DETERMINATIONS OF VARIOUS FORMS OF NITROGEN
IN BOVINE FLESH,
INCLUDING THE PRODUCTS OF HYDROLYSIS
OF SOME OF THE PROTEINS

by

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DETERMINATIONS OF VARIOUS FORMS OF NITROGEN IN BOVINE FLESH,
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When Hausmann¹⁰ introduced his method for nitrogen distribution into protein analysis he opened the way for great progress in that line of investigation. Winterstein¹¹ later showed that phosphotungstic acid would also precipitate cystine. Van Slyke¹² improved the gasometric method of Sachse¹³ and Kormann for the determination of amino nitrogen and from these methods of analysis worked out a combination of them for determining arginine, histidine, lysine, and cystine in proteins. Several improvements in the method, such as removal of the phosphotungstic acid from the precipitate of the bases by shaking it out with amyl alcohol and ether by Van Slyke¹⁴ and the boiling of the bases with alkali of lower concentration for deter-

mination of the arginine by Plimmer¹⁶ have recently been published. The method has been widely used in the analysis of various proteins, tissues, foods and feeding stuffs.

For this study a number of beef proteins were analyzed according to the Van Slyke method. The proteins used represent the fractions of the flesh as developed by Grindley^{1, 2, 3}. The cold water insoluble protein is a mixture of stroma and plasma proteins and contains albuminoids, and nucleo proteins. The cold water soluble, heat coagulable fraction is a mixture of plasma protein, the albumins and globulins of flesh. On page is given an analysis of the flesh of a newborn calf showing just what part of the flesh these fractions represent. A description of the samples and the method of preparing them is given in a later section. Following is a description of the methods employed in this work.

Analysis of Proteins According to the Van Slyke Method

1. Hydrolysis--10 grams of protein are dissolved in ten to

twenty parts of 20 per cent HCl and boiled under a reflux condenser in a tared flask. After 10 hours hydrolysis is stopped and 1 c.c. portions withdrawn for amino nitrogen determinations. The deaminizing reaction is allowed for five minutes standing and one minute shaking. Under these conditions the same proportion of ammonia (15 to 20 per cent) is decomposed in each determination. The flask is weighed and hydrolysis continued for 6 to 8 hours and until the amino nitrogen is constant. The object of the weighing is to allow correction for concentration.

2. Total Nitrogen--The products of hydrolysis are transferred to a graduated flask and total nitrogen is estimated according to the Kjeldahl method on an aliquot of 5 c.c.

3. The ammonia nitrogen determination is made on an aliquot representing 3 grams protein which is placed in a distilling flask and most of the HCl is distilled off in vacuum, diluted to 200 c.c., 100 c.c. alcohol added to prevent frothing, and then an excess of a 10 per cent suspension of Ca(OH)_2 as shown by turbidity and alkaline reaction of the solution. The distillation is carried on at 40° for one-half hour and under 30 to 40 mm. pressure. Alizarin sulphate is used as indicator. A description of the apparatus used is given in the Journal of Biological Chemistry, Vol. 10, page 21, and in Plimmer, The Chemical Constitution of the Proteins, page 74.

4. Humin Nitrogen--The black coloring matter is adsorbed by the lime, which is filtered off on a folded filter paper and washed with water until free from chlorides. The precipitate and paper are submitted to a Kjeldahl analysis, using 35 c.c. H_2SO_4 .

5. Precipitation and Washing of the Phosphotungstic Precipitate.

The filtrate from the humin nitrogen is neutralized with HCl , returned to vacuum distillation apparatus, and concentrated to about 100 c.c. and transferred to a 250 c.c. beaker. 18 c.c. of concentrated HCl and 15 grams of phosphotungstic acid in aqueous solution are added. The entire solution is diluted to 200 c.c. with water and heated on a water bath until the precipitate has nearly redissolved. On cooling the phosphotungstates separate in crystalline form.

After standing at least 48 hours they are filtered off in a 2 inch Buchner funnel on a hardened filter paper. 10 to 15 c.c. of a washing solution containing 2.5 grams of phosphotungstic acid and 3.5 grams HCl per 100 c.c. and which has been cooled to 0°C . are poured into the well packed precipitate, which is stirred up with each portion by means of a flattened stirring rod, so that all parts of the precipitate are reached. Washing is continued until no test for calcium is given. This is made by placing 1 or 2 c.c.

of 10 per cent NaOH containing some oxalate in a test tube and allowing 2 or 3 drops from the Buchner funnel to run down the side of the tube and form a layer on top of the solution. The tube is now gently shaken until just enough alkali has mixed with the upper layer to turn the latter alkaline. The tube is viewed against a dark background with plenty of light going thru the solution.

Removal of phosphotungstic acid. The basic precipitate is removed from the filter by a spatula and transferred to a one-half liter separatory funnel using 200 to 300 c.c. of water in the transfer. 5 to 10 c.c. concentrated HCl and the mixture is shaken with 1:1 amyl alcohol-ether, using so much of the latter that it floats on the water after the precipitate has gone into solution. Usually 100 c.c. of the mixture and one minute shaking are required. If the solution is fouled by humin nitrogen the whole mass without separation can be passed thru a Buchner funnel with suction. The aqueous layer is extracted with three or more successive portions of the mixture (about one-fourth volume of water). Finally the combined alcohol-ether extracts are shaken with water to remove traces of bases and separated and the wash water washed with a fresh portion of the mixture, and combined with the main solution of the bases. The latter should be free from phosphotungstic acid as shown by $\text{Ba}(\text{OH})_2$. The solution is now concentrated in vacuo and washed into a 50 c.c. flask.

The determination of amino nitrogen according to van Slyke¹⁶ can now be performed with the micro apparatus with only one or two c.c. The reaction should be allowed to proceed for one-half hour and a blank should be run. Complete directions for manipulating the apparatus are given in Mathews⁸, page 974, and Hawk⁹, page 88.

Arginine is determined on 20 or 25 c.c. of the solution according to Plimmer's¹⁵ modification. 20 or 25 c.c. of a 40 per cent NaOH solution are added to the solution in a small round bottom flask, a few pieces of pumice stone added. This is then attached to a condenser at the upper end of which is a Folin tube containing 15 c.c. of standard acid. After six hours boiling the water is drawn from the condenser and the solution boiled 15 to 20 minutes to drive off all of the ammonia. One-half of the arginine nitrogen is driven off when treated thus as ammonia. The excess of standard acid in the Folin tube is titrated against NaOH, using Alizarin Sulphonate as indicator.

Cystine is determined by Denis' modification of Benedict's method. 10 c.c. of the solution of bases and cystine are placed in a porcelain dish with 5 c.c. of a solution containing 25 grams of $\text{Cu}(\text{NO}_3)_2$, 25 grams of NaCl and 10 grams of NH_4NO_3 per 100 c.c. The mixture is evaporated to dryness on a water bath and then gradually heated to redness at which temperature it is held for ten minutes. The residue is dissolved in 10 c.c. of a 10 per cent HCl solution and diluted to 150 c.c. 10 c.c. of a 5 per cent BaCl_2 so-

lution are slowly added to the boiling solution and the BaSO_4 is filtered off and washed in the usual way. A blank on the reagents should be run. This should not exceed 1.5 milligrams. One milligram of BaSO_4 corresponds to 0.06 milligrams of cystine nitrogen.

Calculation of Histidine Value

Total nitrogen minus total amino nitrogen gives total non-amino nitrogen--D. This is contained in the arginine and histidine. Since three-fourths of the arginine and two-thirds of the histidine nitrogen are in this form, the total non-amino nitrogen minus three-fourths of the arginine represents two-thirds of the histidine nitrogen. Hence

$$\text{Histidine N} = 3/2(D - 3/4 \text{ Arg.}) = 1.5 D - 1.125 \text{ Arg.}$$

Calculation of Lysine Value

Lysine is found by difference:

$$\text{Lysine N} = \text{Total N} - (\text{Arg.N} + \text{Cyst.N} + \text{Hist.N})$$

Determination of total nitrogen in monoamino acids. To the combined filtrate and washings of the phosphotungstate precipitate 50 per cent NaOH is carefully added until the solution becomes turbid by precipitation of lime. Excess should be avoided. Acetic acid is added to clear the solution. The acid solution is concentrated in vacuo until salt begins to crystallize out. The solution is washed into a graduated flask and made up to 250 c.c. Total nitrogen is estimated in 10 c.c. portions by Kjeldahl's method, 25 c.c. of H_2SO_4 , 10 grams K_2SO_4 , and 0.20 grams CuSO_4 being used. The

acid should be added carefully on account of the evolution of the HCl. The digestion should be continued for three hours after the solution has become clear.

Determination of amino nitrogen in monoamino acids. 10 c.c. portions are used and the reaction allowed to proceed for 6 to 10 minutes.

Corrections for solubilities of phosphotungstates

Table for precipitation in the volume of 200 c.c.

	Total N grams	Amino N grams	Non-Amino N grams
Arginine Nitrogen	0.0032	0.0008	0.0024
Histidine Nitrogen	0.0038	0.0013	0.0025
Lysine Nitrogen	0.0005	0.0005	0.0000
Cystine Nitrogen	<u>0.0026</u>	<u>0.0026</u>	<u>0.0000</u>
Sum		0.0052	0.0049

Preparation of the Samples

The flesh was separated from the fatty tissue and bones by hand. It was then ground finely in a meat chopper and about five kilos placed in a cooler the temperature of which was about 40°F. and mixed thoroly with cold water. The mixture was then filtered thru muslin. If the temperature is not too low labor can be saved by pouring the mixture upon the muslin which has been fastened on a square frame and catching the filtrate by means of a funnel in a bottle

below. The mixing with water and filtering is repeated until the filtrate no longer gives the biuret reaction. The filtrate is treated with a little thymol to prevent bacterial action and filtered thru paper and evaporated on the water bath until quite concentrated. The coagulated proteins are filtered off* by means of suction and washed with water and finally with alcohol. The cold water insoluble residue and the coagulable protein is mixed thoroly with alcohol and preserved that way until used. The filtrate and aqueous washings from the coagulable protein are concentrated on the water bath to a thick syrup and preserved as such. The concentration is such that bacteria will not grow.

Treatment of cold water insoluble residue

The alcohol is filtered from it by suction and it is treated with alcohol several times and filtered. Then it is treated with ether and filtered several times. The alcoholic and ether filtrates are mixed and made up to volume and the nitrogen determined in an aliquot. The protein residue is air dried, weighed, and the nitrogen determined in a sample. Ten gram samples are placed in paper cones and extracted in a soxhlet apparatus. Absolute alcohol is poured upon it first and it is allowed to

*If filtering goes slowly it is best to do it in the cold.

stand for a few days. The alcohol is then siphoned off into the flask and the sample is further extracted with ether in the usual way for about 24 hours. The alcohol-ether mixture is washed into a Kjeldahl flask and the nitrogen in it is determined in the usual way. The quantity of nitrogen obtained in the second extraction is very small. The sum of the weights of the nitrogen in the alcohol-ether extracts and the nitrogen in the residue after extraction divided by the weight of the nitrogen obtained by the two extractions gives the lipoid-protein nitrogen ratio of the cold water insoluble part of the flesh. The extracted samples are then ready for analysis.

The action of water on the proteins during these separations

Van Slyke¹⁶ found by determining the amino nitrogen that digestion of muscle proteins in water at 100°C for three hours did not hydrolyze them. The water extracts of these samples were made in the cold so that enzymes present did not have favorable conditions during that time. They were then brought up to about 80°C and concentrated. There may have been enzymatic action during the time required for the extract to be brought to 80°C and some hydrolysis may have taken place, yielding more non-heat coagulable protein than it should. The extent of this hydrolysis was not determined.

Description of the Samples

The cold water insoluble protein 562C was obtained from

a lean flesh composite of a newborn calf and 549 from a five year old Group III steer (see first part of paper) in the manner described above. The insoluble protein 508B was kindly given me by Dr. Trowbridge. It was prepared as follows: The lean flesh of round of baby beef was extracted with cold water and then with ammonium sulfate, the remaining ammonium sulfate was removed by washing and the sample was treated with alcohol, dried and then extracted with ether. The sample was then air dried. The whitish shreds resulting were sifted and the coarsest sample was 508B.

The coagulable samples were obtained as described above, 560C from the lean flesh composites of a newborn calf and the others from the lean of the round. Steer 505 was full fed from birth and slaughtered at eleven months, 501 was treated as No. 505 but slaughtered at forty-eight months, No. 48 was held as a feeder for three and a half years and then full fed for one and a half years. Nos. 505, 501, and 48 were all fat when slaughtered and analysis of the proteins from the same parts of the carcass ought to show the influence of age upon the composition of the sample. In this paper the cold water insoluble residues are designated with an R before the number of the animal from which it came, and the coagulated samples have the abbreviation coag. before the number of the animal.

In Table I the results of the van Slyke analysis are given.

Table X. Van Slyke Analysis of Seven Beef Flesh Proteins in Per Cent of Total Nitrogen

	Humin Nitrogen	Ammonia Nitrogen	Cystine Nitrogen	Histidine Nitrogen	Lysine Nitrogen	Arginine Nitrogen	Non-Amino N in Filtrate	Amino N in Filtrate	Total Nitrogen
Residue of Lean of Ribs, water extraction followed by alcohol and ether extraction R549 Group III 5 yrs.	1.4	3.9	1.1	1.9 32.3	14.0	15.3	0.9 59.20	58.3	96.8
Coarse Sample Lean Meat extrac- ted with cold water, 10 per cent ammonium sulfate and with ether. Baby Beef R508B	1.7	6.0 ^o	0.7	5.8* 31.3	12.05**	12.8	61.63		100.64
Residue of Lean Flesh Composite after extraction with cold water, alcohol ether. Newborn Calf R562C	1.5	5.4	0.9	4.9 33.1	13.6	13.8	1.8 60.30	58.5	100.35
Coagulated Protein from Lean Flesh Composite of Newborn Calf Coag. 562C	2.3	6.8	1.1	5.3 30.6	13.3	10.9	61.36		101.07
Coagulated Protein of Cold water extract of round of steer Coag. 505 Group I 11 months.	2.2	6.7	1.0	9.1 33.1	13.1	9.9	2.7 60.10	57.4	102.06
Coagulated Protein Lean of Round of NO. 501 Group I 4 yrs.	2.1	6.9	1.2	7.6 34.8	15.5	10.2	56.26		100.07
Coagulated Protein Lean of Round of No. 48 Fat 5 yrs.	2.6	6.0	0.8	5.4 30.0	12.7	11.1	0.3 60.10	59.8	97.88

o During the ammonium sulfate extraction some ammonia may have united
with the protein making the ammonia nitrogen value too high.
*This Value is too high. See discussion of the histidine nitrogen.
**This value is too low. See discussion of the histidine nitrogen.

The Humin Nitrogen

Humin is the name given to the black substance formed during acid hydrolysis of proteins. The amount of humin can be much increased by the addition of sugars, as many investigators have shown. The most thoro study of humin formation has been made by Roxas¹⁸, who found that alanine, leucine, phenylalanine and glutaminic acid gave very little or no humin by boiling them in twenty per cent HCl with glucose, xylose, and fructose. Proline yielded no humin with 4.15 per cent HCl when treated with glucose, but did so when treated with xylose or fructose. Tyrosine gave 15 per cent of its nitrogen to humin formation; cystine, 3.1 per cent; arginine, 2.31 per cent; lysine, 2.62 per cent; hystidine, 1.84 per cent; tryptophane, 71.0 per cent. The addition of proline increased humin formation from tyrosine and cystine. He suggests that humin formation depends upon the presence with the amino acid of groups capable of fairly ready ring formation; this occurs and condensation takes place in the presence of furfural.

The non-amino nitrogen of the filtrate from the bases in these analyses represents the non-amino nitrogen of proline and one-half the nitrogen of tryptophane, not destroyed by acid hydrolysis. The analyses, however, are not accurate enough to allow any conclusion except that non-amino nitrogen is probably present in the filtrates of all samples.

The humin nitrogen is about one per cent lower in cold

water insoluble proteins than in the coagulable proteins. This may be due to differences in carbohydrate, proline and tryptophane contents. Van Slyke found 1.38 per cent humin nitrogen in a hydrolysate of casein.

The Ammonia Nitrogen

The greater part of the ammonia nitrogen formed during the hydrolysis of proteins probably comes from the acid amides. It has been suggested from the fact that in some proteins the amounts of ammonia obtained corresponded with the amount of glutaminic and aspartic acid in the samples that the ammonia and these diamino acids exist in the protein molecule as acid amides. In fact, asparagine and glutamine have been isolated. Andersen¹⁷ and Roed-Mueller have presented strong arguments for believing in the presence of uramino linkings in casein:

This linking would also yield ammonia upon hydrolysis. The uramino acids are strongly acid in character. Other sources of ammonia are the products of secondary decomposition of some of the amino acids. Cystine is one source. Andersen and Roed-Mueller have shown that tryptophane upon drastic hydrolysis in an autoclave does not yield ammonia, but that each successive treatment of casein in an autoclave at 150°C in 3N HCl for one and a half hours would increase the amount

of ammonia by 0.85 per cent in terms of the total nitrogen. In these samples of meat proteins the ammonia nitrogen is higher in the coagulated proteins than in the cold water insoluble samples.

It may be noted that sample R508B has a higher ammonia content than the other cold water insoluble samples. The sample had been extracted with ammonium sulfate and thoroly washed. It may be possible that some ammonia has united with the free amino groups of the protein as the acid salt, $R-NH_2(NH_4)HSO_4$.

The Cystine Nitrogen

The amounts of cystine nitrogen given in the table are lower than the true quantities present, since it is known that cystine gives rise to humin* formation, ammonia¹⁹ formation, and that H_2S ^{19, 20, 21} is given off during hydrolysis. The quantities present, however, are all very close to one per cent and the determinations are of value in calculating the per cent of lysine. The cystine content of these proteins is slightly higher than that obtained by Van Slyke by the same method in casein, which was 0.70 per cent (corrected for solubilities of bases).

*Roxas¹⁸ found that 3.38 per cent of the total nitrogen of a mixture of 0.2 grams cystine and 2.0 grams proline + 2 grams glucose + 20 per cent HCl was changed to humin, while cystine alone with glucose yielded no humin.

Due to the presence of silica the cystine in samples R508B and coag. 48 was not weighed. However, a very small precipitate was noticed. The values given in the table give the amount of cystine nitrogen which would not have been precipitated by the phosphotungstic acid.

The Arginine Nitrogen

The arginine nitrogen in the cold water insoluble samples is higher than in the coagulated samples.

The Lysine Nitrogen

The values given in the table for lysine nitrogen are slightly lower in the cold water insoluble samples. However, a correction for nucleo protein bases would make the values slightly higher so that it is impossible to say from these data that there is any marked difference in amount of lysine nitrogen present.

Summarizing these results we find that all of the cold water insoluble samples differ decidedly from the coagulated samples in their hydrolytic products, that is, in the humin nitrogen formed from them and in the ammonia, the histidine, and the arginine nitrogen content. The differences among the insoluble samples as well as among the coagulated samples must be due to the different sources of the samples.

The three samples of coagulated protein from the round of animals No. 505, No. 501, No. 48, are comparable except as to age, but while the analysis shows differences these differences do not vary with the age of the animal.

The Histidine Nitrogen

The histidine nitrogen is lower in the cold water insoluble samples than in the coagulable samples. The value given in the table for sample R508B is too high since in determining the amino nitrogen in the bases the nitrous acid was allowed to act for only five minutes, while it requires thirty minutes for complete action upon the epsilon amino group of lysine. About four-fifths of the nitrogen of the epsilon amino group of lysine is liberated by nitrous acid in five minutes. Allowing a correction for this the value for histidine becomes 3.9 and that for lysine, 13.9.

Corrections for the Presence of Nucleo-Proteins

The cold water insoluble samples contain small quantities of nucleo-proteins. The nucleic acid^{8, 21} of the nucleo-proteins probably contains the following bases which are precipitated by phosphotungstic acid.

Purine	(	1 molecule of guanine = 5 atoms nitrogen
	{	1 molecule of adenine = 5 atoms nitrogen
Pyrimidine	(	1 molecule of cytosine = 3 atoms nitrogen
	{	1 molecule of thymine = 2 atoms nitrogen

Total = 15 atoms nitrogen

The purine nitrogen in sample R562C was determined according to the method of Hall²² and found to be 0.44 per cent of the total nitrogen. Then the purine and pyrimidine nitrogen precipitated by phosphotungstic acid = $15/10 \times 0.44$, or

0.66 per cent. The formula for calculating the per cent of histidine (see page 50) can be corrected for nucleic acid bases and will be

$$\text{Histidine nitrogen} = 3/2(D - 3/2 \text{ purine nitrogen} - 3/4 \text{ arginine nitrogen}),$$

and for lysine nitrogen

$$\text{Lysine nitrogen} = \text{total nitrogen of bases} - 3/2 \text{ purine nitrogen} - \text{arginine nitrogen} - \text{histidine nitrogen} - \text{cystine nitrogen}.$$

Using these formulae the value of the histidine nitrogen in sample R562C becomes 3.93 and for lysine nitrogen, 13.89. The correction lowers the amount of histidine by 1.0 per cent and raises the amount of lysine by 0.33 per cent. Several assumptions have been made in these calculations, but it is certain that nucleic acid in the proteins which are analyzed according to van Slyke causes the determination of histidine to be too high and that of lysine to be too low. This fact strengthens the conclusion that the cold water insoluble samples have less histidine nitrogen than the coagulated samples.

The non-amino nitrogen of the filtrate from the bases represents one-half the tryptophane and all of the proline and oxyproline nitrogen. The differences shown in the table are not significant because slight errors in the determination will change the results appreciably. In some cases no non-amino nitrogen is given in the table because the amino nitrogen determination was slightly higher than the total

nitrogen determination. This does not mean that no non-amino nitrogen is present, but that the accuracy of the determinations is such that it could not be determined. However, it is certain that in the samples analyzed here the amount of non-amino nitrogen in the filtrate from the bases is not more than about 3 per cent. It may also be remarked that this form of nitrogen--tryptophane, proline and oxyproline--makes up the largest part of the humin nitrogen.¹⁸

Discussion of the Results

The proteins of muscle are generally divided into two groups, those of the muscle plasma and those of the muscle stroma. The former is the thick juice which may be expressed under high pressure from fresh muscle. The plasma proteins may also be obtained by extracting the muscle with 5 per cent sodium chloride or with 5 per cent ammonium chloride. According to Von Fürth there are three coagulable proteins in muscle plasma. They are myogen, myosin, and soluble myogen fibrin.

The greater part of the proteins of the muscle are insoluble in ammonium chloride solution. These are the proteins of the stroma. Nucleo-proteins and phospholipins are also present. The latter are apparently in union with the proteins. The protein is probably an albuminoid or a meta-protein. The heat coagulable proteins used in this work

are of course all plasma proteins, while the cold water insoluble residues contain all of the original stroma proteins and some of the plasma proteins. Mathews (p. 604) says:

'It seems probable from the general phenomena of the squeezing out from gels on contractions of the latter by the process called syneresis of a solution containing the same matter as the gel, but in different concentration, that the plasma may, in reality, contain the same proteins as the stroma but in a more dilute form.' The differences in constitution of the cold water insoluble and the coagulable samples found in this work are evidence against this probability. The amount of nucleo-proteins present does not have an appreciable effect upon the results of the analysis. We have here differences between mixtures of stroma proteins with plasma proteins, and plasma proteins.

Samples R562C and R549

The analysis of these samples shows differences that are greater than the experimental errors. Sample R562C is the cold water residue of the flesh composite of a newborn calf, while R549 is a composite of the lean flesh of the ribs of a five-year-old steer. The latter contained some fatty tissue. It has been found in the analysis of many been animals in this laboratory that the analysis of the lean flesh of the ribs corresponds very closely to the analysis of the lean and fat composite of the carcass. It may be said, then, pending analysis of more samples, that it is probable that

during growth a change takes place in the composition of the cold water insoluble protein of the flesh of beef animals; the products of hydrolysis contain less ammonia nitrogen, less histidine nitrogen, and more arginine nitrogen. The difference in histidine content has been checked by another method (see page 34).

Hartley²³ analyzed ox serum and horse serum proteins according to the method of van Slyke. He found that the serum albumins differed in their chemical constitution from the serum globulins. The analysis of different globulins from the same source showed no marked differences. Below is a table giving Hartley's results on ox serum.

Hartley's analysis of ox serum proteins

	Whole Protein	Albumin	Total Globulin.
Humin nitrogen	1.60	1.1	2.0
Ammonia nitrogen	7.05	5.8	7.7
Cystine nitrogen	2.75	3.5	2.0
Arginine nitrogen	11.05	10.4	10.9
Histidine nitrogen	4.40	6.7	6.3
Lysine nitrogen	13.55	16.3	9.0
Amino nitrogen of fil- trate	56.65	54.2	59.8
Non-amino nitrogen of filtrate	2.15	2.3	2.2
	58.80	56.5	62.0
Sum	99.20	100.3	99.9

Comparing these results with those given in Table XIV for the flesh proteins one is unable to see any close agreement between any of the serum proteins and any of the flesh proteins. The analysis shows differences in the constitution of the proteins but the various forms of nitrogen determined are present in all samples in amounts of the same order of magnitude. The same statement applies to the results given in the following table on fibrin²⁴ and the proteins derived from it and to lactalbumin, casein²⁵ and gelatine. The great difference in constitution of gelatin and the other animal proteins is apparent.

Van Slyke Analysis of Fibrin²⁴, Two Proteins Obtained from
the Fibrin and Lactalbumin and Casein²⁵

	Fibrin	Proto- Albumose	Hetero- Albumose	Lact- Albumin
Humin Nitrogen	3.17	4.11	2.75	2.32
Ammonia Nitrogen	8.32	4.73	6.37	8.50
Cystine Nitrogen	0.99	0.50	0.91	1.21
Arginine Nitrogen	13.86	14.89	14.15	7.11
Histidine Nitrogen	4.83	4.53	6.45	4.27
	31.2	29.50	31.80	25.5
Lysine Nitrogen	11.51	9.60	10.30	12.35
Amino Nitrogen in filtrate	54.20	53.90	54.90	61.50
Non-Amino Nitrogen in Filtrate	2.70	6.95	3.85	1.60
	56.9	60.85	58.75	

(Continued)

	Gelatine	Casein
Humin Nitrogen	0.07	1.28
Ammonia Nitrogen	2.25	10.27
Cystine Nitrogen	0.00	0.73
Arginine Nitrogen	14.74	8.06
Histidine Nitrogen	4.48	6.98
	25.5	
Lysine Nitrogen	6.32	10.40
Amino Nitrogen in Filtrate	55.80	54.75
Non-Amino Nitrogen in Filtrate	14.90	6.13
	70.7	60.88

Several unsuccessful attempts were made to determine the free²⁶ amino nitrogen in these samples. The difficulty lies in the insolubility of the proteins in non-hydrolyzing solvents. An attempt was made to dissolve the samples by rubbing a portion of them in a mortar with glacial acetic acid, also by rubbing up a portion with dry and with wet sodium carbonate and pouring the mixture into glacial acetic acid. The amount of free amino nitrogen corresponds in some proteins with one-half of the amount of lysine present. From this it appears that the epsilon amino group of lysine is free in the protein molecule.

Several unsuccessful attempts were made to separate the proteins in the non-coagulable part of the cold water extract of the newborn calf in large enough quantities for a van Slyke analysis. Alcohol would not precipitate them in a filterable form. The ideal precipitant would be one that would precipitate only the proteoses, peptones, and polypeptides without precipitating other meat bases, and one which could easily be removed.

Tyrosine Determinations

Tyrosine in these samples was determined according to the method of Plimmer and Eaves²⁷. The method depends upon the fact that tyrosine absorbs two atoms of bromine according to the equation:

an observation made by J. H. Millar. The bromine is

absorbed in a nascent form and prepared according to the equation:

$$\text{NaBrO}_3 + 5 \text{ NaBr} + 6 \text{ HCl} = 3 \text{ Br}_2 + 6 \text{ NaCl} + 3 \text{ H}_2\text{O}.$$

An excess of N/5 NaBrO₃ solution + NaBr solution is added and after fifteen minutes absorption the solution is treated with NaI and the iodine which corresponds to the excess bromine is titrated against N/10 Na₂S₂O₃, using starch as indicator. Protein and some of its constituents besides tyrosine, histidine, cystine, tryptophane absorb bromine. Tyrosine is separated from proteins, histidine and cystine by precipitation with phosphotungstic acid and separation from tryptophane is effected by a trypsin hydrolysis since the tryptophane of a protein does not appear in the early stages of trypsin digestion. Plimmer and Eaves have settled upon six hours of digestion with trypsin as the length of time required by trypsin to liberate the tyrosine but not the tryptophane of a protein. They found a close agreement between the figures for tyrosine content obtained by this method from a large number of proteins and the figures obtained by isolation and weighing.

The technique used in the analyses presented here is as follows: 1.25 grams of the air dry protein in a 250 c.c. beaker was treated with 10 c.c. of 23.8 per cent NaOH solution and 50 c.c. of water and boiled for a short time. All but a very small amount of protein went into solution. 4.7 c.c. of 33.7 per cent solution of HCl was added slowly with stirring. The volume of the solution was brought up to 100 c.c., giving it a concentration of 0.2 per cent NaOH. The solu-

tion was warmed to about 37° and 0.1 gram trypsin was added and 2 c.c. of chloroform were added to prevent bacterial action, and the mixture allowed to digest exactly six hours at 37° in an incubator. The solution was then quickly transferred to a 110 c.c. sugar flask, cooled to room temperature and made up to volume. Two 50 c.c. aliquots were treated in a 250 c.c. beaker with exactly 100 c.c. of a 10 per cent phosphotungstic acid in 5 per cent sulphuric acid solution. After stirring, the precipitate consisting of proteins and basic amino acids was filtered thru dry folded filters. 100 c.c. of the filtrate was run into glass stoppered flasks and brominated as follows*: 10 c.c. of a N/5 NaBrO_3 solution was run into the flask from a burette and then 10 c.c. of a 2 per cent NaBr solution was added. The stopper was put into the flask and the flask was gently rotated and allowed to stand for 15 minutes in diffused light. It was then cooled under the water faucet and a few drops of a 4 per cent NaI solution were placed in the depression above the stopper of the flask. When gently opened the slight vacuum in the flask will draw in the NaI solution which will absorb any bromine escaping. 10 c.c. of the NaI solution are added and the stopper is replaced and the flask is gently rotated. Then the iodine

*Plimmer and Eaves recommend the use of NaI and NaBr instead of KI and KBr because phosphotungstic acid is precipitated when K salts are present, making titration difficult.

solution on the stopper and neck of the flask is rinsed with water into the flask and the contents of the flask are titrated against N/10 thiosulfate solution, using starch as indicator. Sometimes the presence of the phosphotungstic acid makes it difficult to recognize the end-point. This is especially so if the original protein solution is dark. In that case the end-point is a change from a distinct blue to a violet. It is, however, readily found after some experience with an error no greater than three drops.

A blank determination on 0.1 gram of trypsin was also made. The thiosulfate solution was titrated against N/10 potassium dichromate. The blank titration minus the titration of the samples gives the amount of bromine absorbed by the sample in terms of N/10 thiosulfate. Following is a table of results of these determinations upon the six samples analyzed.

Table II.

One c.c. of Thiosulfate = 0.008465 gr. Bromine.

Sample	Blank	Thiosulfate Titration		Thiosulfate Used		Wt. Total Nitrogen of Sample
R549	17.75	15.50 15.50	15.50	2.25		0.0874
R508B	17.75	15.45 15.95	15.70	2.05		0.08414
R562C	17.75	16.05 16.10	16.08	2.15 1.67	1.91	0.08560
Coag. 562C	17.75	15.78 15.85	15.82	2.10 1.93	2.02	0.08090
Coag. 505	17.75	15.85 16.00	15.93	1.82		0.08221
Coag. 501	17.75	15.65 15.70	15.68	2.07		0.08430

Table XV, continued.

Sample	Per Cent Tyrosine Nitrogen in Terms of Total Nitrogen		Per Cent Tyrosine in Protein. Wt. of Protein = 6.25 X Wt. of N. of the Sample	
R549	0.955		1.98	
R508B	0.904		1.87	
R562C	0.915 0.720	0.82	1.93 1.50	1.72
Coag. 562C	0.963 0.885	0.924	1.99 1.83	1.91
Coag. 505	0.818		1.69	
Coag. 501	0.907		1.89	

The results of the tyrosine determinations show variations from 0.82 to 0.96 per cent of tyrosine nitrogen in terms of total nitrogen of the proteins, or 1.69 to 1.99 per cent tyrosine in the proteins.

An error of one drop in titration is equal to 0.018 per cent tyrosine nitrogen. These differences are probably within experimental errors, and we may say that the tyrosine nitrogen in all these proteins in terms of total nitrogen is close to 0.9 per cent, or that the tyrosine content of these proteins is close to 1.9 per cent. This latter figure is somewhat lower than the one given for ox muscle protein obtained by isolation and weighing of the tyrosine by Osborn and Jones²⁸ who obtained 2.2 per cent tyrosine. Plimmer and Eaves found differences as great as that by the two methods.

The figures given were calculated upon the assumption that tyrosine absorbs two atoms of bromine (four atoms used) under the conditions of analysis. Plimmer and Eaves obtained very nearly theoretical results under these conditions. However, when a great excess of bromine is present tyrosine absorbs more bromine. Siegfried and Reppin²⁹, who also give valuable hints on the technique of bromine titrations, found that about four and one-half molecules of bromine were used by tyrosine with varying amounts of bromine excess and conditions not greatly different from those of this work.

The coagulated sample 562C absorbed a small amount of

bromine when treated as described but without the addition of trypsin. Sample coag. 505 after 24 hours digestion showed no increase in bromine absorption. Sample R549 after 72 hours digestion showed a marked increase in bromine absorption. The results given here are not to be considered as absolutely reliable because the method of analysis depends upon several assumptions. It is assumed that all of the tyrosine is split off during six hours of tryptic digestion and that none of the tryptophane has been liberated during that time. The verity of these assumptions has so far only been proved in the case of nine vegetable proteins and of casein by Plimmer and Eaves. In these samples they obtained more or less close agreement between the bromination and the isolation method of determining tyrosine. However, if there were any great differences in the tyrosine content of these proteins this method of analysis would show them.

The Bromination of Cystine

Since the writer was unable to find any data in the literature on the bromination of cystine a determination of the bromine consumption of cystine was made under the same conditions which governed the other bromination experiments reported in this paper. One-tenth gram of air dry cystine, kindly furnished by Mr. J. I. Hardy, was dissolved in water and a few c.c. of HCl, and diluted to 100 c.c. The sulfur content of the cystine was found to be 23.631 per cent; the

theoretical content is 26.704 per cent. The purity of the cystine was calculated from the sulfur content and found to be 88.48 per cent. Then, assuming all impurities to be water, the above mentioned 100 c.c. contain 0.08848 grams of dry cystine. Two 25 c.c. aliquots were diluted to about 100 c.c. and brominated (see section under tyrosine for technique). Ten c.c. of bromate added were equivalent to 18.71 c.c. thiosulfate (1 c.c. = 0.008465 gr. Br.). The number of cubic centimeters thiosulfate used to titrate the excess of bromine were 9.25 and 9.23 respectively. From these data it was calculated that one molecule of cystine consumes 10.8 atoms of bromine. An error of 0.8 c.c. in the above titration would account for the 0.8 atom of bromine consumed in excess of ten atoms by one molecule of cystine. This is rather large, but there may be a cumulative error in standardizing solutions and in determining the purity of the cystine. In another determination a somewhat lower bromine absorption was noted. Cystine will very likely consume more bromine if the reaction is allowed to go on for a longer time.

The sulfur in the cystine molecule is that part of it which is very likely to be attacked by bromine. Friedmann²¹ isolated cysteic acid from brominated cystine. The assumption of a formation of cysteic acid agrees well with the consumption of ten atoms of bromine by one of cystine according to the equation:

The oxidation of cystine sulfur was not complete to sulfuric acid since a small quantity which was brominated gave no precipitate with BaCl_2 even after standing a day with an excess of bromine. The possible extent of cystine bromination was no further investigated, the problem of bromine consumption under given conditions only being necessary for the present investigation.

The Bromination of Hydrolytic Products of Beef Flesh Proteins

Siegfried and Reppin²⁹ found that unhydrolyzed proteins absorb more bromine than their hydrolytic cleavage products. They found also that the following acids did not absorb bromine except after long standing with Br_2 , glycocoll, sarcosin, betain, dl-alanine, dl amino valeric, leucin, aspartic acid, glutaminic acid, lysin, arginine, phenyl alanine. By treating tyrosine with an excess of bromine and titrating back with starch and iodine one molecule of tyrosine absorbed 4.5 atoms of bromine; one of tryptophane, about eight atoms; and one of histidine, somewhat over two atoms. Now, cystine consumes bromine (see section on bromination of cystine) and the destruction of it and the tryptophane in acid hydrolysis of the proteins may account for the fact that unhydrolyzed proteins absorb more bromine than their hydrolyates. However, Siegfried and Reppin found

that edestin before trypsin hydrolysis absorbed more bromine than after. The only explanation they offer is that the increased bromine consumption due to liberation of tyrosine is more than balanced by the destruction of bromine consuming linkings during hydrolysis. But this ought not take place during trypsin digestion since tryptophane, cystine, and histidine are not destroyed. It is difficult to explain that.

Brown and Millard³⁰ and J. H. Millar³¹ found an increase in capacity for bromine absorption during the progress of protein hydrolysis, which they attributed to the liberation of tyrosine which is not supposed to absorb bromine in a combined form. However, their results were obtained under different conditions. No excess of bromine was added. Bromate solution was added to the solution of the sample until the latter gave a color reaction with starch-iodine solution.

A Method for Determining Histidine

The following experiments were made to determine whether or not bromine absorption determinations would be of value in conjunction with the van Slyke analysis. Two 5 c.c. aliquots (= 0.0323 g. protein nitrogen) of the solution of the bases of the coagulable protein sample 505 were treated with 10 c.c. of bromate solution (= 18.71 c.c. thiosulfate) and 50 c.c. water, 10 c.c. bromide solution, and 5 c.c. HCl. Bromination was allowed to proceed for 15 minutes. It took

17.0 c.c. of thiosulfate (= 0.008465 g. bromine) to titrate the excess of bromine (see page 69 for technique). The bromine absorbed was 0.01477 grams. Deducting 0.00259 due to cystine (cystine nitrogen = 0.0009 found by determining sulfur in another aliquot) assuming that cystine consumes ten atoms of bromine per molecule, we have a consumption of 0.0119 g. Br_2 by the histidine in solution. One molecule of histidine absorbs two atoms of bromine according to Siegfried and Reppin. Calculating the per cent of histidine nitrogen from the above data we get 9.68 per cent histidine nitrogen. To this must be added 1.17 per cent as a correction for solubility of histidine in presence of phosphotungstic acid as determined by van Slyke, making it 10.86 per cent histidine nitrogen. This is somewhat higher than was obtained by the van Slyke method, 9.08 per cent. By the same method sample R562C had an average of 4.73 per cent histidine nitrogen; by the van Slyke method, 4.97 per cent. The method gave also a value of 3.15 per cent histidine nitrogen for sample R549, while the van Slyke method gave 1.90 per cent.

The accuracy of this method may be considerably increased by using more dilute solutions for the titrations so as to measure larger volumes. A factor for bromine absorption for histidine may be of value since histidine absorbs somewhat more than two atoms of bromine per molecule.

Attention may be called to the fact that this method

requires only two determinations of which the cystine nitrogen is very accurate, while the van Slyke method requires three determinations. Amyl alcohol may interfere by absorbing bromine, but in the van Slyke analysis the solution of the bases after removal of the phosphotungstic acid is evaporated under a vacuum and boiled off. It is not known whether the purine and pyrimidine which are precipitated by phosphotungstic acid will absorb bromine under the conditions employed in these experiments. If further proof of the value of this method for the determination of histidine is obtained a van Slyke apparatus will no longer be necessary for a determination of the hexone bases.

The Bromination of the Filtrate from the Bases

The following table gives the results of brominating 50 c.c. of the filtrates from the bases in the van Slyke determinations. The end point due to the color of the phosphotungstic acid is difficult to recognize until one has had some experience when it can be recognized within a drop or two of the thiosulfate solution.

This bromine absorption may be due to the tyrosine and the tryptophane which has escaped destruction during the acid hydrolysis and the histidine and cystine which has escaped precipitation by the phosphotungstic acid.

The following experiments were made to determine whether or not the correction for the solubility of the cystine can be applied to brominations of the filtrate.

Table III. Bromination of Filtrate from Bases

Sample	R562C	R549	Coag. 505	Coag. 501
Bromate added in terms of c.c. Thiosulfate	18.71	18.71	18.71	18.71
c.c. Thiosulfate titrated	9.9 9.9	7.6 7.9	8.0 7.9	7.2 7.3
	9.9	7.65	7.95	7.25
Weight Br absorbed in terms of c.c. thiosulfate	8.81	11.06	10.76	11.46
Weight Br absorbed after subtract- ing 0.01483 g. for cystine and 0.00289 g. for histidine in solu- tion	0.05686	0.07590	0.07129	0.07929
Wt. of Br absorbed by tyrosine in same weight of sample	0.01650	0.01950	0.01215	0.01845
Weight of Br absorbed by tryptophane	0.04056	0.05640	0.05914	0.06084
Weight of nitrogen in the sample	0.07831	0.08951	0.06460	0.09548
Per cent of tryptophane nitrogen calculated for a bromine absorption 8 atoms per molecule	2.26	2.76	4.01	2.79
Per cent of tryptophane nitrogen calculated for a bromine absorption 6 atoms per molecule	3.02	3.68	3.35	3.72
Per cent of tryptophane. Weight of sample = 6.25 X Wt. N for Bromine Absorption 8 atoms per molecule	2.64	3.21	4.68	3.26
Per cent of tryptophane. Weight of sample = 6.25 X Wt. N for Bromine Absorption 6 atoms per molecule	3.53	4.30	6.24	3.45
N/Br after correction for cystine and histidine	1.37	1.18	0.91	1.20

Two aliquots of a cystine solution which would absorb bromine equivalent to 9.25 c.c. thiosulfate were treated in a volume of 50 c.c. with 4 grams of phosphotungstic acid and 5 c.c. of HCl and heated on a water bath. The solution was allowed to cool and after 36 hours standing the precipitate was filtered off with suction and washed with phosphotungstic acid solution. The filtrate was brominated and consumed an amount of bromine equivalent to 2.9 c.c. thiosulfate. The phosphotungstic acid was removed from the precipitate by extracting it according to van Slyke with amyl alcohol and ether. The solution of the precipitate after subtraction of a blank (2.26 c.c.) due to ether and amyl alcohol in the solution absorbed bromine equivalent to 6.7 ccc. thiosulfate. The precipitate and the filtrate together consumed bromine equivalent to 9.6 c.c. thiosulfate. This slight excess (0.35 c.c.) may be due to inaccuracy of the blanks determined for the bromine consumption of the ether and amyl alcohol in the solution containing the precipitated cystine. Phosphotungstic acid does not consume bromine as solutions of bromine titrate the same amount of thiosulfate in the presence of phosphotungstic acid as without it. If a solution of phosphotungstic acid is colored due to formation of oxides between $W O_2$ and $W O_3$, bromine will destroy the color, but upon titration with thiosulfate it appears again, apparently not affecting the final bromine consumption. The amount of cystine used in the above experiment contained 0.002502 g. nitrogen. According

to van Slyke, $\frac{0.0026}{4}$ or 0.00065 g. cystine nitrogen should be in the filtrate from the phosphotungstic acid precipitate. This would be equivalent to a bromine consumption of 2.4 c.c. in terms of the thiosulfate. The bromine actually consumed was 2.9 c.c., a fair agreement with the theoretical.

If now the correction for unprecipitated cystine and histidine is made, in the bromine absorption determinations upon the filtrates from the bases we have the weight of bromine absorbed by the tyrosine and the tryptophane. The amount of bromine absorbed by the tyrosine has been determined by the method of Plimmer and Eaves (see page 71) on these proteins. Subtracting The bromine absorption due to tyrosine from the corrected value for tyrosine plus tryptophane we get the bromine absorption due to tryptophane. The bromine absorption by tryptophane varies with the time and the excess of bromine added from about six atoms of bromine per molecule according to Plimmer and Eaves²⁷, Homer³², and Siegfried²⁹ and Reppin. The table shows the values for tryptophane nitrogen calculated on the assumption that one molecule of tryptophane absorbs six atoms of bromine and also that it absorbs eight atoms. The values for six atoms of bromine absorption are probably more nearly correct since Plimmer and Eaves, brominating under very similar conditions, obtained a bromine absorption of about six atoms. These values are only offered as indicating the approximate

amount of tryptophane in the proteins, as some tryptophane is destroyed during hydrolysis, and because the method of Plimmer and Eaves is not as reliable as is desirable. The values for sample R562C were calculated on the assumption that the tyrosine content as determined by Plimmer and Eaves' method was too low and that 3.2 per cent as found in ox muscle by Osborn and Jones²⁸ by isolation and weighing was the true tyrosine content. It was found that the 0.5 per cent difference in tyrosine content reduced the value for tryptophane nitrogen by about 0.3 per cent and the tryptophane by about 0.4 per cent.

The values in the table giving the N/Br ratio are obtained by dividing the weight of nitrogen representing the original sample by the weight of the bromine absorbed by the filtrate from the bases after deducting the bromine absorbed by the unprecipitated cystine and histidine. The N/Br ratio varies more than can be accounted for by experimental errors and this must be due to differences in tyrosine and tryptophane content.

The method of Annie Homer³² for the quantitative determination of tryptophane must be mentioned. As far as the writer knows no one besides Miss Homer has employed the method, which no doubt is a good one when one has mastered the technique. However, it has been shown in a previous section of this paper by other methods that differences in tryptophane content in various samples exist.

Foreman³³ has worked out a quantitative method for the

determination of glutaminic and aspartic acids.

The Analysis of a Flesh Composite of a Newborn Calf

The rather complete analysis of the lean flesh of the newborn calf as shown in Table IV was made by the writer in order to show the relation of the proteins which were examined to the fresh flesh. Several gaps in the methods for tissue analyses are evident. The value given for the alcohol-ether soluble nitrogen of the cold water insoluble fraction of the flesh may differ slightly from the true value, since a small part of the sample was removed from it while it was standing in alcohol without previous mixing. However, the result is reliable enough so that one can say unhesitatingly that this sample contains a much larger amount of alcohol-ether soluble nitrogen than the cold water insoluble part of the sample from the five year old steer 549, which contains 2.77 per cent of alcohol-ether soluble nitrogen. Inspection of the table will show that no separations within the alcohol-ether soluble nitrogen of the insoluble residue were made. The mono-amino acid nitrogen in the soluble and coagulated proteins has not been separated. However, approximate values for tyrosine and tryptophane are given on pages 28 and 36 respectively. No separations of the proteose and peptone nitrogen which is precipitated by tannic acid were made. This portion amounts to only two per cent of the total nitrogen

of the flesh. The only constituent of peptide and extractive nitrogen which was determined was creatin and creatinine nitrogen which amounts to 43 per cent of the total peptide and extractive nitrogen.

Summary and Conclusions

I. Cold water insoluble proteins of beef flesh yield on hydrolysis less humin, ammonia, histidine, and more arginine nitrogen than coagulated beef proteins. A possible significance of these results is discussed.

II. From the analyses of cold water insoluble samples from the flesh of a newborn calf and of a five year old steer it seems probable that during growth the insoluble proteins change in composition. The products of hydrolysis contain less ammonia, less histidine, and more arginine nitrogen.

III. The tyrosine nitrogen was determined according to the method of Plimmer and Eaves in a number of cold water insoluble and a number of coagulated beef proteins. No significant differences were found. The reliability of these determinations is discussed.

IV. The amount of bromine which cystine will absorb when treated with an excess of nascent bromine for fifteen minutes was found to be about ten atoms per molecule. Cysteic acid is probably the end product of the oxidation of cystine by bromine under these conditions.

V.. A new quantitative method of determining histidine in proteins by brominating the solution of the bases has been developed. Determinations of histidine on three different proteins by this method agree well with the results obtained by the Van Slyke method. Confirmatory proof of the accuracy of this method would make a Van Slyke apparatus unnecessary for a determination of the hexone bases.

VI. The filtrate from the bases was brominated. From these data approximate values for the tryptophane content were calculated. The N/Br ratio obtained by dividing the weight of the nitrogen in the original aliquot of the sample by the weight of the bromine absorbed varies widely with the different samples, indicating differences in tryptophane content of the samples.

VII. A table giving a rather complete analysis of a flesh composite of a newborn calf is given and discussed.

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