a pH of 8 was measured, and the same amount of sodium hydroxide was added to the enzyme control. The increase in alkalinity in the digest due to the added sodium hydroxide could be corrected for by the increase in alkalinity in the enzyme control. Since the protein control did not contain any glycerol, the pH would not shift, and therefore no alkali was needed. However, in order to compensate for the change in volume, an equivalent amount of water was added to the protein control.

During the course of digestion, the pH of the solutions seldom varied more than one-tenth of a pH. The digestion was carried out until no further changes were observed, which was usually about 150-250 hours. The remaining solutions were used for the subsequent study of intestinal digestion.

3. Intestinal Digestion.

Since the remaining solutions of the pancreatic digest and its controls were already at a pH of about 8.0, no further adjustment of pH was required. 40 ml. of the digest remaining from the pancreatic digestion were mixed with 20 ml. of the intestinal extract, the activity of which had been recently determined. To the same amount of the enzyme control, 20 ml. of the intestinal extract were

added, and to a 40 ml. aliquot of the protein control, 20 ml. of the borate buffer. The increases in acidity and alkalinity were determined in exactly the same manner as previously described. The digestion was carried out until no further changes were observed, usually after 150-250 hours. The pH of the solutions were rather constant during the course of digestion.

The results obtained on the studies of peptic, pancreatic, and intestinal digestions are summarized in Table XV and in Chart I in the discussion.

4. Determination of Peptide Linkages Hydrolyzed by Hydrochloric Acid (1:1).

The digestibility of a protein effected by enzymes may best be expressed in terms of percentage of the nitrogen in the peptide linkages hydrolyzed by acid. It is generally known that a protein can be hydrolyzed completely by acid. If this is true, the increase of free carboxyl and amino groups produced by acid hydrolysis will be equivalent to the total peptide linkages in a protein. Hence, the total peptide linkages of a protein can be estimated by finding the increase in free amino and carboxyl groups following the hydrolysis with hydrochloric acid.

In order to calculate the increase in acidity and alkalinity produced by the acid hydrolysis, the titratable groups of the protein before hydrolysis must be determined.

For ease in taking aliquots it is desirable to have the protein in solution or fine suspension.

Since the proteins isolated in this study were not soluble in neutral salt solution, they were dissolved in alkali, and the resultsnt solutions were adjusted to the nearest possible neutral reaction without precipitating the protein. The solutions were prepared in the following manner. 1 gm. of protein was placed in a 25 ml. volumetric flask, to which 15 ml. of water were added with shaking. The agitation was continued until the protein particles were uniformly suspended in water, then 1.15 ml. of 2 N sodium hydroxide was added, and the agitation continued until no undissolved protein particles were visible. In order to bring the solution as near as possible to the neutral reaction without precipitating the protein, approximately 0.1 N hydrochloric acid was added slowly with shaking until the solution became slightly turbid. It usually took about 9-10 ml. of 0.1 N hydrochloric acid. The resultant solution was diluted to 25 ml. with water. One of the globulin solutions was prepared by rapidly adding an excess of hydrochloric acid to the alkaline protein solution so as to pass the isoelectric point of the protein without precipitating the protein. This was the same method used in preparing solutions for the study of enzymatic digestion. The results obtained on these two solutions checked within the range of experimental error.

A blank was prepared by mixing the same amount of alkali and acid as was used in the preparation of protein solution, and diluting to 25 ml. The acidity and alkalinity of the blank and the protein solution was determined by the Willstätter and the Linderstrøm-Lang titrations. Since the end-point in the case of the protein solutions was difficult because of the turbidity of the solutions, titrations on each sample were done in triplicate. The titrations of the protein solutions were corrected for the amount of acid and base presented in the blank as determined by the same methods. The results were expressed as percentage of the total nitrogen.

For the determination of the acidity and alkalinity of the protein after hydrolysis, 1 gm. of protein was hydrolyzed with 20 ml. of hydrochloric acid (1:1) for 18 hours, at 125° C. The humin was filtered and washed with hot (60-70°) 10 per cent hydrochloric acid. The humin together with the filter paper was analyzed for nitrogen. The filtrate and washings were combined and evaporated on a water bath to dryness. More water was added and the hydrolyzate was evaporated again to dryness. This process was repeated two more times. The residue was dissolved with warm water and transferred into a volumetric flask, and diluted to 100 ml. In the case of albumin, due to the shortage of material, 0.5 gm. protein was used and the residue was diluted to 50 ml.

The acidity and alkalinity of the hydrolyzates were also determined by the Willstätter and Linderstrøm-Lang titrations. The hydrolyzate had a brownish color. This color greatly affected the end-points, the color of the end-point in the Willstätter titration, no longer being blue but green, and that in the Linderstrøm-Lang titration, brownish red. In order to overcome this difficulty, the "block comparator" principle was adopted. All determinations were made in triplicate.

All of the chloride ions were not removed by the repeated evaporations, therefore it was necessary to determine the chloride remaining in the humin-free hydrolyzate and make appropriate corrections in both the acid and base titrations. The chloride was determined by the Volhard method in 2-5 ml. aliquots.

It is usually recognized that the amide groups present in protein are also hydrolyzed by acid. The ammonia thus obtained will act as a base in the Linderstrøm-Lang titration and act as acid in the Willstätter titration. Since these amides are not titrated before the hydrolysis, the ammonia of the hydrolyzate must be determined so as to correct for the increase of titration values due to the presence of ammonia. Ammonia was estimated by distillation under reduced pressure. The procedure is given in the appendix.

If there is phosphoric acid linked to the protein molecule

another correction should be made, as before hydrolysis only two acid groups of the phosphoric acid were titrated, and after hydrolysis all three acid groups could be titrated. A qualitative examination showed that both the globulin and albumin fractions contained phosphorus. The globulin C fraction contained 0.098 per cent as determined by the Fiske-Subbarow method.* Phosphorus determinations were not made on the albumin fraction because of lack of material. Since the amount of phosphorus was found to be small and there was no proof that the phosphorus was an integral part of the globulin molecule, no correction was made.

The total nitrogen in peptide linkages of casein was similarly determined, and the results are included in the appendix. Since it is known that phosphorus in casein is linked with the protein molecule, the titration values were corrected for the phosphorus content, using a factor 0.707 (36). The casein (Hammarsten) was found to contain 0.78 per cent of phosphorus.

In summary, the acidity and alkalinity of a protein hydrolyzate may be attributed to the titratable groups in the protein, to the amino and carboxyl groups liberated by hydrolysis, to the chloride remaining in the hydrolyzate, to the ammonia formed by the cleavage of amide groups, to

*Analyses were made by Dr. R. L. Garner of this department to whom we wish to express our appreciation.

the phosphoric acid existing as an integral part of the protein molecule. In order to determine the amino and carboxyl groups liberated by hydrolysis, corrections for these sources of acidity and alkalinity must be made.

The results are given in the appendix. Values obtained on casein are also included in the same table.

Acid hydrolysis under the conditions used in this study is generally considered as complete, and gives a measure of the total peptide linkages in the protein molecule. The digestibility of a protein by proteolytic enzymes in vitro may be expressed as the percentages of the total nitrogen in peptide linkages of the protein cleaved by acid. The digestibilities of the protein fractions isolated in this study were calculated on this basis.

DISCUSSION

The Proteins of Tung Nuts

The compositions of the globulin and albumin fractions isolated from the defatted tung nut meal are given in Table VIII.

Globulin A represented a composite sample of samples Nos. 12, 14, 17, 20, 22, and 23. The nitrogen contents of these samples ranged from 17.18 to 17.76 per cent on dry basis, and the ash contents ranged from 0.36 to 0.64 per cent. Globulin B was obtained from repurification of samples Nos. 1, 2, 3, 4, 5, 6, 8, 9, and 13. The purification was effected by dissolving the dried samples in 10 per cent sodium chloride solution. Insoluble material was removed by centrifugation. The clear solution was dialyzed and the precipitated protein was dried in the usual manner. Globulin C was a mixture of samples Nos. 25, 27, and 31. It was dried in a vacuum oven at 40° C. for 2 days, and then exposed to air for 24 hours. The resultant material was kept in a tightly stoppered bottle. Albumin A was a composite sample of samples Nos. 7, 18, 19, and 21. The nitrogen contents varied from 13.46 to 13.69 per cent on dry basis, and the ash contents ranged from 1.73 to 4.00

Table VIII

Water, Ash, Nitrogen and Sulfur Contents of Some Protein Preparations

The values for globulin C (1) and albumin B (1) were determined prior to the determinations of amino acids; while those for globulin C (2) and albumin B (2) were determined at the time samples were taken for check determinations of amino acids. Nitrogen and sulfur are given on water and ash-free basis.

Preparations	Preparation	Yield	Moisture	Ash	Nitrogen	Sulfur	N/S
		gm./gm.	per cent	per cent	per cent	per cent	
Globulin 25	Ordinary dialysis	12.0/350	9.37	0.21	17.95	----	----
Globulin 27	" "	20.5/400	10.77	0.34	17.71	----	----
Globulin 31	" "	12.0/300	----	----	-----	----	----
Globulin A	" "	----	8.16	0.56	17.44	1.72	10.14
Globulin B	" "	----	11.41	0.52	18.43	2.72	6.78
Globulin C*	" "	-----	(1) 5.48 (2) 7.90	0.30 0.29	17.55 -----	1.75 ----	10.03 ----
Globulin 33	Dialysis under reduced pressure	15.6/300	8.61	0.82	17.27	1.91	9.04
Albumin 24	Neutral solution, heated to boiling	7.0/350	4.82	0.94	14.10	0.80	17.63
Albumin 26	Acid solution, heated to boiling then neutralized.	8.0/400	5.70	0.00	14.42	0.78	18.49
Albumin 28	Alkaline solution, heated to boiling-	----	4.13	0.08	15.37	1.01	15.22

Globulin B	" "	----	11.41	0.52	18.43	2.72	0.78
Globulin C*	" "	----	(1) 5.48 (2) 7.90	0.30 0.29	17.55 -----	1.75 ----	10.03 ----
Globulin 33	Dialysis under reduced pressure	15.6/300	8.61	0.82	17.27	1.91	9.04
Albumin 24	Neutral solution, heated to boiling	7.0/350	4.82	0.94	14.10	0.80	17.63
Albumin 26	Acid solution, heated to boiling then neutralized.	8.0/400	5.70	0.00	14.42	0.78	18.49
Albumin 28	Alkaline solution, heated to boiling	----	4.13	0.08	15.37	1.01	15.22
Albumin 30	Neutral solution filtered through filter-cel, heated to boiling.	6.5/300	1.39	0.70	14.30	0.79	18.10
Albumin 32	Precipitated with ammonium sulfate, dialyzed, heated to boiling.	5.2/300	4.65	0.27	14.41	0.68	21.19
Albumin A	Neutral solution, heated to boiling.	----	8.14	2.81	14.24	0.98	15.48
Albumin B	Neutral solution, heated to boiling.	----	(1) 4.47 (2) 8.14	0.77 0.63	14.31 -----	0.79 ----	18.11 ----

*The phosphorus content of globulin C was 0.098 per cent.

per cent. Albumin B was obtained by combining samples Nos. 24, 26, and 30. They were mixed by grinding and the resultant material was treated as described in the preparation of globulin C.

All amino acid analyses and digestion studies were made on globulin C and albumin B.*

The yield of globulin and albumin by the procedure described represented about 30 per cent of the total nitrogenous material, extractable by 10 per cent sodium chloride solution found in the extraction experiment. In terms of grams of protein per 100 grams of nut meal, about 4-5 gm. of globulin and 2 gm. of albumin were obtained. Osborne and Campbell (37) obtained 10 per cent globulin from vetch and peas, and 13 per cent from lentil; 2 per cent legumelin from peas, 1.5 per cent from vetch, and 1 per cent from lentil. (Legumelin is an albumin.)

Only 3 per cent of the extractable nitrogen was found in the solution after albumin was removed. The rest of the 60-70 per cent must have been lost in the process of isolation. The dialyzate obtained at the beginning of a dialysis gave a strong biuret test. Substances with small molecular size such as proteose, peptides, and other non-protein nitrogenous material, may go through the dialyzing membrane.

*In the digestion study, on account of shortage of material, albumin A was used to determine the total nitrogen in peptide linkages.

It was noted that much globulin, which was in a finely dispersed condition, could not be centrifuged down. This phenomenon was more marked in the process of repurification. There were, of course, some mechanical loss due to manipulation. The efficiency of extraction when 20 mesh nut meal was used probably would be lower than that when 80 mesh meal was used. In the extraction experiments, 80 mesh nut meal was used. Furthermore, only 3 gm. of meal were extracted in each of the extraction experiments, and the amount used in the isolation of proteins was 50 times as much. This difference may also contribute to the low yield.

The nitrogen content of the globulin fraction of tung nut is very similar to that of ordinary plant globulins as shown in Table IX. The sulfur content of globulin, however, is much higher than most of the globulins reported by Osborne and coworkers (31) (38) (34). The ash contents of the globulin and albumin fractions isolated in this study are also within the range given by Osborne and Campbell (38) (39) (40).

In the preparation of the albumin, no definite coagulating point was observed, turbidity began to appear at about 50° C., and at about 80° C. protein began to coagulate. The coagulating point as stated by Osborne and Campbell (37), is greatly affected by the presence of salts or acids as well as the proportion of dissolved legumelin.

Table IX

Comparison of the Nitrogen, Sulfur, and Ash Contents of the Tung Nut Proteins with Other Plant Proteins

Nitrogen and sulfur values are given on water and ash-free basis.

	Nitrogen	Sulfur	Ash
	per cent	per cent	per cent
Soybean globulin (glycinin)	17.45	0.72	0.30-1.34
Cotton-seed globulin	18.64	0.62	0.11-0.82
Hemp-seed globulin (edestin)	18.84	0.87	----
Tung nut globulin	17.55	1.75	0.21-0.34
Pea legumelin	16.30	1.06	0.21-0.80
Vetch legumelin	16.26	1.11	0.26-0.39
Lentil legumelin	16.25	0.98	0.20-0.74
Tung nut albumin	14.31	0.79	0.00-0.94

When the solution obtained after the removal of the globulin was acidified (Congo red), a small amount of precipitate formed but when heated, no more precipitate was noted. If alkali was added to the resultant solution with stirring, large amounts of protein flocculated when the solution became neutral. If the above solution was made alkaline to litmus, a small amount of precipitate also formed as when acid was added, but this alkaline solution

differed from the acid solution in that a considerable quantity of protein in the solution coagulated upon heating. The albumin isolated by boiling a neutral solution (albumin 24) of the proteins after globulin had been removed, had a lower content of nitrogen and of sulfur than that prepared by boiling an alkaline solution (albumin 28) of the same. The differences of nitrogen and sulfur contents between globulin B and the other preparations, and that between albumin 28 and other albumin preparations seem to indicate that these fractions may be further separated into many fractions. They may consist of groups of proteins rather than pure chemical entities.

Both globulin and albumin fractions of tung nut gave a slightly positive Molisch test. The ash contained some phosphorus. Both fractions as obtained were powdery, and the colors were nearly white.

Amino Acid Content of the Proteins

The amino acid contents as determined by the procedure given in the appendix are summarized in Tables X and XI. In Table X each value represents an average figure obtained from at least two different hydrolyzates. Their ranges are given in parenthesis.

The values given in Table XI are results obtained on a single hydrolyzate,* that is to say threonine, serine,

*Methionine was determined on a separate acid hydrolyzate.

Table X

Amino Acid Content of the Protein Fractions Isolated from Tung Nuts

	Globulin fraction		Albumin fraction	
	Air dried basis	Water- and ash-free basis	Air dried basis	Water- and ash-free basis
	Per cent	per cent	per cent	per cent
Threonine	3.19 (3.19-3.20)	3.39	5.95 (5.81-6.07)	6.28
Serine	5.22 (5.21-5.24)	5.54	6.73 (6.60-6.78)	7.10
Cystine	3.01 (2.98-3.04)	3.19	1.22 (1.19-1.24)	1.29
Methionine	3.95 (3.85-4.01)	4.19	1.98 (1.90-2.02)	2.09
Tyrosine	4.01 (3.94-4.07)	4.26	4.00 (3.97-4.04)	4.22
Tryptophane	1.04 (0.97-1.09)	1.10	1.90 (1.78-1.81)	2.01
Phenylalanine	4.70 (4.48-5.03)	4.99	8.07 (7.61-8.68)	8.52
Arginine	12.47 (12.38-12.53)	13.24	3.54 (3.48-3.60)	3.74
Histidine	2.65 (2.60-2.73)	2.81	2.08 (2.00-2.14)	2.20

cystine, arginine, histidine on one acid hydrolyzate, and tyrosine, tryptophane and phenylalanine on one alkaline hydrolyzate. Table XI also gives the percentages of deviation from the values given in Table X. The percentage of deviation was calculated with reference to the values given in Table XI. The highest deviation thus obtained was 5.8 per cent in the phenylalanine determination. The differences found in the amino acid values given in these two tables are probably the sum of experimental error, inherent error of the methods, and error introduced by the change in moisture contents of the protein fractions during the course of this study. The ash and moisture contents of the protein fractions are given in Table VIII. Since the ash and water contents of these fractions were determined again at the time samples were taken for hydrolysis in preparation for the final check of the amino acid contents, values in Table XI are probably better representatives of the true amino acid contents of these protein fractions. These values will be referred to in all the discussions of amino acids.

The nitrogen content of these nine amino acids represented 44.3 per cent, and 34.7 per cent of the total nitrogen of the globulin and albumin fractions respectively. In terms of sulfur, the methionine and cystine contents of globulin accounted for 96.6 per cent of the total sulfur, and that of albumin accounted for 101.2 per cent. The

Table XI

Amino Acid Content of the Protein Fractions Isolated from Tung Nuts

	Globulin fraction		Albumin fraction		Deviation from the previous result	
	Air dried basis	Water- and ash-free basis	Air dried basis	Water- and ash-free basis	Globulin	Albumin
	per cent	per cent	per cent	per cent	per cent	per cent
Threonine	3.08	3.37	5.96	6.53	-0.6	+3.8
Serine	5.24	5.71	6.30	6.90	+3.0	-2.9
Cystine	2.81	3.06	1.24	1.36	-4.2	+5.1
Methionine	3.72	4.05	1.85	2.03	-3.5	-3.0
Tyrosine	4.04	4.40	4.01	4.40	+3.2	+4.1
Tryptophane	1.05	1.14	1.93	2.12	+3.5	+5.2
Phenylalanine	4.55	4.96	8.24	9.03	-0.6	+5.6
Arginine	12.31	13.41	3.44	3.77	+1.3	+0.8
Histidine	2.65	2.89	2.10	2.30	+2.8	+4.3

data are given in Tables XII and XIII.

The two protein fractions differ greatly in their amino acid contents. The tyrosine value is the same for both fractions but for others the differences are much greater than the limits of error of the methods. In the

Table XII

Amino Acid Nitrogen Expressed as Per Cent of Total Nitrogen in Protein

Amino acid	Globulin (total N--17.55 per cent)		Albumin (total N--14.31 per cent)	
	Gm. of amino acid per 100 gm. protein	Gm. of amino acid N per 100 gm. protein N	Gm. of amino acid per 100 gm. protein	Gm. of amino acid N per 100 gm. protein N
	per cent	per cent	per cent	per cent
Threonine	3.37	1.36	6.53	3.24
Serine	5.71	4.34	6.90	6.43
Cystine	3.06	2.19	1.36	1.11
Methionine	4.05	2.17	2.03	1.33
Tyrosine	4.40	1.94	4.40	2.38
Tryptophane	1.14	0.89	2.12	2.03
Phenylalanine	4.96	2.40	9.03	5.36
Arginine	13.41	24.59	3.77	8.48
Histidine	2.89	4.46	2.30	4.35
Total		44.34		34.71

Table XIII

Cystine-Sulfur and Methionine-Sulfur Expressed as Per Cent of Total Sulfur in Protein

Amino acid	Globulin (total S--1.75 per cent)		Albumin (total S--0.79 per cent)	
	Gm. of amino acid per 100 gm. protein	Gm. of amino acid S per 100 gm. protein S	Gm. of amino acid per 100 gm. protein	Gm. of amino acid S per 100 gm. protein S
	per cent	per cent	per cent	per cent
Cystine	3.06	46.8	1.36	46.0
Methionine	4.05	49.8	2.03	55.2
Total		96.6		101.2

case of hydroxy-amino acids, the albumin fraction has twice as much threonine as the globulin fraction, but these two fractions do not differ very much in serine content. On the other hand, the values of both sulfur-containing amino acids of the globulin fraction are much higher than those of the albumin fraction. The cystine content of globulin is 3.06 per cent as compared with 1.36 per cent in albumin. The difference in methionine content is almost as great, 4.05 per cent against 2.03 per cent. Plant globulins are known usually to have high contents of arginine; the globulin fraction isolated here gives further evidence, however, the albumin fraction has only 3.77 per cent of arginine. The histidine contents of the fractions do not differ very much from each other. The phenylalanine and tryptophane values

of the albumin fraction are almost twice as much as those of the globulin fraction.

On the whole, the amino acid contents of these two protein fractions compare rather well with ordinary plant proteins. For comparison, Table XIV is included.

Table XIV

Comparison of the Amino Acid Content of the Protein Fractions of the Tung Nuts with Some Plant and Animal Proteins

	Globulin fraction	Albumin fraction	Arachin	Glycinin	Casein	
	per cent	per cent	per cent	per cent	per cent	per cent
Threonine	3.37	6.53	2.56	----	3.9	4.6
Serine	5.71	6.90	5.20	----	5.8	---
Cystine	3.06	1.36	1.51	0.74	0.3	0.3
Methionine	4.05	2.03	0.67	1.84	3.6	3.0
Tyrosine	4.40	4.40	5.50	1.86	6.5	5.2
Tryptophane	1.14	2.12	0.88	1.89	2.2	1.2
Phenylalanine	4.96	9.03	2.60	3.86	3.9	5.8
Arginine	13.41	3.77	13.51	5.12	5.2	5.3
Histidine	2.89	2.30	1.88	1.39	2.6	1.9

The threonine, serine, cystine and methionine values of arachin were obtained by Brown (41); the phenylalanine, tyrosine, arginine, and histidine values by Johns and

Jones (42); and the tryptophane value by Jones, Gersdorff, and Moeller (43).

The values of phenylalanine, tyrosine, arginine, and histidine of glycinin were from the data of Osborne and Clapp (44); that of methionine from Baernstein (45); and those of tryptophane and cystine from Csonke and Jones (46). These authors gave values from 1.89 per cent to 2.84 per cent of tryptophane and 0.74 per cent to 1.46 per cent of cystine for the glycinins of different varieties of soybeans.

The values for casein shown in the first column were taken from "Textbook of Biochemistry" by B. Harrow (31), and that in the second column were taken from the second edition of "The Determination of the Amino Acids" by R. J. Block and D. Bolling (13).

The tryptophane figure of the globulin fraction as indicated in Table V is lower than that of casein, nevertheless, 1.02 to 1.03 per cent tryptophane was reported by Brown very recently for casein (Labco), and the value obtained in this study was only 0.97 per cent. The methionine of the albumin fraction is about 56 per cent of that of casein, however, the former has four times as much cystine as there is in the latter.

Digestibility of the Protein Fractions

The results of the digestion studies on the protein fractions are summarized in Table XV and Chart I. The digestibilities of the globulin and albumin fractions by enzymes were found to be 82.4 per cent and 73.9 per cent respectively of that effected by acid. The values* were lower than that of the proteins of milk, lactalbumin 94 per cent (47), lactoglobulin 97 per cent (48), and casein 92 per cent (49).

Although the digestibilities of vegetable proteins are in general lower than those of animal proteins, the values obtained here, especially that of the albumin, are even lower than those reported by Frankel (49) for most of the seed proteins. Frankel determined the digestibilities of these proteins by estimating the increase of amino groups with the Van Slyke amino nitrogen method. He (49) reported 87 per cent for cottonseed globulin and edestin, and 86 per cent for soybean globulin. These values had not been corrected for ammonia. Frankel (49) claimed that the digestibility would be much greater if the correction for ammonia were applied. In this study this correction was made. The actual differences between the digestibilities of the two

*Values from Frankel and Miller and Calvery were not corrected for ammonia, while Hotchkiss applied this correction. Hotchkiss employed the titration methods, Frankel used the Van Slyke amino nitrogen method, and Calvery and Miller employed both procedures.

Table XV

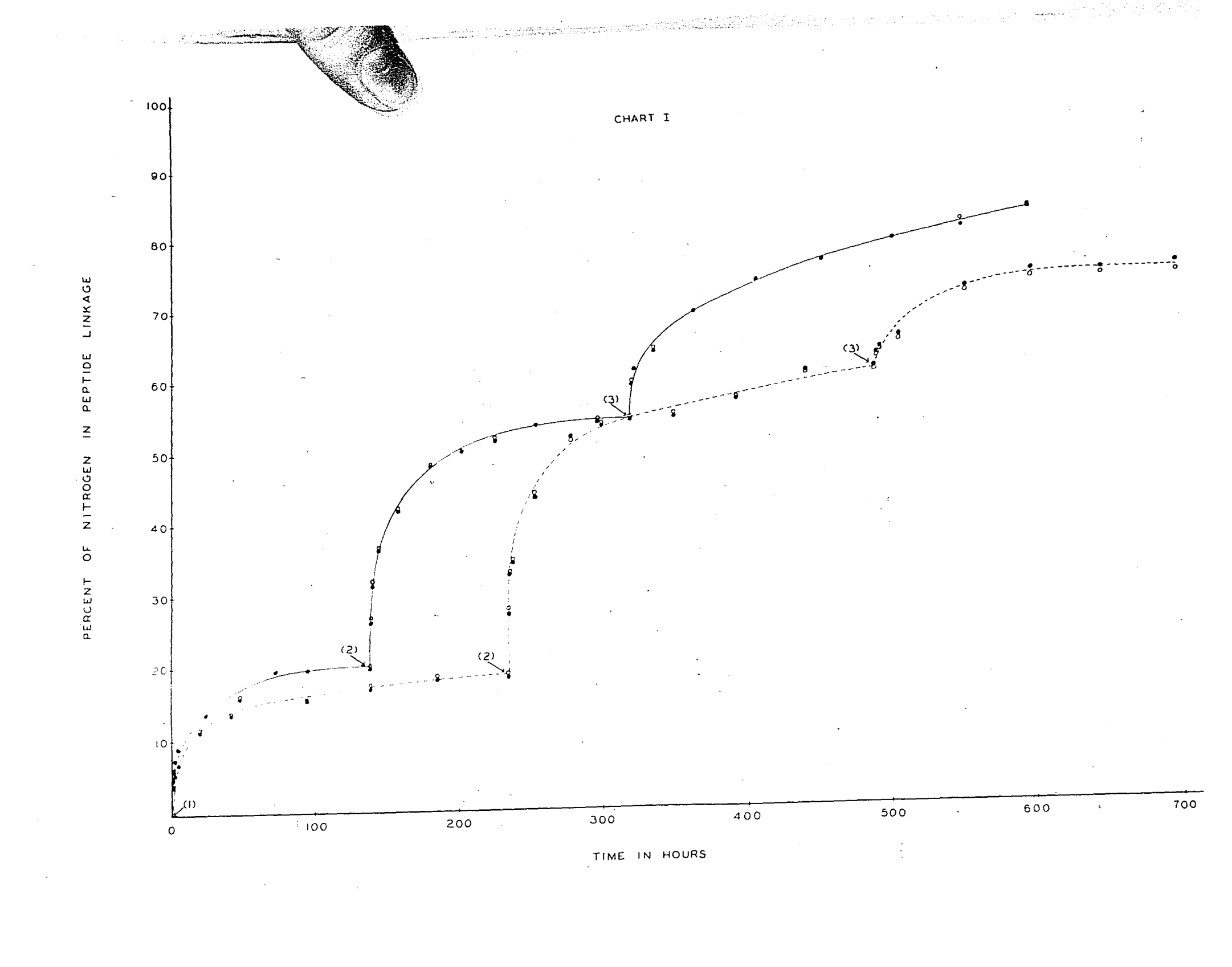
Enzymatic Hydrolysis of the Protein Fractions Isolated from Tung Nuts

All values had been corrected for changes in enzyme controls.

Protein	Enzyme	pH of digest	Time of digestion	Increase of alkalinity in terms of nitrogen		Increase of acidity in terms of nitrogen	
				Protein Nitrogen	Nitrogen in peptide linkage*	Protein Nitrogen	Nitrogen in peptide linkage*
			hours	per cent	per cent	per cent	per cent
Globulin	Commercial pepsin	1.48-1.61	139.5	12.14	19.4	12.41	19.8
	Pancreatic extract	7.90-7.98	184.0	21.43	34.2	21.42	34.2
	Intestinal extract	7.95-7.99	282.0	18.00	28.7	17.85	28.5
	Total		605.5	51.57	82.3	51.68	82.5
Albumin	Commercial pepsin	(a) 1.48-1.71	236.0	14.11	17.8	14.78	18.6
		(b) 1.50-1.74	262.0	14.73	18.6	15.40	19.4
	Pancreatic extract	(a) 8.22-8.21	259.0	33.91	42.7	32.56	41.0
		(b) 7.88-7.98	190.0	34.05	42.9	33.95	42.8
	Intestinal	(a) 8.11-8.16	210.5	10.53	13.3	10.12	12.7

			hours	per cent	per cent	per cent	per cent
Globulin	Commercial pepsin	1.48-1.61	139.5	12.14	19.4	12.41	19.8
	Pancreatic extract	7.90-7.98	184.0	21.43	34.2	21.42	34.2
	Intestinal extract	7.95-7.99	282.0	18.00	28.7	17.85	28.5
	Total		605.5	51.57	82.3	51.68	82.5
Albumin	Commercial pepsin	(a) 1.48-1.71	236.0	14.11	17.8	14.78	18.6
		(b) 1.50-1.74	262.0	14.73	18.6	15.40	19.4
	Pancreatic extract	(a) 8.22-8.21	259.0	33.91	42.7	32.56	41.0
		(b) 7.88-7.98	190.0	34.05	42.9	33.95	42.8
	Intestinal extract	(a) 8.11-8.16	210.5	10.53	13.3	10.12	12.7
		(b) 7.98-8.00	165.0	10.27	12.9	10.27	12.9
	Average Total	(a)	705.5	58.80	74.1	58.55	73.7
		(b)	617.0				

*The total nitrogen in peptide linkages was determined by titrating the amino and carboxyl groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. The calculations are given in the appendix. Under these conditions, 62.7 per cent of the globulin nitrogen, and 79.4 per cent of the albumin nitrogen were liberated.


CHART I
PERCENT OF NITROGEN IN PEPTIDE LINKAGE
TIME IN HOURS
100
90
80
70
60
50
40
30
20
10
0
100
200
300
400
500
600
700
(1)
(2)
(2)
(3)
(3)

Legend for Chart I

Cleavage of the Peptide Linkage of the Proteins of the Tung Nut by Gastro-intestinal Proteolytic Enzymes *in vitro*

The digestibility by enzymes is expressed in terms of percentage of that nitrogen in the peptide linkages hydrolyzed by acid.

Solid line	globulin fraction
Dotted line.	albumin fraction
0	Willstätter titration
●	Linderstrøm-Lang titration
(1)	introduction of pepsin
(2)	introduction of pancreatic extract
(3)	introduction of extract of intestinal mucosa

The data on which this chart is based are given in the appendix.

protein fractions of tung nuts and those of many of the proteins of seeds reported by Frankel (49) are therefore probably higher than they appear to be.

The digestibilities of the proteins isolated from the tung nut may be higher than the values reported in this thesis. Since the protein fractions isolated in this study were only slightly soluble in neutral salt solution, it was necessary to dissolve them in sodium hydroxide. Then, when the solutions were made slightly acid before they were titrated with alkali, the proteins precipitated out. It was quite possible that the precipitated protein did not react as rapidly and as completely as protein in solution. The error introduced in this procedure may have increased the apparent amount of nitrogen in peptide linkages liberated by acid hydrolysis and therefore decreased the percentage of nitrogen in peptide linkages liberated by enzymes. The fact that the nitrogen in peptide linkages liberated as determined by the Willstätter titration was significantly higher than that determined by the Linderstrøm-Lang titration may be explained by this hypothesis. If this difficulty could have been eliminated, much higher values* for digestibility probably would have resulted. However, the significant difference between the digestibilities of these two proteins fractions was probably not to be attributed entirely to this

*Hotchkiss (48) was dealing with a crystalline lactoglobulin which is soluble in neutral salt solution.

factor, for they were studied by exactly the same procedure. It might be a difference in the constitutions of the two protein fractions. The difference between the digestibilities of these two fractions cannot be discussed too critically, for the sample of albumin (albumin B) used in enzymatic digestion was different from that (albumin A) used for acid hydrolysis.

The rate of digestion of these protein fractions by the three groups of gastro-intestinal enzymes as shown in Chart I followed the general pattern of enzymatic hydrolysis. The rate in the first few hours was rapid and progressively decreased. Miller and Calvery (47) reported that in the case of lactalbumin a slow digestion continued for two months or more. In this study, the digestions were followed only to the point where no significant increase of acidity or alkalinity was observed.

The extents to which these two proteins were cleaved by each group of the enzymes were slightly different. Peptic hydrolysis of each was essentially the same. About 19-20 per cent of the nitrogen in peptide linkages of the globulin and the albumin was liberated by commercial pepsin solution. The concentration of pepsin was about 1.25 mg. per mg. of protein nitrogen. Following peptic digestion, the percentage of nitrogen in peptide linkages of the albumin liberated by the pancreatic extract was higher than that of the globulin, 41.9 per cent as compared with 34.2 per cent;

while the amount liberated by intestinal mucosa extract following peptic and pancreatic digestion was much higher in the case of globulin than that in the case of albumin, 28.5 per cent as compared with 13.0 per cent. The total digestibilities by the 3 groups were 82.4 and 73.9 per cent for globulin and albumin respectively. The concentration of pancreatic extract was about 0.8-1.0 Willstätter unit* per mg. of protein nitrogen, and the concentration of intestinal mucosa extract was about 1.2-1.5 ml. activity** per mg. of protein nitrogen. Frankel (49) reported that for many of the seed proteins, peptic activity liberated about 15-20 per cent of the available amino nitrogen in less than 100 hours. Trypsin acting on protein partially digested with pepsin effected a cleavage equivalent to about 70 per cent in 100 to 150 hours, while erepsin acting upon material that had been digested with pepsin and trypsin could disintegrate the protein so that only 10 to 15 per cent of the amino nitrogen was left in peptide linkage. Jones and Waterman (50)

*One unit is that quantity of enzyme under the stated conditions of the test which effects a cleavage equivalent to 1.05 ml. of 0.2 N sodium hydroxide.

**The activity of the enzyme preparation is expressed as ml. of 0.02 N sodium hydroxide per ml. of enzyme solution. That is to say under the stated conditions of the test, 1 ml. of enzyme solution effects a cleavage equivalent to so many ml. of 0.02 N sodium hydroxide.

reported that peptic and tryptic activity liberated about 61.4 per cent of the amino nitrogen in casein, and 48.1 per cent in raw arachin.

SUMMARY

The tung nut has a high content of protein. If the factor 6.25 be used for the calculation of protein content, the nitrogen analyses of two lots of gasoline extracted tung nut meal would indicate a content of approximately 39.1 and 43.6 per cent of protein on the ash-free and moisture-free basis.

Previously reported studies of the toxicity of the defatted tung nut meal have been confirmed in this study.

Two protein fractions, globulin and albumin, were isolated from the tung nut meal. "Globulin" is defined as that fraction of the sodium chloride extract (10 per cent sodium chloride) which was precipitated on removal of the salt by dialysis. "Albumin" is defined as that fraction which was coagulated by heat at approximately neutral reaction after the removal of the globulin. Both of the protein fractions were analyzed for ash, water, nitrogen, sulfur, and nine amino acids. Globulin B was also analyzed for phosphorus.

The average nitrogen content of the globulin and albumin fractions were 17.5 per cent and 14.3 per cent, and the sulfur contents were 1.75 per cent and 0.79 per cent respectively.

The nine amino acids quantitatively determined were threonine, serine, cystine, methionine, arginine, tryptophane, tyrosine, histidine, and phenylalanine. The amino acid contents of the two protein fractions were quite different. The sum of the nitrogen present in these nine amino acids represented 44.3 per cent and 34.7 per cent of the total nitrogen of the globulin and albumin fractions respectively. In terms of sulfur, the methionine and cystine contents of globulin accounted for 96.6 per cent of the total sulfur, and those of albumin accounted for 101.2 per cent.

The digestibilities of these proteins <u>in vitro</u> by the gastro-intestinal enzymes were slightly lower than that of many of the seed proteins, i.e., globulin 82.4 per cent and albumin 73.4 per cent.

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A P P E N D I X

Composition of the Tung Nut Meal Diet

Ingredients	Per cent
Nut meal	20
Casein	10
Starch	35
Glucose	20
Lard	5
Cod liver oil	4
Salt mixture	4
Celluration	2

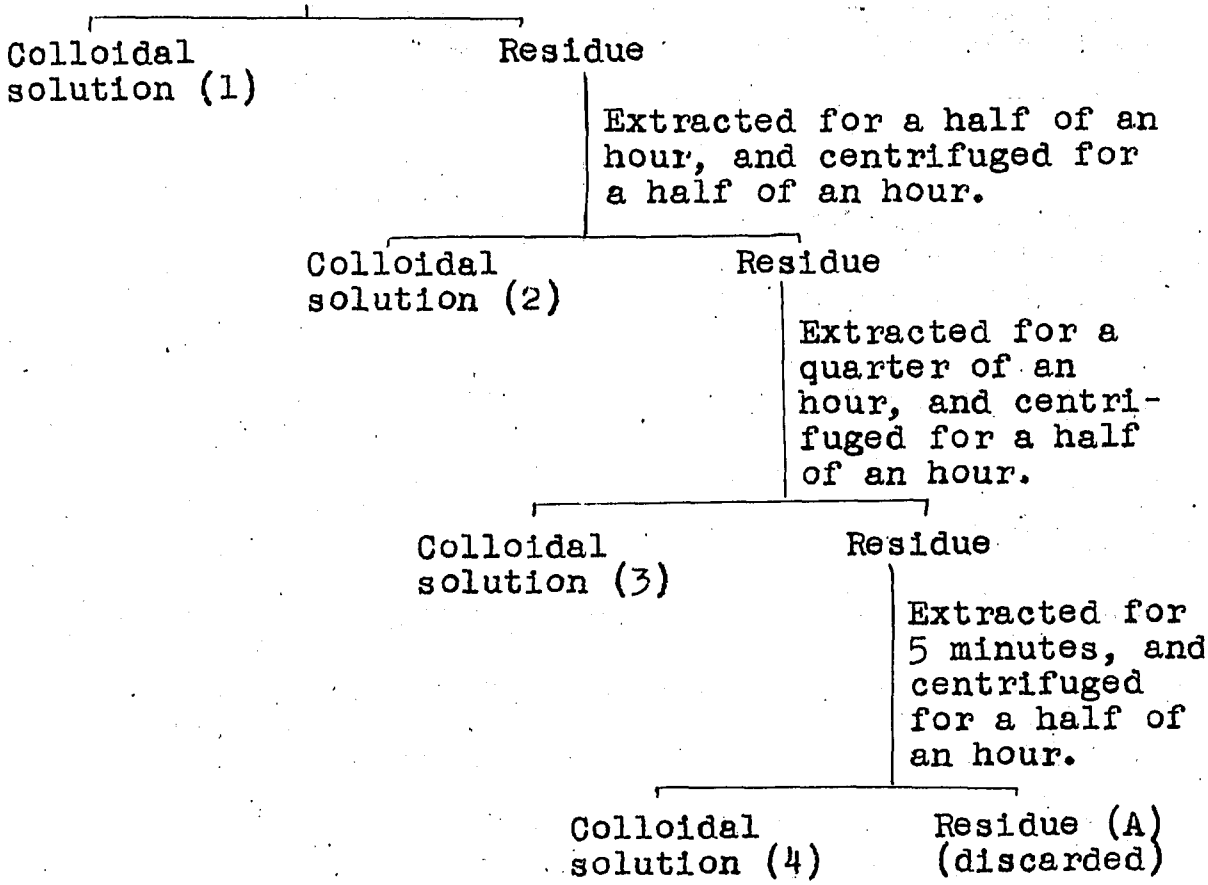
Composition of the Modified Osborne and Mendel Salt Mixture (51)

Salts	Gm.
Calcium carbonate	134.8
Magnesium carbonate	24.2
Sodium carbonate	34.2
Potassium carbonate	141.3
Phosphoric acid	103.2
Hydrochloric acid	53.4
Sulfuric acid	9.2
Citric acid	111.1
Ferric citrate $\cdot \frac{1}{2}H_2O$	6.34
Potassium iodide	0.02
Manganese sulfate	0.079
Sodium fluoride	0.248
Aluminum potassium sulfate $(K_2Al_2(SO_4)_4 \cdot 24H_2O)$	0.0245
Cupric sulfate	2 ml. of 10 per cent cupric sulfate

Procedure Used in the Extraction Experiments

I. Extraction:

3 gm. of the defatted, air dried nut meal (80 mesh) was extracted for 1 hour with 20-30 ml. of 10 per cent sodium chloride solution. The extract was separated by centrifugation for a half of an hour.



Colloidal solutions (1), (2), (3), and (4) were combined.

II. Removal of suspended particles:

The mixed solutions (1), (2), (3), and (4)

50 per cent potassium hydroxide solution was added dropwise until the supernatant liquid became clear (usually about 5 drops were required). The amount depended on the amount of suspended material. The precipitate was centrifuged off.

Solution (5) was pale yellow and very slightly turbid.

Residue

washed twice with 10 per cent sodium chloride solution (pH10). Centrifuged.

Solution (6)

Residue (b)

Solution (5) and (6) were mixed, and the precipitate was filtered, and washed 3 times with 10 per cent sodium chloride solution previously adjusted to a pH of about 10. The filtrate and washings were combined, and diluted to 100 ml., and 10 ml. of it were used for nitrogen determination. Duplicates were made on each extract.

Procedures for Hydrolysis

Alkaline Hydrolysis.

0.2 gm. of protein was weighed out and transferred to a 25 ml. Pyrex test tube fitted with an air condenser. An ordinary glass tubing (35 cm. long) will serve this purpose. The stopper should be wrapped with tin foil.

10 ml. of 5 N sodium hydroxide were used for the hydrolysis, which was carried out in an oil bath, at 100° C. for 24 hours. At the end of the hydrolysis, 2.8 ml. of 18 N sulfuric acid were added to the hot hydrolyzate. The addition of acid was done dropwise with careful shaking. Near the neutral point, a precipitate of silica appeared. The solution was then cooled. It was filtered through an ordinary quantitative filter paper, the paper was washed with N sulfuric acid, and the combined solution and washings were diluted to 50 ml. with N sulfuric acid. The time for filtration sometimes might be very long. Centrifugation of the solution before filtration was usually effective in decreasing the time of filtration. Sometimes the filtrate was colored. In that case, a blank was determined.

Acid Hydrolysis.

One gram of protein was weighed out, and transferred to a 200 ml. round bottom flask connected by a ground glass joint with a water condenser. However, if such an apparatus is not available, an ordinary Kjeldahl flask (300 ml.) fitted with a Liebig condenser and a rubber stopper will also serve the purpose. For one gram of protein 20 ml. of hydrochloric acid (1:1) were used. The hydrolysis was carried out for 24 hours in an oil bath at 120° C. Humin was filtered off and the filtrate was diluted to 100 ml. with water. Aliquots were taken and proper dilutions were

made as required for the determination of each amino acid.

The hydrolyzate prepared in the above manner did not give the same value for methionine as the hydrolyzate prepared by the method described by McCarthy and Sullivan (12). Therefore a separate sample of protein hydrolyzed according to McCarthy and Sullivan's (12) method was used for the determination of methionine. The procedure is briefly as follows:

A sample of 0.2 gm. was hydrolyzed for 10 hours with 2 ml. of hydrochloric acid (1:1) in an oil bath at 125° C. The hydrolyzate was poured into a 50 ml. beaker and the flask was washed with 2 ml. of water. The solution was decolorized by warming with 50 mg. of carboraffin at about 80-90° C. The carboraffin (Carbex E) was washed with 5 ml. of warm (approximately 60° C.) N hydrochloric acid and 5 ml. of cold (room temperature) N hydrochloric acid. The combined washings and filtrate were neutralized to pH 3.5 with 5 N sodium hydroxide (using Congo red paper as an indicator) added dropwise with stirring, and the solution was then diluted with 0.1 N hydrochloric acid to 50 ml. Methionine was determined in 5 ml. portions.

Determination of Threonine

For the determination of threonine in protein hydrolyzates, there are few methods available. These depend on oxidation of the threonine with either periodate or lead tetraacetate. The latter has not been used extensively. In the second edition of "The Determination of of Amino Acids," Block and Bolling (13) recommended the use of sodium periodate as an oxidizing agent instead of the lead tetraacetate which they (52) had suggested previously.

Van Slyke, Hiller, MacFadyen, Hastings, and Klemperer (53) reported that the formation of ammonia by the action of alkaline periodate on hydroxy-amino acids such as hydroxylysine, serine, threonine, i.e., those in which the amino and hydroxy groups are on adjacent carbon atoms, can be used to differentiate these acids from other acids which do not have the amino and hydroxy groups in this relationship.

The method proposed by Shinn and Nicolet (21) in 1941 is based upon the oxidation of threonine with periodate at pH 7 to yield acetaldehyde. Sodium bisulfite is used to trap the volatile acetaldehyde which is removed by aeration. After the excess of bisulfite is removed, the resulting acetaldehyde-bisulfite compound is dissociated with dry sodium bicarbonate, and the bisulfite set free is titrated immediately with standardized iodine solution. The reactions are as follows:

(1) $CH_3CHOHCHNH_2COOH + HIO_4 \longrightarrow CH_3CHO + CHOCOOH + NH_3 + HIO_3$

(2) $CH_3CHO + NaHSO_3 \rightleftharpoons CH_3CHOHSO_3Na$

(3) $H_2O + NaHSO_3 + I_2 \longrightarrow NaHSO_4 + 2\ HI$

For Shinn and Nicolet's (21) method, samples containing 5 mg. or more of threonine are necessary. Winnick (29) in 1942 reported a micro-adaptation of the above method. In this micro-method, the Conway microdiffusion unit is substituted for the aeration apparatus of Shinn and Nicolet (21). Samples containing 0.2 to 0.5 mg. of the amino acid are satisfactory.

Block and Bolling (13) made use of the reaction between acetaldehyde and p-hydroxydiphenyl to yield a red colored compound, in the determination of threonine.

Both Shinn adn Nicolet's (21) and Winnick's (22) methods were studied in order to compare the reliability and advantages of these two procedures. When pure threonine was used, recovery was 96 per cent by Shinn and Nicolet's (21) method and 94 per cent by Winnick's (22) method. When threonine was determined in the presence of serine, Shinn and Nicolet's method gave consistently 96 per cent, and Winnick's method also gave 96 per cent recovery. Casein (Labco) was found to contain 3.69 per cent threonine by Shinn and Nicolet's (21) method, but 4.16 per cent by Winnick's (22) method. The threonine value, 4.10-4.46 per cent, reported by Winnick (22) was also higher than the

figure 3.43-3.68 per cent, reported by Shinn and Nicolet (21). Winnick (22) offered no explanation for the higher results obtained by his procedure. The data of the present investigation are given in Tables XVI and XVII. Details of the procedures as used are presented later.

Table XVI

The Recovery of Threonine from Pure Solution and from Mixtures of Serine and Threonine

Shinn and Nicolet's Method				Winnick's method			
Present		Recovery of threonine		Present		Recovery of threonine	
Threonine	Serine			Threonine	Serine		
mg.	mg.	mg.	per cent	mg.	mg.	mg.	per cent
10.0	0.0	9.62 9.63	96	0.4	0.0	0.374 0.377	94
5.0	10.0	4.83 4.76	96	0.4	0.8	0.386 0.387	97
10.0	10.0	9.63 9.58	96	0.4	0.4	0.379 0.377	95

Table XVII

Threonine Content of Casein (Labco)

All values are calculated on an air dried basis. The nitrogen content of casein is 15.37 per cent on moisture-free and ash-free basis.

Sample Number	Treatment	Shinn and Nicolet's method	Winnick's method
		per cent	per cent
1	0.5 gm. casein per 100 ml., not decolorized with Norit	----	4.00
2	"	----	4.06
3	"	----	3.98
4	3.0 gm. casein per 50 ml., decolorized with 1 mg. of Norite	3.48	3.98
5	"	3.56	----
6	"	3.57	----
7	"	3.47	----
8	"	3.60	----
9	"	3.65	----

Procedure for the Determination of Threonine by Shinn and Nicolet's Method

Reagents.

0.1 N sodium arsenite. Containing 20 gm. of sodium bicarbonate per liter.

1 M sodium bicarbonate.

0.5 M periodic acid.

2 per cent sodium bisulfite. Containing 19 gm. of sodium metabisulfite per liter.

Saturated sodium bicarbonate.

0.1 N sodium thiosulfate. The ultimate standard to which various dilutions of this and of iodine are referred. The solution will be more stable if 10 ml. of 0.1 N sodium hydroxide are added per liter of thiosulfate solution.

0.1 N potassium iodate. For standardizing the sodium thiosulfate.

0.1 N iodine solution. 6.75 gm. iodine plus 12 gm. potassium iodide per 500 ml.

0.02 N iodine solution. 20 ml. of the 0.1 N solution plus 1.5 gm. potassium iodide and diluted to 100 ml. This solution should be prepared and standardized against 0.02 N sodium thiosulfate solution just before use.

1 N iodine solution.

1 per cent starch solution. 0.5 gm. of soluble starch in 50 ml. water. Freshly prepared.

Procedure.

Two to three grams of protein are hydrolyzed with 40 ml. hydrochloric acid (1:1). Most of the acid is removed by evaporation in vacuo after the hydrolysis is completed. The solution is transferred into a 100 ml. beaker, and is warmed with 1 gm. of Norite at about 80-90° C. The Norite is then filtered off, and washed. The filtrate and washings are combined and diluted to such volume that 5 ml. of the solution will contain about 3-10 mg. of threonine.

Before the reaction is started, the three tubes are charged as follows: Into the reaction tube 1 are introduced in the indicated order (1) the aliquot preferably to contain 3 to 10 mg. of threonine, and in a volume not much in excess of 5 ml.* (2) 5 ml. of molar sodium bicarbonate, (3) 10 ml. of sodium arsenite solution.

Tubes 2 and 3 should contain respectively 5 ml. and 3 ml. of 2 per cent sodium bisulfite, diluted in each case to 25 ml. If 2 sets of tubes are connected in series, so that two determinations can be run at the same time, a tube containing 25 ml. saturated sodium bicarbonate should be

*As suggested by the authors, for a relatively pure threonine solution, a quantity of alanine amounting to at least ten times the equivalent of threonine present in the solution should be added.

inserted between the two sets of tubes.

The apparatus is then connected to a source of carbon dioxide, and the gas is passed slowly for several seconds to mix the contents of all the tubes. One to two ml. of 0.5 M periodic acid is pipetted into the introduction tube with its stopcock closed.* Then, the periodic acid is forced down into the reaction tube 1 by blowing, with the stopcock opened. Now the rate of the carbon dioxide gas passing through the system is increased to about one liter per minute, and passage of the gas is continued for one hour.

At the end of the period, the contents of tubes 2 and 3 are quantitatively transferred to a 250 ml. Erlenmeyer flask. Before the "bound" bisulfite is titrated, the excess of free sodium bisulfite must be first removed by titrating with N iodine solution using 1 per cent starch solution as indicator. The end-point (first appearance of purple color) should be carefully adjusted with more dilute iodine solution, as the accuracy of the method depends greatly on the complete removal of the free sodium bisulfite. If the end point should be passed in the process of removing the excess of bisulfite, sodium bisulfite can be used to readjust the end-point.

After the excess of bisulfite is removed, 1-2 gm. of sodium bicarbonate is added to dissociate the acetaldehyde-

*In this study, a tube with stopcock was prepared from a broken burette.

bisulfite compound. The sodium bicarbonate should be added with shaking, and the exact amount necessary is determined by the first disappearance of the purple color of the solution. The colorless solution now is titrated immediately with 0.02 N iodine solution to the same intensity of purple as that used as an end-point in removing the excess of sodium bisulfite. One ml. of 0.02 N iodine solution is equivalent to 1.19 mg. of threonine.

Procedure for the Determination of Threonine by Winnick's Method

Reagents.

0.25 M sodium bisulfite.

0.1 M tripotassium phosphate.

0.2 M periodic acid.

1 N iodine solution.

0.005 N iodine solution. 25 ml. of 0.1 N iodine solution in 500 ml., to which 9.5 gm. of potassium iodide is added. The solution should be standardized just before use.

1 per cent starch solution. Freshly prepared.

3 N sodium hydroxide. For neutralizing hydrolyzate.

Disodium acid phosphate. Dry powdered.

Procedure.

A sample of protein is hydrolyzed with hydrochloric acid (1:1). The acid hydrolyzate is neutralized with 3 N sodium hydroxide, and diluted to such a volume that 2 ml. contain about 0.2-0.5 mg. threonine. In this study an aliquot of the acid hydrolyzate containing 0.1 gm. protein was neutralized with sodium hydroxide to a pH of about 7. The solution was filtered, and diluted to 25 ml. 2 ml. were used for each determination.

1 to 1.5 ml. of 0.25 M sodium bisulfite is pipetted into the central chamber of the diffusion unit. A 2 ml. aliquot of the solution to be analyzed is pipetted into the outer chamber. 1 ml. of approximately 0.1 M tripotassium phosphate is delivered also into the outer chamber. This solution is adjusted so that 1 ml. will just neutralize 1 ml. of 0.2 M periodic acid (to be added in the next step) to a pH of about 7.0. The greased glass cover-plate is placed over the unit, leaving a narrow opening. Through this slit 1 ml. of 0.2 M periodic acid is pipetted quickly into the outer chamber. One slides the cover over immediately so as to seal the vessel completely, and the latter is rotated to mix the solutions in the outer chamber. Usually 15 clockwise and 15 counterclockwise rotations are sufficient.

After the unit has stood for at least 4 hours at room temperature, the cover is removed and the excess bisulfite is

oxidized with 1 N iodine, using a very small drop of starch solution as indicator. Final adjustment may be done with the use of more dilute iodine solution. About 0.3 to 0.4 gm. of powdered disodium acid phosphate is added to dissociate the acetaldehyde-bisulfite compound, and the liberated bisulfite is titrated with standard 0.005 N iodine solution. A light purple color lasting for about 30 seconds is taken as an end-point. 1 ml. of 0.005 N iodine is equivalent to 0.298 mg. of threonine.

Determination of Serine

The existing methods for the determination of serine can be grouped into three classes, namely: (1) isolation, (2) colorimetric, and (3) gravimetric. The isolation method is not much used because it is not quantitative and the process is most tedious.

Two colorimetric methods are available, that of Rapoport (54) and that of Boyd and Logan (19). The method of Rapoport involves the formation of glyceric acid by the deamination of the amino acid and the determination of the glyceric acid either colorimetrically or by oxidation to oxalic acid. In either case, glycine interferes.

The colorimetric procedure of Boyd and Logan (19), and the gravimetric method of Nicolet and Shinn (20) both depend on the oxidation of serine by periodic acid. The formaldehyde liberated is then determined. Boyd and Logan (19) made use

of the color reaction of formaldehyde with chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid). Nicolet and Shinn (20) made use of the reaction between formaldehyde and dimedon to form a precipitate. The colorimetric method is the more sensitive of the two. By this method, amounts of serine from 1 to 5 mg. can be determined, while the gravimetric method of Nicolet and Shinn requires about 10-20 mg. of serine. The reagent is specific for formaldehyde and produces no color with acetaldehyde or higher homologues. 50 to 100 micrograms of formaldehyde in a volume of 50 ml. produced adequate color for estimation in a Duboscq colorimeter. If the reagent is purified and the color developed according to the directions given later, color production is proportional to the concentration when the unknown differs from the standard by not more than 50 per cent. The procedure has an additional advantage in that it is completed in a few minutes. Precipitation with dimedon, on the other hand, is not completed in less than 72 hours. The authors claimed that the error of their colorimetric method would not exceed 1-2 per cent. However, in this study using exactly the same procedure, only 95 to 96 per cent recovery was obtained from either a pure serine solution or a mixture of serine and threonine. Data are shown in Table XVIII.

In the gravimetric determination, serine is oxidized to the formaldehyde and the latter forms a precipitate with

Table XVIII

The Recovery of Serine from Solutions of Pure Serine and of Mixtures of Serine and Threonine

Amino acids present		Nicolet and Shinn's method		Boyd and Logan's method
Serine	Threonine	Uncorrected for solubility	Corrected for solubility	
mg.	mg.	per cent	per cent	per cent
10	0	82	94	95
10	5	87	98	95
10	10	86	97	96

dimedon (5,5-dimethyldihydroresorcinol). Two moles of dimedon react with 1 mole of aldehyde to form 1 mole of dimedon derivative and 1 mole of water. Voländer (55) in 1929 studied dimedon as a reagent for the determination of aldehydes. He recommended a correction for the loss due to the solubility of the dimedon derivative in water, 0.001 gm. formaldehyde-dimedon derivative per each 100 ml. of water solution and per each 100 ml. of wash-water. However, this correction was not used by Nicolet and Shinn (20) in the determination of serine. On reply to the question raised by this laboratory about this correction factor, Dr. Nicolet (private communication) has stated: "We have not used solubility corrections for the reason that we were chiefly interested in getting from nowhere to very good, instead of from very good to "perfect." We are convinced

that the salt content of the solution in which precipitation takes place, makes any previous determination of solubility, pure guesses." In another private communication he said: ". . . As the process is carried out somewhat rapidly, and with suction, it seems clearly unlikely that anything like equilibrium is reached between solid and solvent. Accordingly the full correction for solubility would be clearly dubious, and any correction made would have to be separately justified." They used a very small amount of water for washing, and while in this laboratory, at the beginning of the study, a much greater amount of water was used. Hence, the correction factor though not important in their case, might be necessary in the latter, as it was found that when a large amount of water, say about 200 ml., was used, application of the correction brought the recovery value from 86 per cent to 97 per cent. Data are shown in Table XVIII. The purity of the dimedon derivative was confirmed by melting point determinations--189-189.5° C.* When their detailed procedure (obtained through private communication) was strictly followed, the same amount of serine in casein as reported by the authors was found in this laboratory. The amount of water used here for washing the precipitate was only 30 ml.

It was found that the serine content of casein

*All melting points reported in this study are corrected.

determined by Boyd and Logan's method was nearly the same as that determined by Nicolet and Shinn's method. However, if Boyd and Logan's method was applied to the hydrolyzate which was treated with Norite as recommended by Nicolet and Shinn, the serine value was usually lower than that obtained by Nicolet and Shinn's method on the same solution. Casein Nos. 12 and 13 in Table XIX show this difference clearly.

Table XIX

Serine Content of Casein

All values are on air dried basis.

Sample number	Treatment	Nicolet and Shinn's method	Boyd and Logan's method
		per cent	per cent
2	0.5 gm. casein per 100 ml. No Norite treatment	----	5.24
10	0.5 gm. per 100 ml. No Norite treatment	----	5.13 5.18
10	Same solution as above but treated with Norite	----	4.53
11	3 gm. casein per 50 ml. without Norite treatment	5.47	5.14
12	3 gm. casein per 50 ml. with Norite treatment	5.10	4.57
13	3 gm. casein per 50 ml. with Norite treatment	5.01	4.53

The values obtained by Nicolet and Shinn's method were rather close to that given by Nicolet and Shinn but that determined by Boyd and Logan's method were significantly lower than that reported by Boyd and Logan. Casein No. 11 indicates a similar difference from another angle, when the hydrolyzate was prepared according to Boyd and Logan's direction. Here, the serine value determined by Boyd and Logan's method agreed very well with that reported by Boyd and Logan but that obtained by Nicolet and Shinn's method was higher than that originally reported by these authors. In other words, no matter whether the hydrolyzate was treated with Norite or not, Boyd and Logan's method always gave lower results than Nicolet and Shinn's method if the same solution was used. The data also show that the Norite treatment recommended by Nicolet and Shinn (20) interferes somewhat with the serine determination. Casein sample No. 10 as determined by Boyd and Logan's method, had 5.13-5.18 per cent before the treatment with Norite and had only 4.53 per cent after treatment with Norite. According to Dr. Nicolet (private communication), Norite will not absorb any appreciable amount of serine, if the Norite is washed repeatedly with hot, dilute hydrochloric acid. Here in this study only hot water was used for washing the Norite, and the loss amounted to more than 10 per cent of the total serine. Hot dilute hydrochloric acid might be a more efficient liquid for washing but might not be able to elute all the

adsorbed serine easily, and the consequent loss might be just enough to compensate for the difference, so that the results obtained by these two methods appear to check with each other.

The average value of serine determined by Nicolet and Shinn's method (sample Nos. 12 and 13) with Norite treatment is 5.06 per cent on air dried basis, and 5.23 per cent on moisture-free and ash-free basis. With Boyd and Logan's method without Norite treatment, the average value (samples Nos. 2, 10 and 11) is 5.17 per cent on air dried basis and 5.35 per cent on moisture-free and ash-free basis.

In another private communication, Dr. Nicolet stated that acid hydrolysis would destroy something like one-half of the portion of serine combined in ester linkage with phosphoric acid. This discovery throws doubt on the determination of the serine content of many proteins, especially that of casein.

Since all aldehydes form derivatives with dimedon, it is necessary to remove aldehydes which form precipitates with dimedon in the determination of serine. In the procedure given for the determination of threonine, acetaldehyde is aerated quantitatively into bisulfite solution. The solution left behind will contain only formaldehyde if no other

hydroxy-amino acids are present.* This solution can be conveniently used for the determination of serine.

Procedure for the Determination of Serine by Nicolet and Shinn's Method

Reagents.

0.4 per cent aqueous 5,5-dimethyldihydroresorcinol solution.

0.1 N sodium arsenite. Containing 20 gm. of sodium bicarbonate per liter.

1 M sodium bicarbonate.

0.5 M periodic acid.

2 per cent sodium bisulfite. Containing in addition 19 gm. of sodium metabisulfite per liter.

Saturated sodium bicarbonate.

Saturated methyl red in 0.05 N hydrochloric acid.

Glacial acetic acid.

Procedure.

As was mentioned previously, with Nicolet and Shinn's method, threonine and serine can be determined advantageously in the same sample, for in order to determine serine,

*If hydroxylysine is a constituent of the protein hydrolyzate, formaldehyde would be formed by periodate oxidation and thus calculated as serine. Dr. Nicolet recently devised an improved method (private communication) for determination of serine in a solution which also contains hydroxylysine.

acetaldehyde must be removed.

A sample, which should preferably contain 10 to 20 mg. of serine, is treated exactly as described for the determination of threonine. After removal of the acetaldehyde the solution is transferred to a 250 ml. Erlenmeyer flask, 1 drop of methyl red indicator is added, and then acetic acid drop by drop until the color changes from yellow to a faint red. The volume is now usually about 50 ml.

The formaldehyde is now precipitated as the dimedon derivative, by adding 100 ml. of 0.4 per cent dimedon solution. Oxidation of 1 mole of serine would give 1 mole of formaldehyde, and 1 mole of glyoxylic acid, and each of them reacts with 2 moles of dimedon. The dimedon derivative of glyoxylic acid is soluble, hence only the formaldehyde is determined. In order to precipitate the formaldehyde completely, at least 4 moles of dimedon must be added. Upon periodate oxidation, 1 mole of threonine also gives rise to 1 mole of glyoxylic acid in addition to acetaldehyde. Therefore if a solution contains 1 mole of serine and 1 mole of threonine, 6 moles of dimedon is necessary for the complete precipitation of formaldehyde.

The precipitation should take place at room temperature in the dark for 72 hours. The precipitate is collected on a sintered glass crucible, washed three times with water, and dried in a vacuum desiccator. The total amount of water used for transferring the precipitate and washing

should not exceed 50 ml. After the precipitate is weighed, its purity may be tested by determination of melting point and of mixed melting point. The melting point of the pure derivative has been found here to be 189-189.5° C., which is the same as given by Nicolet and Shinn.

For the calculation of results, 1 mg. of the dimedon derivative is equal to 0.3596 mg. of serine.

Procedure for the Determination of Serine by Boyd and Logan's method

Reagents.

25 per cent potassium arsenite. 25 gm. in 100 ml. water.

0.5 M periodic acid. 56.99 gm. per 500 ml.

Saturated methyl red in 0.05 N hydrochloric acid.

0.12 N potassium cyanide.

0.1 N ammonium thiocyanate.

Saturated ferric ammonium sulfate solution.

Stannous chloride.

Hydrogen sulfide gas.

Talcum powder.

0.100 N silver nitrate.

0.1 M chromotropic acid. 0.9 gm. of the purified crystals is dissolved in water and diluted to 25 ml. in a volumetric flask. Approximately 50 mg. of stannous chloride are added, and the solution shaken and filtered through an ordinary quantitative filter

paper. If not clear, refilter through the same filter paper. The filtrate usually has a slightly yellow color. The solution should be stoppered immediately. If it is not exposed to the air except for withdrawal of samples, the solution should be good for at least two days.

Formaldehyde working standard. A portion of the stock standard is diluted with water to contain 15 micrograms of formaldehyde per ml. This standard should be freshly prepared on the day it is used.

Purification of Chromotropic Acid.

25 gm. of the impure, colored chromotropic acid, purchased from Eastman Kodak Company, are dissolved in 100 ml. of water in a flask on a steam bath. 2 gm. of lead carbonate are added. After solution has occurred, hydrogen sulfide is bubbled through the solution until all the lead is precipitated. The flask is stoppered and the lead sulfide allowed to settle. If the supernatant liquid is not pale yellow, more lead carbonate and hydrogen sulfide are added. The solution is filtered hot without contact with air. This can be done by previously flushing the receiver with carbon dioxide gas, and filtering in a carbon dioxide atmosphere. The flask is stoppered and the substance crystallizes out at 4° C. The crystals are filtered with suction, washed with alcohol and ether, and dried in a vacuum desiccator. The product should be nearly white.

Preparation of Formaldehyde Stock Standard.

100 gm. of paraformaldehyde in 100 ml. of water are hydrolyzed with 20 ml. of 2 N sulfuric acid by heating at 90° C. until no precipitate of paraformaldehyde remains. It is then steam distilled. This distillate is returned to the original flask and redistilled after the addition of 10 ml. of 2 N sulfuric acid. The stock formaldehyde solution thus obtained should be approximately 6 M. If 1 ml. of 2 N sulfuric acid per liter is added to the stock solution, no perceptible change in the concentration of the formaldehyde takes place over a period of a month. It is standardized essentially according to the procedure of Romijn (56).

An aliquot equivalent to 0.2-0.3 ml. of the stock solution is added to 20 ml. of 0.12 N potassium cyanide. To this are added first 3 ml. of concentrated nitric acid and then 25 ml. of standard 0.100 N silver nitrate. The solution is diluted to 100 ml. in a volumetric flask, filtered, and 75 ml. of the filtrate are titrated with 0.1 N ammonium thiocyanate, 5 ml. of saturated ferric ammonium sulfate being used to detect the end point. The potassium cyanide solution is standardized by the same procedure except that no aldehyde is added.

The titration depends upon the fact that potassium cyanide forms a complex with formaldehyde. A certain amount of formaldehyde is added to a known amount of potassium cyanide, and the excess of potassium cyanide is precipitated

by a known amount of silver nitrate. Finally, the amount of silver nitrate left is titrated with thiocyanate.

1. $KCN + HCHO \rightleftarrows KCNHCHO + KCN$ (excess)

2. KCN (excess) $+ AgNO_3 \longrightarrow AgCN + KNO_3 + AgNO_3$ (excess)

3. $AgNO_3$ (excess) $+ NH_4SCN \longrightarrow AgSCN + NH_4NO_3$

If the same known amount of potassium cyanide is treated in the same way but without the addition of formaldehyde, one can easily calculate the amount of potassium cyanide reacted with formaldehyde. 1 ml. of 0.1 N thiocyanate is equal to 3 mg. of formaldehyde.

4. $KCN + AgNO_3 \longrightarrow AgCN + KNO_3 + AgNO_3$ (excess)

5. $AgNO_3$ (excess) $+ NH_4SCN \longrightarrow AgSCN + NH_4NO_3$

The number of ml. of thiocyanate used in equation (3) minus that used in equation (5) multiplied by 3 will give the number of mg. of formaldehyde in the solution used. Since here only 75 ml. out of the 100 ml. solution is used, correction must be made.

Procedure.

The acid hydrolyzate used for the determination of threonine by Winnick's method is suitable for this determination. An aliquot containing 1 to 5 mg. of serine is introduced into a 300 ml. Kjeldahl flask. To this, 3 drops of methyl red indicator are added to make sure that the solution is neutral. If not, a final adjustment should be made by very dilute acid or base. 4 ml. of 25 per cent

potassium arsenite and 2.5 to 2.8 ml. of 0.5 N periodic acid are added. The last 0.5 ml. of periodic acid is added drop by drop with continuous gentle shaking. The addition of periodic acid should be stopped when the mixture is acid to methyl red. Sometimes the change is difficult to see, in that case one can judge by the initial liberation of iodine, which imparts a brown color to the solution. This color will disappear after shaking. However, if too much of periodic acid has been added as indicated by a permanent brown color, the solution should be discarded. A pinch of talcum powder and enough water to make the final volume of 70 ml. are now added. The Kjeldahl flask is placed in an upright position and connected to a vertical water condenser, the cooling surface of which is 25 to 35 cm. in length. Sufficient water to cover the end of the condenser tube (5-10 ml.) is placed in a receiver and the solution distilled over until about 5 ml. remain in the Kjeldahl flask. Several times during the distillation the contents of the flask are given a swirl to wash down the iodate, which splatters and becomes dried on the sides of the flask. This precaution should be observed particularly near the end of the distillation. Distillation is complete after 10 to 12 minutes of vigorous boiling. The volume of the distillate is made up to 100 ml.

Colorimetric estimation.--5 ml. of standard formaldehyde solution (containing 15 micrograms per ml.) are pipetted into

a 1 by 8 inch test tube graduated at 50 ml. An amount of the distillate containing from 50 to 100 micrograms of formaldehyde is added to a similar tube. To each tube is added 0.5 ml. of 0.1 M chromotropic acid and enough water to bring the final volume to 17 ml. The tubes are cooled in an ice bath and 10 ml. of concentrated sulfuric acid are added to each during 40 to 45 seconds with gentle shaking. The tubes are kept in the bath until the solutions are again cooled to the temperature of the bath. They are then diluted to the mark by allowing concentrated sulfuric acid to flow down the center of the tubes. The temperature of the contents rises to about 80° C. The tubes are now heated for 10 minutes in a boiling water bath, cooled to room temperature, and the color comparison is made in a colorimeter equipped with all-glass cups, within an hour. 3.50 micrograms of serine are equivalent to 1 microgram of formaldehyde.

Determination of Cystine

Several methods for the determination of cystine have been suggested in recent years and these fall into three classes.

(1) Gravimetric. In 1933, Vickery and White (57) employed cuprous oxide as a precipitating agent to determine cystine in proteins. Since chloride ions interfere with

the precipitation, sulfuric acid is used for the hydrolysis of the protein. Cystine in the hydrolyzate is reduced to cysteine by tin filings. Under the conditions described by the authors, cysteine can be precipitated to the extent of at least 98 per cent. From their analytical data, they thought the precipitate had the composition $C_3H_6O_2NSCu \cdot CuSO_4 \cdot 2H_2O$. The mercaptide was decomposed with hydrogen sulfide. After the removal of copper sulfide and sulfate ions, the organic sulfur was determined, and the cystine content was calculated. They found that the values obtained by this method checked with those obtained by the methods of Sullivan, and Folin-Marenzi. This gravimetric method requires 20 mg. of cystine, i.e., usually 2 gm. of protein. Therefore it is not a very desirable procedure if the protein to be studied is not available in relatively large amounts.

Graff, Maculla, and Graff (58), in 1937, suggested the use of zinc for the reduction of cystine to cysteine instead of tin as recommended by Vickery and White (57). The cysteine is then precipitated with cuprous oxide. The cystine content is determined either by a sulfur or nitrogen estimation of the mercaptide. This method requires only 2-5 mg. of cystine.

In 1941, Zittle and O'Dell (59) suggested a further modification of the cuprous precipitation method. Direct use of cuprous oxide to reduce cystine and to precipitate

cysteine is recommended. The cysteine in the mercaptide precipitate can be determined gravimetrically by the estimation of sulfur, or after removal of the copper with thiocyanate, by the colorimetric method of Sullivan. They emphasized the dangers in the use of zinc as reducing agent, for zinc phosphate may adsorb some cysteine.

(2) Colorimetric. There are three colorimetric methods commonly used for the determination of cystine and cysteine.

(a) The Folin-Marenzi method. The method of Folin and Looney (60) is based on the fact that cysteine is the only known amino acid which reduces the uric acid reagent of Folin and Denis in alkaline solution and gives rise to a blue color. For the determination of cystine, sulfite is added to an alkaline solution of cystine to convert it into cysteine. According to Clarke (61) the reaction is a double decomposition. One mole of cysteine and one mole of sodium S-cysteine-sulfonate are formed from one mole of cystine and one mole of sodium sulfite according to the following equation:

$$R\text{-}SS\text{-}R + Na_2SO_3 = R\text{-}SNa + R\text{-}SSO_3Na$$

Formerly this reaction was thought to be a simple reduction. In 1929, Folin and Marenzi (28) introduced a modification of the Folin-Looney method. The phosphotungstic acid reagent was perfected which would eliminate the

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influence of tyrosine in this method, as tyrosine will not react with this new reagent. Furthermore, they found that addition of sulfite to acid solution instead of alkaline solution as described by Folin and Looney reduces the amount of sulfite needed, thereby reducing the reading for the blank produced by the sulfite.

According to Lugg (62), the reaction of cystine with sulfite is slightly different from that suggested by Clarke (61). He suggested that first, one mole of cystine is decomposed to give one mole of cysteine and other decomposition products. The resultant cysteine reacts with the color reagent and gives half mole of cystine plus the reduced color reagent. In form of equation, they will be:

$$R\text{-}SS\text{-}R + SO_2 + H_2O \longrightarrow RSH + RHS_2O_3 \qquad (1)$$

$$RSH + \text{reagent} \longrightarrow \frac{1}{2}\ R\text{-}SS\text{-}R + \text{reduced reagent} \qquad (2)$$

Since sulfite is present, the resulting half mole of cystine in equation (2) will repeat the course as shown in equation (1) and yield half mole of cysteine which again reacts with the color reagent as shown in equation (2) and gives a quarter of a mole of cystine. The process repeats, each time the amount of cystine formed is only half of that in the preceeding reaction. Eventually a point is arrived where practically no cystine is left and the reactions come to a stop. The amount of reduced reagent would by then have attained a total of two moles.

It is therefore evident that the color developed with the reagent by one mole of cystine alone is zero, and by one mole of cystine plus sulfite is two moles. The fact that one mole of cysteine gives two moles of color can be similarly explained.

Lugg (62) found that the reaction is best carried out at pH 5.0-6.5. If the amount of reducing substance is not too large and there is sufficient reagent present, the color produced is strictly proportional to the amount of cystine or cysteine. However, the color developed by a reducing substance may vary with the time allowed for the reaction, the salt concentration and the presence of substances which may react with a particular reducing substance or with the reagent. An increase in the concentration of salt usually increases the color developed by sulfite alone. Mercuric chloride inhibits the color development of cysteine but not that of sulfite. It is therefore useful in estimating non-cystine reducing substances. It also offers a clue to the determination of cystine in the presence of cysteine or vice versa.

(b) Sullivan's method. This has been claimed to be the most specific method for cystine, 0.5-1.0 mg. cystine can be estimated by this procedure. This reaction is based on the formation of a red color when cysteine is treated with sodium β-naphthoquinone-4-sulfonate in the presence of alkali and a reducing agent such as sodium sulfite. For

the determination of cystine, it must be first reduced to cysteine before the color is developed. The reaction is:

$$R\text{-}SS\text{-}R + NaCN \longrightarrow RSNa + RCNS$$

Sodium hydrosulfite is added to destroy the color due to the presence of substances other than cysteine, and this renders the method more specific for cysteine.

It is known that the colored decomposition products and the presence of other amino acids render the colorimetric determination of cystine in protein hydrolyzates rather difficult. Hence if cystine is isolated from a hydrolyzate before color is developed, the interference due to other amino acids, etc., can be eliminated. In 1935, Rossouw and Wilken-Jorden (30) combined the cuprous mercaptide precipitation and Sullivan's (63) colorimetric method. Cuprous chloride was used both to reduce the cystine to cysteine, and to precipitate the cysteine. The resultant mercaptide is dissolved in acid. Cuprous ions are removed by potassium thiocyanate and pyridine. Cysteine in the solution is determined by Sullivan's method (63).

Evans (64), and Csonka et al. (65), recently reported the use of the spectrophotometer in the determination of cystine by Sullivan's method.

(c) Vassel's method. (66). The basis of this method is the formation of a blue color when cystine is heated with p-aminodimethylaniline in the presence of hydrochloric

acid, ferric ions, and zinc. Here, zinc acts not only as a reducing agent but probably also as a condensation agent. This procedure can be used to estimate from 0.01 to 0.20 mg. of cystine or cysteine per ml. of solution, with an average error of ± 3 per cent.

In 1943 Mecham (67) introduced a modification for Vassel's method. He suggested the combination of all the reagents to form a single reagent solution. With modification, the error of the original Vassel's method can be decreased from ± 3 per cent to ± 1.4 per cent.

(3) Iodimetric. Okuda (68) titrated cysteine in the presence of bromide and acid with a bromate solution, under which conditions the cysteine is oxidized to cystine and then to cysteic acid. This is the so-called bromine method. He also titrated cysteine in the presence of iodide and acid with a iodate solution. This method could be applied to the estimation of cystine after reduction under suitable conditions. Yamazaki (69) described a potentiometric method for the determination of cystine based on the same principle.

Baernstein (70) introduced another modification of the same method. Cystine was reduced in hydrochloric acid with Devarda's alloy* and the cysteine was reoxidized with excess iodine in potassium iodide, the excess of iodine was

*Later he used zinc instead of the alloy.

estimated by measuring the increase in pressure produced by the evolution of nitrogen from hydrazine sulfate in the Van Slyke manometric apparatus.

In the present study the method of Folin and Marenzi and two of its modifications were tried. In Lugg's modification, as given by Block and Bolling (13), mercuric chloride was used to inhibit the color production of cysteine, so that color developed by substances other than cysteine can be corrected for. The recovery of added cystine by this procedure was found to be low. The Rossouw and Wilken-Jorden's precipitation method followed by Folin-Marenzi's method was found to give satisfactory recovery when the last trace of copper was removed by pyridine. For this procedure sulfuric acid hydrolyzate is better because chloride ions interfere with the precipitation.

The method of Folin and Marenzi when applied to protein hydrolyzate has been observed repeatedly to give higher cystine values than those obtained by other methods. We also obtained slightly higher values with this method as shown by the following values of casein. However, if the protein hydrolyzate is clarified or the cystine is isolated before color is developed, the values obtained by the two methods are essentially the same. Acid hydrolysis of protein for the determination of cystine may lead to a loss of cystine due to adsorption of cystine on the humin formed. Sullivan (23) therefore has suggested the use of titanous

chloride to prevent the formation of humin in acid hydrolysis. This procedure was tried and the cystine content of casein was found to be very close to that obtained with an ordinary hydrochloric acid hydrolyzate decolorized with Carbex E as recommended also by Sullivan (12). Furthermore, this value is almost the same as that found by Rossouw and Wilken-Jorden's precipitation method followed by Folin-Marenzi's method. The values are shown in Table XX.

Table XX

Treatment of casein before color development	Lugg's modification of Folin-Marenzi's method as adopted by Block and Bolling	Folin-Marenzi's method
	per cent	per cent
Hydrolyzed with hydrochloric acid for 20 hours and decolorized by neutralization.	0.27	0.33
Hydrolyzed with hydrochloric acid for 20 hours, but decolorized with Carbex E.	0.23	0.25
Hydrolyzed in presence of titaneous chloride	0.24	0.26
Hydrolyzed with sulfuric acid and precipitated by Rossouw and Wilken-Jorden's method.	0.25	0.26

The k value found according to the method of Folin and Marenzi is constant within a limit of 0.4-1.2 mg. of cystine. The k value found according to Lugg's modification as adapted by Block and Bolling is not constant. The data are shown in Tables XXI and XXII. The cystine solution was prepared by dissolving 100 mg. of cystine in 20 ml. water plus 5 ml. concentrated hydrochloric acid and diluting to 500 ml. An aliquot containing the desirable amount of pure cystine was pipetted into a 50 ml. graduated

Table XXI

k Value for the Folin-Marenzi Method

Cystine present	Galvanometer reading	Corrected galvanometer reading	L value	k value
mg.				
0.2	80^{2}	80^{1}	0.0955	23.88
0.2	81^{0}	80^{3}	0.0928	23.20
0.4	67^{2}	67^{1}	0.1723	21.54
0.4	67^{1}	67^{0}	0.1739	21.74
0.4	67^{0}	66^{3}	0.1756	21.95
0.6	56^{2}	56^{2}	0.2480	20.67
0.6	56^{0}	56^{0}	0.2518	20.98
0.6	55^{2}	55^{2}	0.2557	21.31
0.8	46^{0}	46^{0}	0.3370	21.06
0.8	45^{1}	45^{1}	0.344	21.50
0.8	45^{3}	45^{3}	0.340	21.26
1.0	38^{0}	38^{0}	0.420	21.00
1.0	37^{3}	37^{3}	0.423	21.15
1.0	31^{0}	31^{0}	0.509	21.38

The average k value is 21.26

Table XXII

k Value for Lugg's Modification as Adapted by Block and Bolling

Since the k value is not constant, a curve must be used instead of a definite k value.

Cystine present	Galvanometer reading	Corrected galvanometer reading	L value	k value
0.4	72^{1}_{2}	72^{0}	0.1427	17.84
0.4	71^{2}	71^{1}	0.1472	18.40
0.6	60^{0}	60^{0}	0.2218	18.48
0.6	59^{1}	59^{1}	0.2273	18.94
0.8	50^{1}	50^{1}	0.299	18.69
0.8	48^{3}	48^{3}	0.312	19.50
1.0	38^{1}	38^{1}	0.417	20.85
1.0	37^{1}	37^{1}	0.429	21.45
1.2	28^{1}_{3}	28^{1}_{3}	0.549	22.83
1.2	28^{3}	28^{3}	0.542	22.58

cylinder. The blank was prepared by omitting sodium sulfite solution in the Folin-Marenzi method. For the Lugg modification, the blank was prepared by using mercuric chloride to inhibit the color development due to cysteine.

In this study, the method of Folin and Marenzi was adopted on a hydrochloric acid hydrolyzate decolorized with Carbex E for the determination of cystine. This method has another advantage, for methionine can also be determined on the same solution. The detailed procedure is given below.

Procedure for the Determination of Cystine by the Method of Folin and Marenzi

Reagents.

10 per cent lithium sulfate. 14 gm. in 126 ml. water.

Saturated sodium bicarbonate.

10 per cent sodium sulfite. 1 gm. in 10 ml. water. Freshly prepared.

1:1 hydrochloric acid.

1 N hydrochloric acid.

0.1 N hydrochloric acid.

5 N sodium hydroxide.

Cystine solution. 100 mg. of cystine in 20 ml. water plus 5 ml. concentrated hydrochloric acid and dilute to 500 ml. with water.

Phosphotungstic reagent. Prepared by diluting the stock phosphotungtic reagent with an equal volume of water.

Preparation of the stock phosphotungstic reagent. 50 gm. of sodium tungstate are dissolved in 100 ml. of water using a 250 ml. flask. If hydrated sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) is used, 56.1 gm. is dissolved in 94 ml. water. 10 ml. of 85 per cent phosphoric acid are added slowly with shaking and cooling under the tap. The solution must not be allowed to become warm. Hydrogen sulfide is passed into the solution at a very moderate rate for 20 minutes. At the end of the first 3 or 4 minutes, another

5 ml. of phosphoric acid are added slowly without interruption of the passage of the hydrogen sulfide. The 15 ml. of acid should be sufficient to render the solution slightly acid to Congo red paper. If the solution is not acid enough, more phosphoric acid must be added. At the end of the 20 minutes, the solution is filtered and the first 20 ml. of the filtrate are returned to the filter paper. If the conditions of preparation are right, the filtrate should be clear and slightly greenish in color. The filtrate is placed in a 500 ml. separatory funnel and 150 ml. of 95 per cent ethyl alcohol are added with shaking. The mixture separates at once into a reddish or greenish supernatant solution and a bluish subnatant layer. The latter contains all the phosphotungstic acid. The subnatant layer is drawn off into a weighed 500 ml. flask. No solid particles are allowed to pass into the flask. Water is then added to the phosphotungstic acid solution until the weight of the contents amounts to 150 gm. The solution is boiled over a microburner for a few minutes until all the hydrogen sulfide has been removed as tested with lead acetate paper. The flame is diminished and 10 ml. of 85 per cent phosphoric acid are added. The contents of the flask are boiled under a reflux condenser (glass connections only) for 1 hour. The flame is reduced, the condenser is removed, and a few drops of bromine are added to destroy the blue color of the solution. When the blue

color has disappeared, the solution is again boiled rapidly for a few minutes to remove the bromine. The flask is cooled under the tap.

12.5 gm. of lithium carbonate are placed in a 600 ml. beaker and 25 ml. of phosphoric acid are added slowly, then 125 ml. of water are added and the solution is boiled to dispel the carbon dioxide.

When the solution of lithium phosphate is cool, it is added to the phospho-18-tungstic acid and the resulting solution is diluted to 500 ml.

Procedure.

About 0.2 gm. of protein is hydrolyzed with 4 ml. of hydrochloric acid (1:1) for 22 hours at 125° C. The hydrolyzate is decolorized by warming with 20 mg. of Carbex E. The solution is filtered, and refiltered several times through the same filter. It is then washed with 5 ml. of warm (about 80° C.) 1 N hydrochloric acid, followed by 5 ml. (room temperature) 1 N hydrochloric acid. It is neutralized with 5 N sodium hydroxide, using Congo red paper as indicator. Finally the solution is diluted to 50 ml. with 0.1 N hydrochloric acid.

Four aliquots of the hydrolyzate, not exceeding 5 ml., are pipetted into 50 ml. stoppered and graduated cylinders. The solutions are brought to the 5 ml. mark with water. One ml. of 10 per cent lithium sulfate and 5 ml. of

saturated sodium bicarbonate are now added and the solutions are mixed. 2 ml. of phosphotungstic reagent, followed immediately by either 1 ml. of water (for the blank) or 1 ml. of 10 per cent sodium sulfite are added. The solutions are mixed and the flasks are placed in a water bath at 30° for 5 to 8 minutes. At the end of this time, the solutions are diluted to 50 ml. and the color is read in the Evelyn colorimeter. As recommended by Block and Bolling, filter 520 is used. The readings are corrected for the blank. A k value of 21.26 is used to calculate the cystine concentration.

Determination of Methionine

Baernstein (45) first developed a method for the determination of methionine. It is based on the reaction of the methyl-thiol group of methionine and hydroiodic acid to liberate methyl iodide. The iodide is precipitated as silver iodide by adding excess silver nitrate. The amount of methyl iodide liberated is determined indirectly by a volumetric estimation of the excess silver. In 1934, he (71) modified the method by using a mixture of glacial acetic acid, potassium acetate, and bromine to absorb methyl iodide instead of alcoholic silver nitrate. Mercuric chloride has been introduced into the absorption train to remove phosphine arising from the hypophosphite

used to keep the hydroiodic acid reduced.

In 1936, Baernstein (72) introduced a new method for the determination of methionine. It is based also on the demethylation of methionine, but does not depend on the estimation of methyl iodide formed. In this new method, the sulfydryl group of homocysteine is titrated with iodine. In 1938, Kassell and Brand (73) adapted the hydroiodic acid hydrolysis method of Baernstein to the determination of methionine, cysteine, and sulfate. Slight modifications were introduced.

Employing the same hydroiodic acid hydrolysis, Beach and Teague (74) developed a gravimetric method. The homocysteine formed from methionine in the hydroiodic acid hydrolysis changes into homocystine thiolactone which does not react with cuprous oxide. If the hydrolyzate is treated with this reagent only cysteine formed by reduction of cystine and that originally existing as cysteine are precipitated by cuprous oxide. The sulfur content of the cuprous mercaptide is determined gravimetrically after oxidation to sulfate. When the solution is made alkaline with sodium hydroxide, the thiolactone ring opens, and now total cysteine both that originally existing as cysteine, and cysteine formed by reduction of cystine, and homocysteine formed by the decomposition of methionine are precipitated by cuprous oxide. The total sulfur of the mercaptides of cysteine and homocysteine is determined in

the same way. The sulfur content of the mixed mercaptides minus the sulfur content of mercaptide of cysteine will give the sulfur content of methionine.

Lavine (75) in 1943, proposed a micro iodometric method. Methionine is treated with halogen, to form a perhalide of the type $R_2S \cdot X_2$ according to the equation below:

$$R_2S + X_2 \rightleftharpoons R_2S \cdot X_2$$

Since the periodide is stable against such reducing agents as thiosulfate or arsenite, any excess iodine is easily removed. Acidification of the resulting colorless solution results in the reversal of the reaction shown by the equation with the liberation of iodine from the periodide which now may be titrated with thiosulfate.

In 1944, Albanese and coworkers (76) adapted Toennies' method for the estimation of methionine in protein hydrolyzates and human urine. The method depends on the selective oxidation of methionine by hydrogen peroxide in the presence of perchloric acid.

McCarthy and Sullivan (12) in 1941, proposed a colorimetric method for the estimation of methionine. The color is developed when methionine is treated with unoxidized nitroprusside in a strongly alkaline medium. Histidine and tryptophane will also give color with this reagent. However, in an acid hydrolyzate, most tryptophane has been changed

to humin so it should not interfere significantly. Furthermore, if the solution is kept cold at the time the acid is added, there is no color reaction given by tryptophane. In presence of glycine and a strongly alkaline medium, histidine gives no color. The authors claimed that this reaction has a high degree of specificity. It is negative with all other amino acids found in acid hydrolyzates of protein. Methionine sulfoxide is negative, as are homocystine, cystine, and cysteine, but glycylmethionine is positive. They also claimed that the reaction is sensitive to 50 parts of methionine per million in the Duboscq colorimeter and to 20 parts per million in the Klett-Summerson photoelectric colorimeter with filter No. 54. This method was studied and adapted in this study.

A methionine solution in 0.1 N hydrochloric acid was prepared; aliquots were taken, and color was developed according to McCarthy and Sullivan's procedure with slight modification. The detailed procedure is given later. A rather linear relation was found between color produced and methionine concentration within a narrow range. When the methionine concentration was limited to 0.4-0.8 mg., the k value was found to be 176 at the beginning of the study. Later in the final check it was found to be 157. This difference might be due to some changes in the reagents, etc. However, color developed in solutions of the

same concentrations at different times sometimes varied considerably. These are shown in Table XXIII.

Table XXIII

Concentration of methionine	G reading of solutions			Color corrected for blank of solutions				k value
mg.	(1)	(2)	(3)	(1)	(2)	(3)	Average	
0.0 Blank	139	148	149					
0.2	183	159	189	44	?	40	42	210
0.4	207	218	222	68	70	73	70	175
0.6	240	256	260	101	108	111	107	178
0.8	277	286	295	138	138	146	141	176
1.0	309	320	294	170	172	?	171	171

Dr. Salo (unpublished data) and Miss Pfeiffer (unpublished data) had similar difficulties with this method. McCarthy and Sullivan (12) obtained values for methionine with one sample of casein, which varied from 2.90 per cent to 3.12 per cent. Because of the lack of uniform color development, in this study, four determinations with at least two blanks were carried out at the same time. The k value was checked, about once a week.

With this method, the methionine content of casein (Labco) was found to be 3.44 per cent on air dry basis and 3.56 per cent on water and ash-free basis. This value, as mentioned previously, agreed reasonably well with that

given in the literature. Therefore the methionine contents of the protein fractions isolated in this study were estimated by this method.

Procedure for the Determination of Methionine by McCarthy and Sullivan's Method

Reagents.

1 per cent glycine solution. 1 gm. in 100 ml. water. Keep in refrigerator when not in use.

14.3 N sodium hydroxide. 57.2 gm. diluted to 100 ml. with water.

10 per cent sodium nitroprusside. ($Na_2Fe(CN)_5NO \cdot 2H_2O$) Should be freshly prepared, and not older than four hours. 0.3 gm. nitroprusside are dissolved in 3 ml. water in a graduated glass stoppered test tube. The solution should be kept away from direct sunlight.

Hydrochloric acid-phosphoric acid mixture. 450 ml. concentrated hydrochloric acid plus 50 ml. 85 per cent phosphoric acid.

Methionine standard. 100 mg. in 500 ml. 0.1 N hydrochloric acid.

Carboraffin.

Procedure.

The protein hydrolyzate is prepared as given in the procedure for the determination of cystine. 5 ml. of the

solution to be tested are introduced into a 2.5 cm. by 15 cm.* test tube, and to this 1 ml. of 14.3 N sodium hydroxide, 1 ml. of a 1 per cent aqueous solution of glycine, and 0.3 ml. of a 10 per cent aqueous solution sodium nitroprusside are added with mixing after the addition of each. The tube is placed in a water bath at a temperature of 35-40° for 5-10 minutes. This is followed by cooling for two minutes in an ice bath. 5 ml. of hydrochloric acid-phosphoric acid mixture are then added with shaking. The mixture is stoppered immediately and allowed to stand for 5-10 minutes. The tube is then shaken continuously for one and a half minutes. During the shaking the stopper should be kept open in order to expose the contents to air. At the end of the shaking period, the tube is cooled in an ice bath for 10-15 seconds. The color developed is reddish. It does not faint easily, but it is preferable to read the color within half an hour. For Klett-Summerson photoelectric colorimeter, filter No. 54 is desirable. A k value is used for the calculation of methionine concentration. As mentioned before, the k value should be checked once a week.

*Sullivan recommended 2.5 by 25 cm.

Determination of Arginine

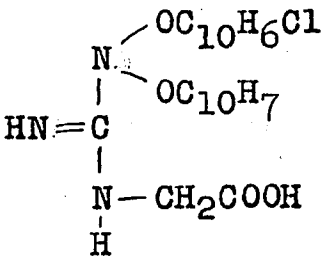
It is known that arginine is the only amino acid present in protein which yields urea on treatment with arginase. Jansen (77) in 1917, first proposed the use of this reaction for the determination of arginine. Hunter and Dauphinée (78) perfected the original procedure. They gave a very comprehensive discussion and detailed procedure including the method of preparing arginase. The urea formed was determined by converting to ammonia and the latter was titrated. Urea can also be determined by precipitation as dixanthydryl urea. Graff and coworkers (79) devised a method for the determination of arginine based upon this reaction.

Classically arginine is separated from other basic amino acids by silver precipitation at pH 8.5 according to Kossel's procedure of 1924 (80). The arginine silver salt is then decomposed by hydrogen sulfide. The arginine is precipitated as arginine flavianate. In general, this phosphotungstic acid and silver precipitation method is not very satisfactory for the procedure requires a relatively large quantity of protein and is known to be difficult to carry out successfully.

Sixteen years later, Vickery (81) published a method by which arginine is precipitated as the impure diflavianate directly from a protein hydrolyzate. The diflavianate is

then converted into monoflavianate and weighed as such. No doubt the latter is a better method but it still requires too much material to be generally useful.

For the estimation of small amounts of arginine, Sakaguchi devised a colorimetric method in 1925 (35). He claimed that the color reaction between arginine and α-naphthol and hypochlorite in presence of alkali is specific, and can be used to detect the presence of arginine in 1 to 1,000,000 dilution. Guanidine, when substituted by one ω-carboxyl-alkyl radical, provides the structure responsible for the formation of the colored compound with the reagents. The author found that glycocyamine ($HNCNH_2NHCH_2COOH$) but not creatine gives the color. Sakaguchi suggested that the colored compound formed by the reaction of glycocyamine and the color reagents might have the following formula.



Weber (82) modified this method. He substituted hypobromite for hypochlorite and added urea to stabilize the color. In 1932, Jorpes and Thorén (83) proposed a further modification. These methods require the separation of arginine from certain other amino acids and

ammonium salts, if they are present, since they interfere with the full development of color by arginine. In 1939, Thomas et al. (26) modified the method so that it could be applied directly to protein hydrolyzates. They used larger amounts of α-naphthol to prevent the interference of other amino acids, but not enough to give the strong yellow color produced by the reaction of α-naphthol and hypobromite. On the other hand, the yellow color, which interferes with the color measurement, develops slowly, and is inversely proportional to the amount of hypobromite. With the greater amount of hypobromite, the rate of fading will increase but the yellow color at a certain time is decreased. Therefore, in order to reduce the development of the yellow color, and the rate of fading, a suitable amount of hypobromite with a constant amount of α-naphthol should be used. This is what Thomas and coworkers suggested in their modification of Weber's method. This modification of Thomas et al. was adopted in this study and the details will be given later.

Dubnoff (84) in 1941, devised a micro method based on the Sakaguchi reaction. Here arginine and glycocyamine are separated by the selective adsorption of the arginine on permutit. This modification is useful for the determination of arginine in biological fluids, but is of little value in the determination of arginine in protein hydrolyzate, for the latter does not contain glycocyamine. He

also suggested that if urea is added before the addition of hypobromite, the interference due to ammonia, histidine, tyrosine and tryptophane can be prevented. In plant proteins, arginine usually occurs in large amounts, and since the Sakaguchi reagent is sensitive for arginine, the amount of these other substances present will not generally interfere with the determination. Macpherson (85) suggested that urea should be added before the hypobromite, since this will ensure maximum and consistent color development, and the result will not be affected by a slight change in the quantity of reagents used.

Brand and Kassell (86) in 1942, incorporated many modifications of the Sakaguchi reaction in a photometric procedure for the determination of arginine. They found that the arginine value as determined on a larger aliquot of protein hydrolyzate is lower than that obtained on a smaller aliquot. They suggested that the inhibition of color development is due to the presence of ammonia and histidine, and may be eliminated by extrapolating the arginine value to zero protein concentration. In order to plot the curve, the arginine concentration of a protein must be determined at different levels. In this study, the amount of protein needed for each determination was usually a fraction of a milligram, so that the value found should not be too far from that obtained by extrapolating to zero protein concentration.

It is known that the intensity of the color developed by arginine in the Sakaguchi reaction decreases with an increasing amount of arginine. Both Dr. Salo's experimental data (unpublished) and that obtained by us showed that with an Evelyn photoelectric colorimeter, a constant k value can be obtained only within concentrations of from 0.007 mg. to 0.03 mg. of arginine. Dr. Salo also showed that histidine and lysine present in the same amount as arginine did not interfere with the color development. However, the color obtained differed considerably with varying amounts of hypobromite. Therefore it is necessary to determine a k value at different hypobromite levels, and whenever a new hypobromite solution is prepared. We have determined three k values, one with 0.15 ml., two with 0.20 ml. hypobromite solution. Filter 520 was found to be best. The average k values with 0.20 ml. hypobromite were 19.22 and 20.12; and with 0.15 ml., 20.22. They agree very well with that found by Dr. Salo (20.2) with 0.20 ml. hypobromite. The procedure used is that of Thomas et al. (26) slightly modified by Dr. Salo. The data are given in Table XXIV.

With the method given below casein (Labco) was found to contain 3.54 per cent arginine with approximately 1 per cent error. This value though similar to that reported by Thomas et al. (26), is much lower than that given by Block and Bolling (13). The arginine contents of the

Table XXIV

k Values Established with Different Quantities of Hypobromite Solution

Concentration of arginine	G reading	L value	k value
mg.			
I. With 0.15 ml. of Hypobromite Solution (1)			
0.007	72^{2}	0.1412	20.17
	72^{0}	0.1442	20.60
	72^{0}	0.1442	20.60
	71^{3}	0.1457	20.81
0.01	62^{0}	0.2076	20.76
	62^{0}	0.2076	20.76
	62^{3}	0.2024	20.24
	62^{3}	0.2024	20.24
0.02	39^{0}	0.409	20.45
	39^{1}	0.406	20.30
	40^{3}	0.390	19.50
	40^{3}	0.390	19.50
0.03	25^{1}	0.598	19.93
	25^{1}	0.598	19.93
	25^{0}	0.602	20.06
	25^{3}	0.589	19.63
		Average	20.22
II. With 0.20 ml. Hypobromite Solution (1)			
0.01	64^{3}	0.1880	18.88
	64^{2}	0.1905	19.05
	64^{0}	0.1939	19.39
0.02	40^{3}	0.390	19.50
	41^{1}	0.385	19.25
	41^{1}	0.385	19.25
0.03	26^{1}	0.581	19.37
	26^{3}	0.573	19.10
		Average	19.22

Table XXIV (Continued)

Concentration of arginine	G reading	L value	k value
mg.			
III. With 0.20 ml. Hypobromite Solution (2)			
0.01	63^{1}	0.1990	19.90
	64^{1}	0.1922	19.22
	63^{0}	0.2007	20.07
0.02	39^{0}	0.409	20.45
	39^{0}	0.409	20.45
	38^{2}	0.414	20.70
		Average	20.12

protein fractions isolated in this study are 13.41 per cent for globulin, and 3.77 per cent for albumin.

Procedure for the Determination of Arginine by the Sakaguchi Reaction

Reagents.

0.05 per cent α-naphthol solution. Prepared by diluting with water a 0.5 per cent alcoholic solution of α-naphthol. If the alcoholic solution is kept in a brown bottle and stored in a refrigerator, it is stable for one month. α-naphthol (EK #170) is purified by redistillation.

Sodium hypobromite solution. 2.0 gm. (0.64 ml.) of bromine are dissolved in cold 5 per cent sodium hydroxide and diluted to 100 ml. with 5 per cent

sodium hydroxide. It is kept in a dark brown bottle in the cold and is stable for about two months.

Urea solution. 40 gm. per 100 ml. of water, and is kept in a refrigerator.

10 per cent sodium hydroxide. 10 gm. in 100 ml. water.

Arginine standard. 12.1 mg. of arginine monohydrochloride is dissolved in 1000 ml. of water. This gives 0.01 mg. arginine per ml.

Procedure.

Approximately 0.1 gm. of protein is hydrolyzed with 2 ml. 20 per cent hydrochloric acid or 25 per cent sulfuric acid for 20-24 hours at 125°. Humin is filtered off. The filtrate is diluted to the proper volume with water so that from 1 to 5 ml. will contain from 0.007 mg. to 0.03 mg. of arginine.

An aliquot of diluted hydrolyzate is placed in a Pyrex test tube, and the final volume is adjusted to 5 ml. The content is cooled in an ice bath for 30 minutes. Other reagents should be taken out from the refrigerator and cooled to ice cold at the same time. 1 ml. of the 10 per cent sodium hydroxide and 1 ml. of α-naphthol solution are added. The content is shaken and cooled for 5 more minutes. 0.2 ml. of hypobromite solution is added, the mixture is shaken for exactly 15 seconds. Then, 1 ml. of cold urea solution is added to stop the reaction. To the solution

are added 3 ml. of ice cold water, with shaking. After the color development is completed, the solution is warmed by holding in palm of hand. Color is read at intervals of 3, 5, and 7 minutes, in the Evelyn photoelectric colorimeter using filter 520. If the reading is rather constant, the reading at 5 minutes is used for calculating the arginine value. If not, the same procedure is repeated with 0.15 ml. or 0.25 ml. of hypobromite solution. In the solutions containing insufficient hypobromite, the absorption of light will increase with time and in those containing too much hypobromite it will decrease. After selecting the amount of hypobromite which gives the most nearly constant light absorption, several determination are made on the same arginine solution with the use of the optimal amount of hypobromite. A k value should be determined with the same amount of hypobromite solution as used in the determination. The allowable range of arginine concentration is from 0.007 to 0.03 mg. in 5 ml. of solution. It is necessary to work within a very narrow range of arginine concentration, since the concentration is not strictly proportional to the color over a wide range of concentration.

Determination of Tyrosine and Tryptophane

All the better and recent methods for the determination of tyrosine and tryptophane are colorimetric. When both tyrosine and tryptophane are to be determined in the same aliquot, the procedure developed by Lugg (27) as a modification of the Folin and Ciocalteu method is probably the best available at present. In one paper, Lugg (27) gave a very detailed discussion of the mechanism and factors which affect the reaction. In another paper (8), he reported the result of his investigations of sources of error in the estimation of tyrosine and tryptophane in protein hydrolyzates; he also suggested corrections for these errors.

There are many other modifications of the original Folin-Ciocalteu method, such as those of Arnow (87), Urban (88), and Zurwerkalow (89).

For a direct determination of tryptophane, May and Rose (90) adapted the reaction of tryptophane with p-dimethylaminobenzaldehyde to form a blue colored compound. In 1938, Sullivan et al. (91) introduced the use of hydrogen peroxide to hasten the reaction. This modification shortened the time necessary for one determination from 7-8 days to 30 minutes. In 1945, Horn and Jones (92) reported another modification of the same method which they claimed to be not only more rapid but also more accurate.

The glyoxylic acid method (93) can also be directly applied to a protein hydrolyzate without the removal of tyrosine and other phenolic compounds. Shaw and McFarlane (94) made a critical study of the p-dimethylaminobenzaldehyde method as contrasted with the glyoxylic acid method. They found that the tryptophane content of casein determined by the aldehyde method was approximately twice that obtained by the glyoxylic acid method. The erroneous result was thought to be due to the use of free tryptophane as the standard in the aldehyde method. Tryptophane as combined in the protein molecule gives more color with the aldehyde reagent than does an equivalent amount of free tryptophane.

In 1943, Eckert (95) published a new micro method for the determination of tryptophane. Tryptophane reacts with N-(1-Naphthyl)ethylene diamine to give a red colored compound. The procedure is stated to estimate 0.01-0.25 mg. of tryptophane. No other amino acids give the same reaction except tyrosine in large amount. Ordinarily it is possible to perform the determination directly on the hydrolyzate without removing other amino acids. Brown (11) reported that the tryptophane content of casein (Labco) as determined by the Eckert method was the same as that determined by the glyoxylic acid method.

For the direct determination of tyrosine in protein hydrolyzate, there are not many methods available. Gerngross et al. (96) employed nitroso-naphthol for the

estimation of tyrosine. When tyrosine is treated with 1.2-nitroso-naphthol followed by concentrated nitric acid, a dark purple color is produced. The reaction is highly sensitive, as 1 mg. of tyrosine in 1 liter of water gives a distinct color. It is highly specific since diiodo-tyrosine is the only other amino acid known as a constituent of proteins which gives color with the reagent. However, the color fades gradually, especially in the presence of an excess of nitric acid. Thomas (97) modified the procedure slightly to take care of the fading. The modifications are: (1) Increase the acidity of the reaction by hydrochloric acid and use more dilute nitric acid. When this is done the development and fading of the color take place much more slowly. (2) Cool the solution under the proper conditions. By doing so, the fading can be delayed.

In this study, Lugg's modification of the Folin-Ciocalteu method was used, and it was found to be very satisfactory, except that some difficulty was experienced with "floating" of the precipitate. By using filter-cel, as suggested by Dr. Salo (unpublished), "floating" was prevented easily. The detailed procedure adapted in this study will be given later.

Standard tyrosine solution was prepared by dissolving 25 mg. of pure tyrosine in 250 ml. N sulfuric acid. The average k value found for tyrosine with filter 520 was 1.172. Data are shown in Table XXV.

Table XXV

Concentration of tyrosine	G reading	Corrected G	L value	k value
mg.				
0.1	76^{0}	75^{3}	0.1206	1.206
	76^{0}	75^{3}	0.1206	1.206
	76^{3}	76^{2}	0.1163	1.163
	76^{3}	76^{2}	0.1163	1.163
0.2	58^{2}	58^{2}	0.2328	1.164
	58^{3}	58^{3}	0.2310	1.155
	58^{1}	58^{1}	0.2347	1.174
	58^{2}	58^{2}	0.2328	1.164
0.3	43^{0}	43^{0}	0.367	1.224
	45^{2}	45^{2}	0.342	1.140
	45^{0}	45^{0}	0.347	1.157
	45^{2}	45^{2}	0.342	1.140
0.4	34^{0}	34^{0}	0.469	1.173
	34^{1}	34^{1}	0.465	1.163
	34^{1}	34^{1}	0.465	1.163
	34^{3}	34^{3}	0.457	1.143
0.5	25^{3}	25^{3}	0.589	1.178
	25^{2}	25^{2}	0.594	1.190
	26^{0}	26^{0}	0.585	1.170
	25^{1}	25^{1}	0.598	1.196
			Average	1.172

In order to establish a satisfactory k value for the determination of tryptophane, pure tryptophane is dissolved in "solution D" instead of water. "Solution D" * is a solvent for the tryptophane mercurial precipitate obtained in the separation of tryptophane and tyrosine. Lugg (27)

*The composition of "solution D" is given in the procedure for the determination of tyrosine.

stated that the tryptophane mercurial solution in absence of nitrite deteriorates by some 8 or 9 per cent in 20 hours at 20°. It is therefore necessary to allow the tryptophane mercurial solution to stand for exactly the same length of time as that is needed to carry out all the processes between the solution of the tryptophane mercurial precipitate and the development of color. In this study, 2 hours were found to elapse during the procedures between the solution of the precipitate and the development of color. Hence, a standard solution of tryptophane dissolved in "solution D" was allowed to stand for 2 hours, before the color was developed. The k value was found to be rather constant with filter 520, as shown in Table XXVI.

Table XXVI

Concentration of tryptophane	G reading	Corrected G	L value	k value
mg.				
0.1	60^{3}	60^{3}	0.2164	2.164
0.1	61^{0}	61^{0}	0.2147	2.147
0.1	61^{2}	61^{2}	0.2111	2.111
0.2	37^{1}	37^{1}	0.429	2.145
0.2	37^{2}	37^{2}	0.426	2.130
0.2	38^{0}	38^{0}	0.420	2.100
0.3	23^{0}	23^{0}	0.638	2.127
0.3	22^{3}	22^{3}	0.643	2.143
0.3	23^{1}	23^{1}	0.634	2.113
0.4	14^{0}	14^{0}	0.854	2.135
0.4	14^{0}	14^{0}	0.854	2.135
0.4	14^{2}	14^{2}	0.836	2.095
			Average	2.127

From a mixture of pure tyrosine and tryptophane, 100 per cent of the tyrosine and 98 per cent of the tryptophane could be recovered. Data are given in Table XXVII.

Table XXVII

Recovery Experiments with Mixtures of Tyrosine and Tryptophane

Treatment of sample	Tyrosine			Tryptophane		
	Added	Recovered		Added	Recovered	
	mg.	mg.	per cent	mg.	mg.	per cent
100 mg. filter-cel were used, and washed twice.	0.4	0.397	99	0.4	0.400	100
	0.4	0.400	100	0.4	0.393	98
20 mg. filter-cel, and washed twice.	0.4	0.387	97	0.4	0.386	97
	0.4	0.389	97	0.4	0.386	97
Solution was stirred with 3-5 mg. filter-cel until no visible ppt. was climbing up the tube, washed once with same amount of solution.	0.1	0.100	100	0.1	0.091	91
	0.4	0.405	101	0.4	0.409	102
	0.2	0.205	103	0.2	0.191	96
	0.2	0.205	103	0.2	0.191	96
Same as above.	0.1	0.102	102	0.1	0.100	100
	0.3	0.306	102	0.3	0.307	102
Same as above.	0.1	0.099	99	0.1	0.095	95
	0.2	0.198	99	0.2	0.199	100
	0.3	0.292	97	0.3	0.300	100
	0.4	0.392	98	0.4	0.388	97
		Average	100		Average	98

When the techniques as developed were applied to casein (Labco), the values were found to be very close to those reported in the literature. For tyrosine it was 5.69 per cent as compared to 5.2 per cent given by Block and Bolling (13) for casein and to 5.7 per cent as given by Brown (11) for casein (Labco). For tryptophane it was 0.97 per cent as compared to 1.03 per cent reported by Brown (11). All values given here for tryptophane include the 6 per cent correction for the loss of tryptophane in hydrolysis, as suggested by Lugg (8). Data are given in Table XXVIII.

Table XXVIII

Tyrosine and Tryptophane Content of Casein (Labco)

Treatment of sample	Tyrosine		Tryptophane	
	On air dried basis	On ash-H_2O-free basis	On air dried basis	On ash-H_2O-free basis
	per cent	per cent	per cent	per cent
0.2 gm. casein hydrolyzate was diluted to 25 ml. 1 ml. was taken for analysis. 100 mg. filter-cel were used to prevent floating.	5.50 5.50 5.50	5.69 5.69 5.69	0.93 0.93	0.96 0.96
Same as above but only 20 mg. filter-cel were used.	5.50 5.50	5.69 5.69	0.93 0.95	0.96 0.98
Average		5.69		0.97

With the same procedures, the tyrosine and tryptophane contents of the isolated globulin were found to be 4.40 per cent, and 1.14 per cent respectively; and those of the albumin fraction were 4.40 per cent and 2.12 per cent respectively.

Procedure for the Determination of Tyrosine and Tryptophane by the Lugg Modification of Folin-Ciocalteu Method

Reagents.

Solution A. (N sulfuric acid.) 128 gm. of 95-96 per cent sulfuric acid per 2.5 liters.

Solution B. 75 gm. mercuric sulfate, 55 gm. mercuric chloride, and 70 gm. anhydrous sodium sulfate are dissolved in 850 ml. water plus 128 gm. 95-96 per cent sulfuric acid and the solution is diluted to 1 liter.

Solution C. This is made by diluting a volume of solution B with an equal volume of solution A.

Solution D. 12 gm. mercuric sulfate and 9 gm. mercuric chloride are dissolved in 600 ml. water plus 103 gm. of 95-96 per cent sulfuric acid. A further 512 gm. of sulfuric acid are added with cooling, and the mixture is diluted to 1 liter.

Solution E. (M sodium nitrite). 6.9 gm. sodium nitrite per 100 ml.

Standard solutions:

Tyrosine. 25 mg. are dissolved in 250 ml. N sulfuric acid.

Tryptophane. 10 mg. are dissolved in 100 ml. solution D.

Filter-cel.

Procedure.

An alkaline protein hydrolyzate should be made acid equivalent to N sulfuric acid. An aliquot of the hydrolyzate is introduced into a 15 ml. centrifuge tube, and made to 5 ml. with N sulfuric acid. 5 ml. of solution B are added and the tube is maintained at 60-65° in a water bath for 30 minutes. It is then cooled in a water-bath for 1 hour at 1 or 2° below room temperature, and after centrifuging, the clear liquid is drained into a 25 ml. graduated cylinder. After 10 ml. of solution C are run into the centrifuge tube, any precipitate is well stirred up for a minute or so. At this stage one usually finds a small amount of precipitate floating on top of the solution and climbing up the side of the tube. In order to prevent the floating, 5-10 mg. of filter-cel are stirred into the solution. The procedure is repeated two more times. The stirring rod is rinsed with solution C, and centrifuged. At this stage, one sometimes still sees a small amount of the precipitate on top of the solution, and there might be some

precipitate dried up on the side. In that case it is safer to wet the side of the tube with the contents and stir in another 5 mg. filter-cel. The stirring rod and the side of the tube are rinsed with solution C, and the contents are again centrifuged. The liquid is drained into the cylinder and the decanted fluid is diluted with solution C to 24.5 ml. in readiness for the tyrosine estimation.

For the estimation of the tyrosine, the contents of the graduated cylinders should be employed within an hour, as cloudiness may develop on long standing. 0.5 ml. of solution E is run slowly into each cylinder so as to prevent mixing, and the contents are shaken immediately. Color intensity is measured at exactly 3 minutes after the mixing, in a photoelectric colorimeter with filter 520.

For the estimation of the tryptophane the solid mercurial is well rubbed up with 10 ml. of solution D, and the tube is maintained at 40-45° in a water-bath for 15 minutes with occasional rubbing of any solid that has not yet dissolved. It is then cooled in a water-bath for 30 minutes at 1 or 2° below room temperature, and after centrifuging, the clear liquid is drained into a 25 ml. graduated cylinder. A further 10 ml. of solution D are run into the tube, the contents are stirred and any solid is well rubbed for a few minutes, and after centrifuging, the liquid is drained into the cylinder and the volume made up to 24.5 ml. with solution D. 0.5 ml. of solution is run into

each cylinder so as to float on top, and the contents are mixed by inverting the tube three times. Color intensity is measured with the least delay. The use of filter 520 gives best results. The time elapsed between mixing and color measurement usually amounts to about 15 seconds.

Substances in the test solutions may contribute adventitious color to the tyrosine and tryptophane unknowns before the addition of the nitrite. Correction can be made by employing appropriate blanks (substituting water for nitrite). For calculating the concentration of tyrosine, a k value of 1.172 was used. For tryptophane, 2.127 was used.

Determination of Histidine

All methods available for the estimation of histidine require separation of histidine or histidine together with other basic amino acids from an amino acid mixture. It can be separated from a mixture of amino acids by adding excess of a soluble salt of silver and neutralizing to pH 7.4 according to the procedure developed by Kossel and Kutscher (98); by adding an excess of mercuric chloride and neutralizing to pH 7.0 to 7.4 (99); or together with other basic amino acids, by adding an excess of phosphotungstic acid at strongly acid reaction (100). Basic acids can be also separated by means of electrical transport as originally

suggested by Foster and Schmidt (101), and later perfected on a quantitative basis by Albanese (102). Histidine is precipitated from the basic acids fraction as mercuric chloride complex.

The first gravimetric method advanced for the determination of histidine is that of Kossel and Kutscher (98) in 1900. This depends upon repeated precipitation and purification of the silver compound of histidine. Kossel and Patten (103) later found that histidine could be further purified by precipitation with mercuric sulfate in acid solution. The precipitate was dissolved in water and decomposed by hydrogen sulfide. Mercuric sulfide was filtered off. The histidine content could then be estimated by determining nitrogen in the filtrate. In 1927, Vickery (104) suggested the use of flavianic acid* as a reagent for the isolation of histidine. In 1937, Block (105) introduced nitranilic acid as a precipitant for histidine. In 1942, Vickery (106) reported the use of 3,4-dichlorobenzene-sulfonic acid for the gravimetric estimation of histidine in hemoglobin. More recently he and Wintermitz (109) have published the details of the method, and discussed not only the desirability of this new precipitant but also commented on the limited value of flavianic acid as a reagent for the

*This had been used successfully by Kossel and Gross previously as a reagent for the gravimetric determination of arginine.

quantitative isolation of histidine.

As is generally known, one of the short-comings of gravimetric methods is the need of a comparatively large sample. The latest method proposed by Vickery and co-workers (107) requires about five grams of ordinary proteins. It is very evident that this method is not desirable in many cases. For small amounts of histidine, a suitable colorimetric method would usually be the method of choice.

In 1919, Koessler and Hanke (39) proposed a colorimetric method based on the reaction of histidine with diazonium compounds. Histidine is first precipitated from a protein hydrolyzate with silver at pH 7.4 in order to separate it from interfering substances such as tyrosine etc. Lang (33) precipitated histidine in an approximately neutral reaction with mercuric chloride, dissolved the precipitate in cyanide, and then determined the histidine according to Koessler and Hanke's method. This method is employed in this study. In 1942, Macpherson (85) offered a modification of this method. In the modified procedure, the reaction is carried out at room temperature instead of zero as recommended by Koessler and Hanke, and the color is rendered stable by addition of alcohol. Recently Saidel and Brand (34) reported a modification of Lang's procedure. This method will be discussed in more detail later.

Kapeller-Adler (108) in 1933, advanced another

colorimetric method for the determination of histidine. When histidine is brominated and the resulting compound is warmed with alkali, a blue violet color develops. Tyrosine, tryptophane, glycine, methionine, proline, hydroxyproline, alanine, and uracil interfere with the reaction. Therefore, as in other methods, histidine has to be separated from a protein hydrolyzate with mercuric chloride or other precipitants.

Lang's (33) procedure with slight modifications was adapted in this study for the determination of histidine. As mentioned by Lang, the color developed by the reaction of histidine with the diazotized solution depends on the amount of sodium cyanide and mercuric ions present in the solution. The amount of mercury varies with the amount of histidine and other amino acids that are precipitable by mercury. It is rather difficult to control the amount of mercury. Therefore in order to get around this, a smaller sample was used. Here, determinations were usually limited to about 0.05 to 0.15 mg. histidine in protein hydrolyzates, or about 4 mg. protein in each determination. For pure histidine solutions the allowable range is greater, say 0.05 to 0.4 mg. The amount of 5 per cent sodium cyanide necessary to develop a maximum color should be determined with each type of material. Usually the smallest amount of sodium cyanide that is necessary to dissolve the precipitate is the optimum quantity for a maximum color

development, as sodium cyanide inhibits color development. For ordinary purposes, 2 to 3 drops are sufficient. Dr. Salo (unpublished) found that 1 ml. sodium cyanide completely inhibits the color formation. For an aliquot containing about 4 mg. of protein, 3 drops of 5 per cent sodium cyanide was found to be satisfactory. After the amount of sodium cyanide necessary is estimated, a k value should be determined with the same amount of sodium cyanide. Table XXIX will show the linear relationship between color and concentration of histidine with different amounts of sodium cyanide, and constant quantities of other reagents. For concentrations greater than 0.15 mg. of histidine, the k value was not constant, and a standard curve had to be employed.

The accuracy of the method depends also on the separation of histidine from tyrosine. It is found in this study, that a careful control of the pH of the solution is desirable. A drop of methyl red is added to the solution as indicator. According to the original method, if the volume at which histidine is precipitated varies somewhat, the pH will differ significantly, and subsequently the separation will not be complete. In Brand's modification,* he not only suggested the use of a limited amount of

*The details of this method have not been published, and the preliminary report came out after this part of the work was completed.

Table XXIX

k Values Established with Different Amounts of

5 Per Cent Sodium Cyanide

Concentration of histidine	G reading	L value	k value
mg.			
(1) With 2 drops of 5 per cent sodium cyanide solution			
0.05	73^{2}	0.1352	2.70
0.05	73^{3}	0.1337	2.65
0.075	63^{1}	0.1990	2.65
0.10	54^{2}	0.2636	2.64
0.125	47^{3}	0.321	2.57
0.125	47^{2}	0.323	2.58
0.125	47^{2}	0.323	2.58
0.15	40^{2}	0.392	2.61
		Average	2.62
(2) With 3 drops of 5 per cent sodium cyanide solution			
0.05	73^{3}	0.1322	2.64
0.075	64^{1}	0.1922	2.56
0.10	56^{1}	0.2499	2.50
0.125	48^{3}	0.312	2.50
0.15	42^{0}	0.377	2.51
		Average	2.54

histidine but also stated the exact pH (6.5-6.7) at which histidine should be precipitated. The reprecipitation of histidine as suggested by Brand certainly would render the method more sensitive and accurate.

With Lang's method as modified, we were able to obtain 100 per cent recovery either when the method was applied to pure histidine or to pure histidine added to

a casein hydrolyzate. The data given in Tables XXX and XXI will support the above statement.

Table XXX

Recovery of Histidine from a Pure Histidine Solution

Histidine present	Histidine found	Recovery
mg.	mg.	per cent
0.1	0.101	101
0.1	0.101	101
0.2	0.200	100
0.2	0.201	100
0.3	0.393	98
0.3	0.388	97

Table XXXI

Recovery of Histidine from a Protein Hydrolyzate

Solution analyzed	Histidine found	Recovery
	mg.	per cent
0.5 ml. casein hydrolyzate (4 mg. casein).	0.140	
0.5 ml. casein hydrolyzate plus 0.05 mg. histidine	0.190	100
0.3 ml. casein hydrolyzate plus 0.1 mg. histidine	0.182	98

With this method, the histidine content of casein was found to be 3.52 per cent. This value is higher than most of the figures reported in the literature, but agrees

rather well with 4.1 per cent and 3.6 per cent given by Kapeller-Adler (108) (24), 3.4-3.8 per cent by Lautenschläger (109), and approximately 3.1 per cent* given by Albanese (102). The histidine content of the protein fractions isolated in this study were found to be 2.89 per cent for globulin, and 2.30 per cent for albumin.

The details of the procedure employed are given below.

Procedure for the Determination of Histidine

Reagents.

Mercuric chloride mixture. 70 gm. of crystalline sodium acetate, or 42.2 gm. of the anhydrous salt, and 10 gm. of sodium chloride are added to 150 ml. of saturated mercuric chloride solution.

Diazotizing solution. 2 ml. of 2 per cent sodium nitrite are added to 20 ml. of 0.5 per cent sulfanilic acid in 1.75 per cent hydrochloric acid at 0°. After 5 minutes, 2 more ml. of the sodium nitrite solution are added and after another 5 minutes, 2 more ml. are added. Finally, the mixture is diluted to 50 ml. This solution should be kept in ice water for at least 15 minutes before use.

4 per cent sodium borate (tetra) solution.

*Albanese reported the histidine value as 5.36 per cent of the total nitrogen.

Saturated sodium carbonate solution.

N hydrochloric acid.

5 per cent sodium cyanide solution.

Standard histidine solution. 61.7 mg. of histidine monohydrochloride is dissolved in 1000 ml. of water. Each ml. contains 0.05 mg. of histidine.

Procedure.

From 0.5 ml. to 2 ml. of an acid hydrolyzate of protein estimated to contain 0.05 mg. to 0.15 mg. of histidine is placed in a 15 ml. graduated centrifuged tube. A drop of methyl red solution (saturated in 0.05 N hydrochloric acid solution) is then added. The solution is stirred well with a pointed glass rod. The solution is adjusted to the neutral point with saturated sodium carbonate and N hydrochloric acid. Finally the solution is made up to 2.5 ml., and acidified with 2 drops of N hydrochloric acid. 1 ml. of mercuric chloride mixture is added with stirring. The stirring rod is rinsed with a few drops of water, and the precipitate is separated by centrifugation. The precipitate is washed with water once by breaking up the precipitate with a stirring rod. The solution is centrifuged again. The precipitate is dissolved with 2 to 3 drops of 5 per cent sodium cyanide solution. The solution is rinsed into a 50 ml. graduated cylinder with water. Two ml. of saturated carbonate are

now added, followed by 4 ml. of diazotizing solution. The solution is brought to the 50 ml. mark with water. The color is read employing filter 540, and at 20 minutes after the color is developed. A k value is used for calculating the histidine concentration. For concentrations greater than 0.15 mg. of histidine, the k value is not constant, and a standard curve has to be employed.

Determination of Phenylalanine

There are not many methods available for the determination of phenylalanine. Several other amino acids present in protein hydrolyzates interfere with the chemical determination. If phenylalanine is separated from the interferent substances before determination is made, chemical methods are satisfactory. Small amounts of phenylalanine can be separated from other amino acids chromatographically. Recently, biological methods are also available for the determination of phenylalanine.

The best known isolation method is that suggested by Baptist and Robson (110). In this method phenylalanine is salted out together with leucine from a hydrochloric acid hydrolyzate of protein at pH 6 by saturating the solution with sodium chloride. Phenylalanine is separated from leucine by precipitation as picrolonate. The phenylaline isolated by this method is pure, but the isolation

is not quantitative.

Kapeller-Adler's (17) reaction for the estimation of phenylalanine was the only colorimetric method known until the appearance of Sullivan's (18) method in October, 1944. Phenylalanine after nitration will give a violet color when mixed with hydroxylamine in the presence of ammonium hydroxide. Kapeller-Adler thought that the color was due to the formation of either 3-nitroso-4-nitrobenzoic acid or 3-nitro-4-nitrosobenzoic acid. The chemistry of this reaction was studied by Block and Bolling (111). They thought the color was due to the formation of a derivative of diaci-o-dihydrodinitrobenzene.

Histidine, tyrosine, and tryptophane are known to interfere with the color reaction. Kapeller-Adler got rid of the tryptophane by using a sulfuric acid hydrolyzate, histidine by precipitation with phosphotungstate, and tyrosine by oxidation with potassium permanganate. In 1940, Block et al. (10) reported than when color filter 560 was used, it was unnecessary to remove tyrosine and histidine as recommended by Kapeller-Adler. They advised the use of an alkaline hydrolyzate, for they found that the acid destroyed some phenylalanine, and that the addition of an alkaline protein hydrolyzate to a phenylalanine solution did not interfere with the determination. Knight and Stanley (112) found that tryptophane gave color with this reagent. These authors corrected for the interference

by subtracting from the colorimeter reading of the unknown a value corresponding to the amount of tryptophane present in the aliquot of the solution taken for analysis. Brown (14) also found that tryptophane interfered with the color reaction. He assumed that the use of a color filter would avoid the interference due to the presence of tyrosine and histidine, and hence suggested a modification of the Block and Bolling procedure, in which tryptophane was removed by precipitation with mercuric sulfate from an alkaline hydrolyzate before nitration was carried out. In the same number of the Journal of Biological Chemistry, Albanese (15) reported a modification of the Kapeller-Adler method in which adsorption of histidine on permutit replaced the precipitation of histidine with phosphotungstate. He found that when phosphotungstate was used as a precipitant, excess of reagent led to low results, and an insufficient quantity gave values for phenylalanine which were too high. He assumed, as did Kapeller-Adler, that tryptophane was destroyed in acid hydrolysis.

The Block and Bolling (13) procedure based on the Kapeller-Adler reaction was adopted in this study with only two slight modifications. They recommended a nitration period of 20 minutes. It was found in this study that when nitration was carried out for 25 minutes with swirling every five minutes, the results were more consistent than when a nitration period of 20 minutes was used.

Therefore the nitration period was changed from 20 to 25 minutes. Later, Albanese (15) used a hot air bath as a substitute for steam bath and nitrated for 30 minutes instead of 20 to ensure complete nitration. Color filter 540 was found to give a slightly higher absorption than filter 560, hence the former was used in this study.

In his thesis, Dr. Salo recorded that this procedure has an inherent error of 8 per cent. It was found in this study that with pure phenylalanine duplicates may vary as much as 6-10 per cent. The accuracy of this method can probably be increased by more thorough studies. The introduction of the use of hydroxylamine-ammonium sulfate reagent as substitute for hydroxylamine and ammonium hydroxide by Albanese (15) would certainly increase the consistency. A change of the final volume from 25 ml. to 50 ml. would also increase the accuracy.

Only the effects of tyrosine and histidine on the estimation of phenylalanine were studied, for the determinations were to be carried out on acid hydrolyzates. Both histidine and tyrosine were found to give a yellow color with the reagent, but the intensity of color produced by histidine was not deep enough to affect the result significantly. On the other hand, tyrosine seemed to give about 4 per cent of the color intensity of phenylalanine. However, when tyrosine was added to a phenylalanine solution, the effect was different. When the concentration of

phenylalanine was low, the presence of tyrosine increased the phenylalanine value; but when the concentration was high, the presence of tyrosine decreased it. If the amount of tyrosine present in the aliquot taken was 1 mg. or lower, the phenylalanine value was found to fall in the range obtained with pure phenylalanine. The effect of histidine was also studied in the same manner, and it was also found that if the concentration of histidine was 1 mg. or less it did not alter the color intensity of a phenylalanine solution significantly. So, in order to get a better approximation of the phenylalanine value of a protein, it is advisable when possible to use an aliquot containing 1 mg. or less of tyrosine and of histidine.

A k value was determined with pure phenylalanine according to the procedure developed. The data are shown in Table XXXII. When histidine and tyrosine were added

Table XXXII

Concentration of phenylalanine	G reading	L value	k value
mg.			
0.5	67^{1}	0.1739	0.348
	67	0.1756	0.351
1.0	46	0.337	0.337
	44^{1}	0.354	0.354
1.5	32^{1}	0.491	0.327
	30^{0}	0.520	0.347
2.0	22^{0}	0.658	0.329
	20^{0}	0.699	0.350
		Average	0.343

to phenylalanine in an amount not greater than 1 mg., the average k value was found to be almost exactly the same as that for pure phenylalanine, as indicated in Table XXXIII.

Table XXXIII

Concentration of phenylalanine	Concentration of tyrosine	Concentration of histidine	k value
mg.	mg.	mg.	
1	0.5	0.5	0.332
1	0.5	0.5	0.347
1	0.5	1.0	0.337
1	0.5	1.0	0.347
1	0.0	0.5	0.340
1	0.0	0.5	0.337
1	0.0	1.0	0.337
0.5	1.0	0.0	0.348
0.5	1.0	0.0	0.342
1.0	1.0	0.0	0.314
1.0	1.0	0.0	0.340
1.5	1.0	0.0	0.325
1.5	1.0	0.0	0.320
2.0	1.0	0.0	0.350
2.0	1.0	0.0	0.347
0.5	0.5	0.0	0.351
0.5	0.5	0.0	0.364
1.0	0.5	0.0	0.328
1.5	0.5	0.0	0.347
1.5	0.5	0.0	0.339
2.0	0.5	0.0	0.339
3.0	0.5	0.0	0.326
			Average 0.340

A k value of 0.340 was therefore used in all the calculations. When analyzed by this procedure, casein, hydrolyzed with sulfuric acid, was found to contain 5.67 per cent phenylalanine. This value checks with that given by Block and Bolling (13). In order to confirm whether tryptophane

present in an alkaline hydrolyzate will affect the determination or not, the phenylalanine contents of the protein fractions isolated in this study were estimated on both acid and alkaline hydrolyzates. Values as determined check with each other within the limit of error of this method. The phenylalanine values given in Table X in the text are averages of values obtained on acid and alkaline hydrolyzates, and those given in Table XI are averages obtained on alkaline hydrolyzates.

The phenylalanine contents of casein reported in the literature vary greatly. Brown (14) reported only 3.99 per cent of phenylalanine in casein with his procedure in which tryptophane was removed by mercuric sulfate. Kapeller-Adler (17) reported 4.52-5.33 per cent phenylalanine in casein with her method in which tyrosine and histidine were removed prior to the determination of phenylalanine. Isolation procedure usually gives lower value such as that of 2.9 per cent given by Baptist and Robson (110).

Recently Hess and Sullivan (18) used an entirely different method. Their method depends on the reaction of phenylalanine with 1,2-naphthoquinone-4-sodium sulfonate to give a red color. Tryptophane but not tyrosine or histidine, interferes with the determination. Tryptophane is destroyed by oxidation with potassium permanganate in this procedure. This method was claimed by the authors to be very sensitive. The smallest amount of phenylalanine

that can be estimated is approximately 6 micrograms. The values obtained by Hess and Sullivan seem to check with those given by Block and Bolling (13). The results obtained by Hess and Sullivan confirm the suggestion made by Block and Bolling, that for the determination of phenylalanine, hydrolysis with 5 N sodium hydroxide should be used, and that hydrolysis with hydrochloric acid destroys part of the amino acid.

The detailed procedure adapted in this study is given below.

Procedure for the Determination of Phenylalanine

Reagents.

Nitrating mixture. Dissolve 20 gm. of powdered potassium nitrate in 100 ml. of concentrated sulfuric acid.

Hydroxylamine hydrochloride. Dissolve 30 gm. of hydroxylamine hydrochloride in 100 ml. of water.

Concentrated ammonium hydroxide.

Procedure.

Sulfuric acid or sodium hydroxide hydrolyzates can be used for this determination. Four aliquots that contain 0.5-2.0 mg. of phenylalanine are pipetted into 10 cm. porcelain evaporating dishes. The solutions are evaporated to an oily consistency or to dryness on a steam bath. The

that can be estimated is approximately 6 micrograms. The values obtained by Hess and Sullivan seem to check with those given by Block and Bolling (13). The results obtained by Hess and Sullivan confirm the suggestion made by Block and Bolling, that for the determination of phenylalanine, hydrolysis with 5 N sodium hydroxide should be used, and that hydrolysis with hydrochloric acid destroys part of the amino acid.

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Hydroxylamine hydrochloride. Dissolve 30 gm. of hydroxylamine hydrochloride in 100 ml. of water.

Concentrated ammonium hydroxide.

Procedure.

Sulfuric acid or sodium hydroxide hydrolyzates can be used for this determination. Four aliquots that contain 0.5-2.0 mg. of phenylalanine are pipetted into 10 cm. porcelain evaporating dishes. The solutions are evaporated to an oily consistency or to dryness on a steam bath. The

dishes should be removed from the bath soon after they are dried. After cooling, 2 ml. of the nitrating mixture are added and the dishes are warmed on a steam bath for 25 minutes, and the solution should be stirred by swirling every 5 minutes. At the end of 25 minutes, the dishes are removed from the bath and the solution is transferred as quickly as possible into a 25 ml. stoppered graduated cylinder. The quantitative transfer is effected by washing with small portions of water. The final volume in the cylinders should not be over 9 to 10 ml.

The solutions should be mixed thoroughly and cooled in ice bath for 30 minutes. Then, 2.5 ml. of hydroxylamine hydrochloride are added to three of the graduates. One aliquot is kept as a protein blank. Each of the solutions is shaken for 10 seconds immediately after the addition of hydroxylamine. The cylinder is put back to the ice bath and cooled for exactly one minute (not longer), and the solution is diluted to the 25 ml. mark with ice cold concentrated ammonia. Ammonia should be added slowly and with shaking. The contents are then mixed by inverting the cylinder several times. The color is allowed to develop at room temperature for 45 minutes. When determinations are made on alkaline hydrolyzates, it is often desirable to filter the colored solutions 15 or 20 minutes before the end of this period. Any fast paper (dry) may be used.

The colors are read in the Evelyn colorimeter with filter 540. The solution which no hydroxylamine hydrochloride was added is used as the "blank." The k value is determined with pure phenylalanine solution. The value 0.340 was found to be applicable under the conditions of these determinations as established.

Preparation of Enzyme Solutions

Pepsin Solution.

0.9 gm. of commercial pepsin (Difco 1:20,000) was dissolved in 60 ml. of a sodium hydroxide-hydrochloric acid mixture, prepared by mixing 15 ml. of 2 N sodium hydroxide with 30 ml. of 2.26 N hydrochloric acid and diluting to 500 ml. The concentration of sodium hydroxide and hydrochloric acid were the same as that used in preparing the protein solutions. This mixture has a pH of 1.28.

Pancreatic Extract (113).

Hog pancreas, freed from fat and most of the connective tissue was immersed in acetone. The tissue was minced several times in a meat grinder, and then shaken with three times its weight of acetone. The suspension was allowed to stand for two hours with frequent shaking. The solvent was removed by filtration, and the solid residue was again shaken with acetone. After filtration, the residue was shaken with an acetone-ether mixture (1:1). The extracted

material was dried by shaking twice with ether. The tissue containing some residual ether was spread out in a thin layer on filter paper and dried at room temperature. It was then powdered and sifted through a 40 mesh seive. The dry pancreas powder was stored in a tightly stoppered bottle and kept at room temperature. The dry material was extracted with glycerol as needed for the digestion experiments.

To prepare a glycerol extract, the powder was shaken for two hours with 10 times its weight of 87 per cent * glycerol. This extract was separated from the residue by filtration which was best carried out in a refrigerator, as it usually took almost 2 weeks to filter about 100 ml. of extract. Just before use, the extract was diluted and activated by an enterokinase preparation.

The enterokinase was prepared in the following way. The upper third of the small intestine of hog was washed with distilled water, then split, and scraped with a spatula. These scrapings were dropped into acetone, an amount equal to three times the weight of mucosa. The mixture was allowed to stand for two hours with frequent shaking and then filtered. The extraction was repeated. The residue was dried with ether in the usual manner, and then exposed to the air to remove the residual ether. The

*This was prepared by mixing 87 gm. of 100 per cent glycerol with 13 gm. of water.

material was pulverized, and stored at room temperature.

Just before use, the powdered mucosa was extracted with a slightly alkaline solution. 0.25 gm. of the dry material was suspended in 12.5 ml. of water. The mixture was made just alkaline to litmus with 0.1 N sodium hydroxide (usually 3-5 drops), and then incubated at 37° C. for three hours. At the end of this period the residue was removed by centrifugation.

10 ml. of the enterokinase extract were added to 50 ml. of the glycerol extract of powdered pancreas which had been diluted with 40 ml. of water. This mixture was incubated at 30° C. over night. The activity of the activated pancreatic extract was tested by the action of 0.5 ml. of the enzyme solution on 5 ml. of casein solution (approximately 3 gm. of casein per 50 ml.), in the presence of 0.2 ml. of an ammonia-ammonium chloride buffer (0.1 M pH 8.2). The mixture was incubated at 30° C for exactly 20 minutes. At the end of this period, the digestion was stopped by pouring the test solution into a flask containing 25 ml. of 95 per cent alcohol, and the test tube was rinsed with 4 ml. of water. Controls were run in exactly the same manner except that enzyme solution was added after the protein-buffer mixture had been rinsed into the flask containing the alcohol. These alcoholic solutions were then titrated with approximately 0.1 N sodium hydroxide using thymolphthalein as indicator. When the first blue

color appeared, 50 ml. of boiling 95 per cent alcohol were added, and the aliquots were titrated to the same end-point again. The test solutions were also titrated in the same way. The difference between the control and the test solution showed the action of the enzyme. The unit of activity is expressed as that quantity of enzyme under the stated conditions of the test which effects a cleavage as shown by increase in acidity equivalent to 1.05 ml. of 0.2 N sodium hydroxide (113).

Extract of Intestinal Mucosa.

The upper third of the small intestine of hog was used. When the intestine was still warm, it was cleaned and then rinsed with distilled water. The mucosa scraped from the walls of the intestine was suspended in 5 times its weight of 87* per cent glycerol and the mixture was stored in the refrigerator.

The extract was diluted as needed for the digestion experiments. 50 ml. of the glycerol extract of intestine were pipetted as free of tissue as possible and diluted with an equal volume of water. 1.35 ml. of N acetic acid were added to the diluted extract, while agitating. The precipitate was removed by centrifugation, and then filtered through cotton to remove any material floating

*This was prepared by mixing 87 gm. of 100 per cent glycerol with 13 gm. of water.

on top of the solution.. The pH was adjusted to 8.0 with sodium hydroxide. The adjustment had to be made carefully to avoid inactivation of the enzyme. The activity of the preparation was determined in the following way.

The substrates used for testing the activity of peptidases were dl-leucyl-glycine and dl-leucyl-glycyl-glycine. The concentration of the leucyl-glycine was 0.1882 gm. per 50 ml. and that of the leucyl-glycyl-glycine, 0.2450 gm. per 50 ml. These solutions were adjusted to 8.0-8.1, by dissolving the peptide in 2 ml. of ammonia-ammonium chloride buffer (1 N, and pH 9.26) and diluting to 50 ml. The activity of the extract was determined by incubating 5 ml. of the substrate with 0.3 ml. of the diluted enzyme extract at 30° C. for 1 hour. At the end of one hour, the digestion was stopped by adding 25 ml. 95 per cent alcohol. The groups liberated by the enzyme were titrated with 0.1 N sodium hydroxide. When the first permanent blue of the indicator appeared 50 ml. of boiling 95 per cent alcohol were added, and the titration was completed. Controls were carried out at the same time, in the same manner except that alcohol was added before the introduction of enzyme. The difference between the control and the test solution gave the activity of the enzyme, expressed as ml. of 0.02 N sodium hydroxide per ml. enzyme solution.

Table XXXIV

Activity of Pancreatic Extract Before and After Mixing with Borate Buffer

1. Before mixing with borate buffer:

	Test Solution ml.	Control ml.
Casein	5	5
Ammonia-ammonium chloride buffer	2	2
Diluted pancreatic extract	0.5	0.5*
Titration with 0.1158N sodium hydroxide using thymolphthalein as indicator	13.94	11.55

The activity of the diluted pancreatic extract is equal to 2.64 units** per ml.

2. After mixing with borate buffer: (30 ml. of buffer solution plus 90 ml. diluted pancreatic extract. 0.7 ml. of this mixture equals to 0.525 ml. of the diluted pancreatic extract.)

	Test solution ml.	Control ml.
Casein	5	5
Ammonia-ammonium chloride buffer	2	2
Diluted pancreatic extract plus buffer	0.7	0.7*
Titration with 0.1158 N sodium hydroxide using thymolphthalein as indicator	13.85	11.55

The activity of the buffer-enzyme mixture is equal to 1.81 units** per ml., but on the basis of the original diluted pancreatic extract, the activity is equal 2.74 units** per ml.

*In the control, enzyme solution was added after the casein-buffer mixture had been mixed with alcohol.

**One unit is that quantity of enzyme under the stated conditions of the test which effects a cleavage equivalent to 1.05 ml. of 0.2 N sodium hydroxide.

Preparation of Protein Solutions for the Study of Digestion

About 5 gm. of protein were placed in a 250 ml. Erlenmeyer flask, fitted with a rubber stopper, and mixed with water. The total amount of water used was 78.4 ml., which had to be added in 10 ml. portions with 5 minutes interval of agitation between each addition. Then, 0.2 ml. of caprylic alcohol, an anti-foam agent, and 0.2 ml. toluene, a preservative, were added followed by 3.7 ml. of 2 N sodium hydroxide. The suspension was shaken continuously until all particles dissolved, (usually about an hour). Now, 7.5 ml. of 2.26 N hydrochloric acid mixed with 35 ml. of water were poured quickly into the protein solution while it was being vigorously whirled. After the acid was added, the protein usually precipitated out, but the precipitate would disappear upon further shaking. The final volume of this solution was 125 ml. and the pH, 1.5. This colloidal solution was then diluted to 200 ml. with 75 ml. of sodium hydroxide-hydrochloric acid mixture.*

The compositions of the digest and controls were as given in Table XXXV.

The composition of the pancreatic digest, intestinal mucosa digest, and their controls are given in the text.

*15 ml. of 2 N sodium hydroxide and 30 ml. of 2.26 N hydrochloric acid are diluted to 500 ml.

Table XXXV

	Protein solution (pH 1.39)	Pepsin solution (pH 1.45)	Sodium hydroxide-hydrochloric acid mixture* (pH 1.26)	pH of the final mixture
	ml.	ml.	ml.	
Protein-enzyme mixture	75	25	--	1.48
Enzyme control	--	25	75	1.29
Protein control	75	--	25	1.33

*15 ml. of 2 N sodium hydroxide and 30 ml. of 2.26 N hydrochloric acid are diluted to 500 ml.

Titrimetric Methods

Willstätter titration (114).

The 1 ml. aliquots were placed in 50 ml. Erlenmeyer flasks, each of which contained 2 ml. of 95 per cent ethyl alcohol, 0.4 ml. of 0.5 per cent alcoholic (95 per cent) thymolphthalein, and 1 ml. of water. Alcoholic potassium hydroxide (0.04 N in 90 per cent aldehyde-free alcohol) was added until the first blue color appeared. To this solution, 18 ml. of 95 per cent alcohol were added, and titrated to the same blue color as that of a standard prepared by mixing 20 ml. of 95 per cent alcohol, 0.4 ml. of thymolphthalein, 2 ml. of water and 0.23 ml. of 0.0418 N alcoholic potassium hydroxide. When there was much precipitate, the end-point was difficult to determine. In

that case, the flask, tightly stoppered, was allowed to stand for about two minutes. The colors of the supernatant liquids were compared.

Linderstrøm-Lang Titration (115).

The 1 ml. aliquots for titration were mixed with 3 ml. of acetone (99.5 per cent), 3 drops or about 0.05 ml. of naphthyl red (0.1 per cent in 95 per cent alcohol), and 1 ml. of water. The titration was carried out with 0.03 N alcoholic hydrochloric acid (90 per cent alcohol) to a distinct red. To this solution, 14 ml. of acetone (99.5 per cent) were added, and the diluted solution was titrated to the end-point of the standard. The standard was prepared by mixing 17 ml. of acetone, 3 drops of indicator, 0.7 ml. of 0.0284 N alcoholic hydrochloric acid and 2 ml. of water. The titration was difficult with hydrolyzates which formed precipitates in the presence of acetone. The use of a "block comparator" was found to assist in correcting for the precipitate.

Determination of Ammonia (Amide) Nitrogen

The procedure given by Linderstrøm-Lang (116) was employed for the determination of ammonia. An aliquot, usually 1.2 mg. nitrogen in 5-10 ml., of hydrolyzate and 80 ml. of water were introduced into a 300 ml. Kjeldahl flask, and this mixture was made alkaline by the addition

of 12-15 ml. of 10 per cent calcium hydroxide. By partial vacuum distillation, ammonia was driven into boric acid,* and then titrated as described in the micro Kjeldahl procedure.

The Micro Kjeldahl Procedure

The exact procedure depends on the particular apparatus employed. In this study, a Parnas type apparatus was used, and the following procedure was found to give satisfactory results.

A sample containing about 0.5-1.0 mg. of nitrogen was digested with 500 mg. of potassium sulfate, 50 mg. of mercuric oxide, and 1.5 ml. of sulfuric acid, in a micro Kjeldahl flask of 30 ml. capacity, for 6 hours. For material which had higher contents of carbon and nitrogen, a sample containing 5-10 mg. of nitrogen was digested similarly but with 2 gm. of potassium sulfate, 200 mg. of mercuric oxide, and 10 ml. of sulfuric acid in a 100 ml. Kjeldahl flask. The digest was cooled, diluted to 50 ml. and 5 ml. aliquots were taken for distillation.

Preparatory to distillations, the apparatus was steamed for 10 minutes. At the beginning of each determination, the steam generator was about 2/3 full. A mark

*11 ml. of brom cresol green-methyl red indicator (117) were mixed with 1 liter of 2 per cent boric acid.

was put on the flask, so that the water could be brought to the same level each time.

Before the introduction of the sample, the flask (125 ml.) containing 10 ml. boric acid* was placed in such a position that the tip of the silver delivery tube was below the surface of the liquid. After the clamp of the steam trap was opened, the sample was introduced. The digestion flask and funnel were rinsed at least three times with water. Then, 5 ml. of sodium hydroxide-thiosulfate mixture** were added, followed by rinsing. Two micro burners were placed under the steam generator, and the steam trap was closed immediately. The flames were adjusted so that the steam was generating at such a rate that 15 seconds after the bulb of the distilling head became hot, the boric acid* began to change color. The period of distillation was counted from the time at which the small distilling head became hot, and continued for 3 minutes. At the end of that time, the flask was lowered so that the tip of the delivery tube was 1-1.5 cm. above the surface of the liquid. Immediately after this, the water in the condenser was emptied. The distillation was continued for 2 more minutes. Then, the flask was lowered further so that the

*11 ml. of brom cresol green-methyl red indicator (117) were mixed with 1 liter of 2 per cent boric acid.

**40 gm. of sodium hydroxide were dissolved in 60 ml. of boiled water. Cooled. 5 gm. of sodium thiosulfate were added.

tip of the delivery tube was at the neck of the flask. In this position the distillation was continued for 1 more minute, giving a total period of 6 minutes for distillation. Finally, the delivery tube was rinsed with water. The distillates were diluted to approximately 45 ml. and titrated with 0.01 N hydrochloric acid. Blanks were carried out in exactly the same manner.

Calculation of Digestibility of Protein in the Enzymatic Digestion *in vitro*

I. Hydrolysis by Enzymes

$$I = \frac{100\ [(D_t - D_o) - (E_t - E_o)] \times 14 \times C}{N}$$

All values for titration are expressed as ml. of standard acid or alkali.

I . . Increase of acidity or alkalinity in terms of per cent of total nitrogen.

D_t. . Titration of the digest at time t.

D_o. . Titration of the digest at zero time.

E_t. . Titration of the enzyme control at time t.

E_o. . Titration of the enzyme control at zero time.

C . . Normality of the alcoholic hydrochloric acid or alcoholic potassium hydroxide.

N . . Total nitrogen content of the protein in mg. per ml. of digest.

The protein control showed such slight increase in acidity at the end of digestion, that it was not included in the calculation.

II. Hydrolysis by Acid

$$P = 14(A_a - Cl + H - Am - A_o) \times 100$$

or

$$= 14(B_a + Cl + H - Am - B_o) \times 100$$

All values are expressed in ml. of 0.01 N alkali or acid per mg. of total nitrogen.

P . . Nitrogen in peptide linkage expressed as percentage of the total nitrogen.

A_a. . Willstätter titration of the humin-free hydrolyzate.

Cl. . Chloride of the humin-free hydrolyzate.

H . . Potential titratable acidity or alkalinity in the humin, i.e., 1/2 of the humin nitrogen.

Am. . Ammonia in humin-free hydrolyzate.

A_o. . Willstätter titration of the unhydrolyzed protein.

B_a. . Linderstrøm-Lang titration of the humin-free hydrolyzate.

B_o. . Linderstrøm-Lang titration of the unhydrolyzed protein.

III. Digestibility

$$D = \frac{I}{P} \times 100$$

D . . Digestibility.

Table XXX

Determination of the Increase of Acidity of Peptide Linkages by Hydroc

All values are expressed as ml. of 0.01 N acid or base per mg. nitrogen.

	Observed		Titratable acidity and basicity in the humin	Corrected for humin		Obser
	Acidity	Alkalinity		Acidity	Alkalinity	Acidi
	ml.	ml.	ml.	ml.	ml.	ml.
Unhydrolyzed globulin I	0.434	0.771				
Unhydrolyzed globulin II	0.437	0.767				
Average	0.436	0.769				
HCl hydrolyzed globulin I	5.797	5.788	0.036	5.833	5.824	5.39
HCl hydrolyzed globulin II	5.847	5.800	0.035	5.882	5.835	5.44
Average						
Unhydrolyzed albumin	0.719	0.628				
HCl hydrolyzed albumin	7.299	6.748	0.084	7.383	6.832	6.66
Unhydrolyzed casein	0.968	0.547				
HCl hydrolyzed casein*	6.349	5.522	0.017	6.366	5.539	5.38
Correction for Phosphorus						

* nach Hammarsten. Phosphorus content equals 0.78 per cent. The correction f

Table XXXVI

e of Acidity and Alkalinity Due to the Cleavage
es by Hydrochloric Acid Hydrolysis
n.

for	Observed increase		Ammonia	Increase corrected for ammonia		Increase expressed as percentage of total nitrogen		
linity	Acidity	Alkalinity		Acidity	Alkalinity	Acidity	Alkalinity	Average of acidity and alkalinity
l.	ml.	ml.	ml.	ml.	ml.	per cent	per cent	per cent
824	5.397	5.055	0.755	4.642	4.300	65.0	60.2	
835	5.446	5.066	0.770	4.676	4.296	65.5	60.1	
						65.25	60.15	62.7
832	6.664	6.204	0.771	5.893	5.433	82.6	76.1	79.4
539	5.381	4.975	0.984	4.397	3.991	61.6	55.9	
						59.8	55.9	57.9

correction factor used was 0.707 (36).

Table XXXVII

Digestion of Tung Nut Globulin with Commercial Pepsin

All values given in the table had been corrected for any increase found in the enzyme control. Alkalinity was determined by the Linderstrøm-Lang method and acidity by the Willstätter method. 1.0 ml. of the digest contained 2.917 mg. of protein nitrogen (equivalent to 16.6 mg. protein), and 3.75 mg. of pepsin.

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage *	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage *
hours		mg.	per cent	per cent	mg.	per cent	per cent
1	1.48	0.115	3.94	6.3	0.115	3.94	6.3
2		0.134	4.59	7.3	0.138	4.73	7.5
4		0.164	5.62	9.0	0.167	5.73	9.1
24		0.248	8.47	13.5	0.250	8.57	13.7
48		0.289	9.91	15.8	0.298	10.22	16.3
73		0.351	12.03	19.2	0.356	12.20	19.5
96		0.354	12.14	19.4	0.361	12.38	19.7
139.5	1.61	0.354	12.14	19.4	0.362	12.41	19.8

*The nitrogen in peptide linkages was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent).

Table XXXVIII

Digestion of Tung-Nut Globulin with Pancreatic Extract

Following Peptic Digestion

All values given in the table had been corrected for any increase found in the enzyme control. Alkalinity was determined by the Linderstrøm-Land method, and acidity by the Willstätter method. 1.0 ml. of the digest contained 1.361 mg. of protein nitrogen (equivalent to 7.8 mg. protein) and 1.104 Willstätter units of tryptic activity.**

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*
hours		mg.	per cent	per cent	mg.	per cent	per cent
1	7.49 7.90	0.0549	4.03	6.4	0.0576	4.23	6.7
2		0.1000	7.35	11.7	0.1014	7.45	11.9
6		0.1422	10.45	16.7	0.1423	10.44	16.6
20		0.1895	13.92	22.2	0.1912	14.05	22.4
44		0.2448	17.99	28.7	0.2436	17.90	28.5
66		0.2591	19.04	30.4	0.2559	18.80	30.0
89		0.2714	19.94	31.8	0.2705	19.88	31.7
118		0.2885	21.20	33.8	0.2852	20.96	33.4
162		0.2877	21.14	33.7	0.2904	21.34	34.0
184	7.98	0.2917	21.43	34.2	0.2915	21.42	34.2

*The nitrogen in peptide linkages was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

**One unit is that quantity of enzyme under the stated conditions of the test which effects a cleavage of casein equivalent to 1.05 ml. of 0.2 N sodium hydroxide.

Table XXXIX

Digestion of Tung Nut Globulin with Extract of Intestinal Mucosa Following Peptic and Pancreatic Digestion

All values given in the table had been corrected for any increase found in the enzyme control. Alkalinity was determined by the Linderstrøm-Land method, and acidity by the Willstätter method. 1.0 ml. of the digest contained 0.689 mg. protein nitrogen (equivalent to 3.9 mg. protein), and 1.00 ml. of peptidases activity.**

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage *	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage *
hours		mg.	per cent	per cent	mg.	per cent	per cent
2 1/3	7.95	0.024	3.48	5.5	0.020	2.90	4.6
4 1/2		0.031	4.50	7.2	0.029	4.20	6.7
18		0.042	6.10	9.7	0.042	6.20	9.9
46		0.066	9.58	15.3	0.064	9.29	14.8
91 1/2		0.084	12.19	19.4	0.082	11.90	19.0
137 3/4		0.095	13.79	22.0	0.093	13.50	21.5
187 1/2		0.108	15.67	25.0	0.105	15.24	24.3
235		0.113	16.40	26.2	0.116	16.95	27.0
282	7.99	0.124	18.00	28.7	0.123	17.85	28.5

*The nitrogen in peptide linkages was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

**The activity of the enzyme preparation is expressed as ml. of 0.02 N sodium hydroxide per ml. enzyme solution.

Table XL

Digestion of Tung Nut Albumin with Commercial Pepsin

I

All values given in the table have been corrected for increase found in the enzyme controls. The alkalinity was determined by the Linderstrøm-Lang method, and the acidity was by the Willstätter method. 1.0 ml. of the digest contained 2.949 mg. of protein nitrogen (equivalent to 20.6 mg. protein), and 3.75 mg. of pepsin.

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*
hours		mg.	per cent	per cent	mg.	per cent	per cent
1	1.48	0.111	3.80	4.8	0.112	3.80	4.8
2		0.130	4.41	5.6	0.133	4.51	5.7
4 1/2		0.165	5.60	7.1	0.168	5.71	7.2
20		0.250	8.48	10.7	0.272	9.22	11.6
42		0.318	10.78	13.6	0.324	10.99	13.8
95		0.363	12.31	15.5	0.364	12.31	15.5
140		0.390	13.22	16.7	0.401	13.60	17.1
186		0.417	14.14	17.8	0.432	14.65	18.5
236	1.71	0.416	14.11	17.8	0.436	14.78	18.6

*The nitrogen in peptide linkages was determined by titrating the acid and basic groups by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

Table XL (Continued)

II

1.0 ml. of the digest contained 3.375 mg. of protein nitrogen (equivalent to 23.6 mg. protein), and 3.75 mg. of pepsin.

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage *	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage *
hours		mg.	per cent	per cent	mg.	per cent	per cent
1	1.50	0.121	3.59	4.5	0.116	3.44	4.3
2		0.162	4.80	6.0	0.160	4.74	6.0
4		0.211	6.25	7.9	0.217	6.43	8.1
21		0.308	9.13	11.5	0.324	9.60	12.1
46		0.358	10.61	13.3	0.394	11.68	14.7
68		0.390	11.56	14.6	0.418	12.36	15.6
117		0.443	13.13	16.5	0.462	13.69	17.2
166		0.482	14.28	18.0	0.510	15.05	19.0
215		0.504	14.93	18.8	0.535	15.85	20.0
262	1.74	0.497	14.73	18.6	0.520	15.40	19.4

* The nitrogen in peptide linkages was determined by titrating the acid and basic groups by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

Table XLI

Digestion of Tung Nut Albumin with Pancreatic Extract

Following Peptic Digestion

I

All values given in the table have been corrected for any increase found in the enzyme control. Alkalinity was determined by the Linderstrøm-Lang method, and acidity by the Willstätter method. 1.0 ml. of the digest contained 1.032 mg. of protein nitrogen (equivalent to 7.0 mg. protein) and 0.968 Willstätter units of tryptic activity.**

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*
hours		mg.	per cent	per cent	mg.	per cent	per cent
1	7.32						
	8.22	0.072	6.98	8.8	0.074	7.17	9.0
2		0.120	11.63	14.6	0.115	11.14	14.0
4		0.130	12.50	15.7	0.129	12.50	15.7
20		0.206	19.96	25.1	0.204	19.77	24.9
46		0.271	26.26	33.7	0.265	25.68	32.3
67		0.286	27.71	34.9	0.285	27.62	34.8
118		0.295	28.59	36.0	0.293	28.39	35.8
162		0.315	30.52	38.4	0.311	30.14	38.0
211		0.346	33.52	42.2	0.335	32.46	40.9
259	8.21	0.350	33.91	42.7	0.336	32.56	41.0

*The nitrogen in peptide linkages was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

**One unit is that quantity of enzyme under the stated conditions of the test which effects a cleavage equivalent to 0.2 N sodium hydroxide.

Table XLI (Continued)

II

1.0 ml. of the digest contained 1.152 mg. of protein nitrogen (equivalent to 8.1 mg. protein), and 1.177 Willstätter units of tryptic activity.**

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*
hours		mg.	per cent	per cent	mg.	per cent	per cent
1	7.88	0.083	7.21	9.1	0.085	7.39	9.3
2		0.105	9.13	11.5	0.101	8.78	11.1
4		0.139	12.08	15.2	0.132	11.47	14.4
18		0.225	19.55	24.6	0.223	19.37	24.4
46		0.301	26.15	32.9	0.300	26.06	32.8
94		0.359	31.19	39.3	0.361	31.36	39.5
143		0.388	33.71	42.5	0.388	33.71	42.5
190	7.98	0.392	34.05	42.9	0.391	33.95	42.8

*The nitrogen in peptide linkages was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

**One unit is that quantity of enzyme under the stated conditions of the test which effects a cleavage equivalent to 1.05 ml. of 0.2 N sodium hydroxide.

Table XLII

Digestion of Tung Nut Albumin with the Extract of Intestinal Mucosa Following Peptic and Pancreatic Digestion

I

All values given in the table have been corrected for any increase found in the enzyme control. Alkalinity was determined by the Linderstrøm-Lang method, and acidity by the Willstätter method. 1.0 ml. of the digest contained 0.731 mg. protein nitrogen (equivalent to 5.1 mg. protein), and 1.11 ml. of peptidases activity.**

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*
hours		mg.	per cent	per cent	mg.	per cent	per cent
2	8.11	0.009	1.23	1.5	0.012	1.64	2.1
4		0.014	1.92	2.4	0.015	2.05	2.6
18		0.024	3.28	4.1	0.024	3.28	4.1
66		0.064	8.76	11.0	0.063	8.62	10.9
112		0.076	10.40	13.1	0.075	10.26	12.9
161 1/2		0.074	10.12	12.7	0.075	10.26	12.9
210 1/2	8.16	0.077	10.53	13.3	0.074	10.12	12.7

*The total nitrogen in peptide linkage was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

**The activity of the enzyme preparation is expressed as ml. of 0.02 N sodium hydroxide per ml. enzyme solution.

Table XLII (Continued)

II

1.0 ml. of the digest contained 0.711 mg. of protein nitrogen (equivalent to 5.0 mg. protein), and 0.90 ml. of peptidases activity.**

Digestion time	pH of digest	Increase of alkalinity in terms of nitrogen			Increase of acidity in terms of nitrogen		
		Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*	Per ml. hydrolyzate	Protein nitrogen	Nitrogen in peptide linkage*
hours		mg.	per cent	per cent	mg.	per cent	per cent
2	7.98	0.006	0.84	1.1	0.005	0.70	0.9
4		0.008	1.13	1.4	0.007	0.98	1.2
20		0.030	4.22	5.3	0.030	4.22	5.3
68		0.045	6.33	8.0	0.047	6.61	8.3
117		0.070	9.85	12.5	0.070	9.85	12.5
165	8.00	0.073	10.27	12.9	0.073	10.27	12.9

*The total nitrogen in peptide linkage was determined by titrating the acid and basic groups liberated by hydrolysis with hydrochloric acid (1:1) for 18 hours at 125° C. (100 per cent)

**The activity of the enzyme preparation is expressed as ml. of 0.02 N sodium hydroxide per ml. enzyme solution.

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