A thin glass bulb is blown at the end of a 3-mm. tube in the usual manner, but just before it becomes rigid it is squeezed lightly by a U-shaped piece of spring brass. This operation forms a banjo-shaped bulb. The flattened sides possess concentric corrugations which give superior clicking characteristics to the diaphragm. Usually only one side clicks well. However, in case there is confusion because both sides click near the same pressure difference, one of them can be heated until the corrugations are destroyed.

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THE MOLECULAR WEIGHT OF CASEIN. III[1]

BY D. C. CARPENTER

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In the two previous papers of this series, Svedberg, Carpenter and Carpenter[2] have described their experiments dealing with the determination of the molecular weight of casein by use of the ultracentrifuge. Casein prepared either by the method of Hammarsten[3] or the method of Van Slyke and Baker[4] was shown to consist of a mixture of protein molecules of different molecular weights. Furthermore, different specimens prepared by the latter method were shown to consist of different mixtures.

By extracting Hammarsten casein with warm acidified alcohol we have separated a protein which behaved as a monomolecular substance and which had a molecular weight of 375,000. This was found to constitute about 30% of the crude Hammarsten casein. The other chief constituent of crude casein was studied in several samples of Van Slyke and Baker casein and it was shown that the molecular weight of this constituent lay between 75,000 and 100,000.

That the protein of molecular weight between 75,000 and 100,000, the one of 188,000 and the acid-alcohol soluble one of molecular weight 375,000 are separate and distinct species has been shown by the serological studies of Carpenter and Hucker.[5]

This paper deals with the estimation of the molecular weight of the

[1] Read before the Meeting of the American Chemical Society at Cincinnati, Ohio, on September 9, 1930.

[2] Svedberg, Carpenter and Carpenter, THIS JOURNAL, **52**, 241, 701 (1930).

[3] Hammarsten, "Handbuch der biochemischen Arbeitsmethoden," E. Abderhalden, Berlin u. Wien, 1910, Vol. II, p. 384.

[4] Van Slyke and Baker, *J. Biol. Chem.*, **35**, 127 (1918).

[5] Carpenter and Hucker, *J. Inf. Diseases*, **47**, 435 (1930).

simplest of these proteins, the one which is the chief constituent of crude casein prepared by either of the foregoing methods and which may be fractionated by potassium oxalate. We propose to find the molecular weight of this protein through calculations based upon the sulfur, phosphorus and various amino acid contents of the protein. This idea is not particularly new, as it has been used by Cohn and his co-workers[6] in attempts to calculate the probable molecular weight of several proteins. The method of attack, however, presupposes (a) a monomolecular protein and (b) accurate analytical procedure for the estimation of the desired constituent. The former requisite is most easily fulfilled when we are dealing with a crystallizable protein and is most difficult of fulfilment when we are concerned with the amorphous proteins. The second requirement, of methods which yield the correct amino acid content after hydrolysis of the protein, must take into account the amount of any given amino acid which is destroyed by the hydrolytic process or lost in other ways. Losses by the Fischer ester method have been examined on known mixtures of amino acids by Osborne and Jones[7] and found to range from 20 to 80% for various amino acids.

In determining the molecular weight of a protein from the amino acids which it contains, it is obvious that the larger the individual molecular weight of a given amino acid and the smaller the number of times it occurs in the parent protein, the greater will be the accuracy of the molecular weight estimation. From the older analyses of casein[8] which were based on what we now know to have been a mixture of material, it seems probable that the determination of cystine, tryptophane and histidine will be of most service to us in selecting the molecular weight. Methods for estimating these amino acids have been worked out for use on relatively small quantities of protein and have been reported as satisfactory. As tyrosine can be estimated in the same hydrolysate after removal of the tryptophane, we propose to carry out this determination also, but will bear in mind at the outset that as there is considerable tyrosine in casein, the molecular weight of the parent protein based on the tyrosine content can scarcely have the same degree of certainty as the results of the cystine, tryptophane and histidine analyses.

Part I. Analysis of the Protein

Sulfur.—The various methods proposed for the estimation of sulfur in proteins have been critically examined by Redfield and Huckle.[9] Of these methods the Liebig and Koch, and the Liebig methods gave the maximum recovery of sulfur from a sample of peptone. In brief the Liebig

[6] Cohn and Hendry, *J. Gen. Physiol.*, **5**, 521 (1923).

[7] Osborne and Jones, *Am. J. Physiol.*, **26**, 305–28 (1910).

[8] Osborne and Guest, *J. Biol. Chem.*, **9**, 333 (1911).

[9] Redfield and Huckle, THIS JOURNAL, **37**, 607 (1915).

and Koch method[10] consists of a preliminary digestion of the protein with nitric acid followed by a fusion with potassium hydroxide and potassium nitrate, while the Liebig method is the same except that it omits the preliminary digestion with nitric acid.[11]

Determination of Sulfur.—For the estimation of sulfur in casein we have employed the Liebig and Koch method of oxidation. About one gram of casein was digested in a Kjeldahl flask with 25 cc. of concentrated nitric acid at a brisk boil for two hours. The solution was cooled in running water and 20 cc. of a 40% potassium hydroxide solution added slowly to the contents of the flask, which turned deep brown in color. The solution was transferred to a nickel crucible containing 0.5 g. of potassium nitrate and evaporated over an alcohol lamp, the flask being rinsed several times and the rinsings added to the contents of the crucible as the solution volume in the latter was reduced by evaporation. The contents of the crucible were then brought to gentle fusion over an alcohol lamp to complete oxidation of the protein. After cooling, the contents of the crucible were dissolved in water, made acid with hydrochloric acid, evaporated twice to dryness with hydrochloric acid to expel nitric acid, baked to dehydrate silica, dissolved in dilute hydrochloric acid, filtered and the sulfate in the filtrate precipitated in the usual way with barium chloride, filtered, washed, ignited and weighed as barium sulfate. The results of the sulfur analysis are reported in Table I and show that the protein under examination contains 0.785% of sulfur.

Phosphorus.—Hillebrand and Lundell[12] have shown that no volatilization of phosphates occurs when heated with excess sulfuric acid below 150°. Prolonged heating at higher temperatures, however, caused conversion of H_3PO_4 to HPO_2 and $H_4P_2O_7$, which resulted in large phosphorus losses.

Baumann[13] and Martland and Robison[14] appear to be the first workers to recognize the above findings and the significance they had in the phosphorus estimation in phosphorus-containing organic compounds. Both of these workers decomposed organic materials in the presence of concentrated sulfuric acid and emphasized that phosphorus losses occurred when the temperature rose sufficiently for white sulfur trioxide fumes to appear. The former used small amounts of 30% hydrogen peroxide and the latter used nitric acid in the presence of sulfuric acid to carry out the oxidation. Both finally estimated the phosphorus by colorimetric methods.

Determination of Phosphorus.—In our own experiments we have oxidized the protein with sulfuric acid and hydrogen peroxide as recommended by Baumann,[13] adding hydrogen peroxide and heating until no further charring occurred, being careful to so restrict the heating that no sulfur trioxide fumes were lost. The solution was then diluted with water made neutral with ammonia, then acidified with nitric acid and the phosphorus precipitated as phosphomolybdate under the conditions recommended by McCandless and Burton.[15] The yellow precipitate was filtered off, washed with 5%

[10] Liebig and Koch, *Chem. Zentr.*, **1886**, 894.

[11] Sherman, "Organic Analysis," The Macmillan Co., New York, 1912, p. 298.

[12] Hillebrand and Lundell, THIS JOURNAL, **42**, 2609 (1920).

[13] Baumann, *J. Biol. Chem.*, **59**, 667 (1924).

[14] Martland and Robison, *Biochem. J.*, **18**, 765 (1924).

[15] McCandless and Burton, *Ind. Eng. Chem.*, **16**, 1267 (1924).

ammonium nitrate until free from molybdenum, dissolved in ammonia and the solution filtered. The solution was then neutralized to litmus paper by dropwise addition of 1:1 hydrochloric acid, stirring continuously, and the phosphorus precipitated in the cold with magnesia mixture, followed after fifteen minutes by the addition of 12 cc. of concentrated ammonia per 100 cc. of solution volume. After standing overnight the precipitate was filtered off, washed with 2.5% ammonia, and finally with a little ammonium nitrate solution to assist in burning the filter paper. The precipitate was dried and heated carefully over a low flame and then ignited to constant weight with strong ignition and weighed as $Mg_2P_2O_7$. Blanks were run on the reagents used in both the sulfur and phosphorus estimations and the results corrected therefor. The results of the phosphorus determinations are given in Table I and show that the protein contains 0.856% of phosphorus.

TABLE I

SULFUR AND PHOSPHORUS CONTENT OF CASEIN

Expt.	Casein, g.	$BaSO_4$ corr., g.	$Mg_2P_2O_7$ corr., g.	S, %	P, %
1	1.336	0.07640	....	0.7854	...
2	1.336	.07642	....	.7856	...
3	1.336	....	0.04084	...	0.8519
4	1.336	....	.04113	...	.8599
Mean				.7855	.8559

Recovery of Cystine.—Hoffman and Gortner[16] have followed the effect of boiling cystine with 20% hydrochloric acid for different lengths of time by the bromate titration of Okuda[17] and found that after boiling periods of twenty-four and forty-eight hours the titratable cystine recovered was 93.99 and 92.52%, respectively, of the initial amount. These authors also record the fact that the recovered cystine was optically inactive and its phosphotungstate was more soluble than that of the original plate cystine used in the experiment, and concluded that an isomeric form of cystine had been produced. Plimmer and Lowndes[18] record that cystine loses 7% of its nitrogen by boiling with acids and that only 40% of the resultant material is precipitated by phosphotungstic acid, whereas about 97% of the original cystine is precipitated by this reagent.

For the purposes of this paper we are not concerned with what products are formed from cystine under the acid treatment but only with the matter of how much cystine escapes this change for a definite period. We have accordingly followed the change during the boiling process by the iodate method of Okuda.[19] This method is applicable for the determination of cystine and cysteine in the presence of all protein cleavage products.

In short the method consists of reducing cystine to cysteine with zinc dust and acid, and oxidizing the cysteine back to cystine with potassium iodate. The reactions involved are

[16] Hoffman and Gortner, THIS JOURNAL, **44**, 341 (1922).

[17] Okuda, *J. Col. Agr. Imp. Univ. Tokyo*, **7**, No. 1, 69 (1919).

[18] Plimmer and Lowndes, *Biochem. J.*, **21**, 247–53 (1927).

[19] Okuda, *J. Biochem. (Japan)*, **5**, 201, 217 (1925).

$$5KI + KIO_3 + 6HCl = 6I + 6KCl + 3H_2O \quad (1)$$

$$6HSCH_2CHNH_2COOH + 6I = 3COOHNH_2CHCH_2SSCH_2CHNH_2COOH + 6HI \quad (2)$$

Okuda recommends a $M/300$ potassium iodate solution for the titration, 1 cc. of which theoretically equals 0.0024 g. of cystine. At 17.5° Okuda found that 1 cc. corresponded to 0.00215 g. of cystine and the writer has found 0.00226 g. at 18.5°. Acid concentration and particularly the temperature must be fairly well controlled if concordant results are to be obtained. Starch indicator was used to mark the end-point of the titration more clearly.

Experimental

Three hundred and fifty milligrams of cystine four times recrystallized by the method of Folin[20] and dried *in vacuo* at 61°, was weighed out, dissolved in 2% hydrochloric acid and made up to 100 cc.; 10-cc. portions of this standard solution were then placed in Kjeldahl flasks and the calculated amount of concd. hydrochloric acid and distilled water added to make 100 cc. of 20% hydrochloric acid. Test-tube condensers closely fitting the necks of the Kjeldahl flasks were inserted and the entire set of flasks (with the exception of No. 1) were heated by an air-bath to the boiling temperature. At intervals of twenty-four hours one of the series of flasks was removed from the bath, the condensers washed off with a little water, the contents of the flask transferred to a distilling flask and the bulk of the hydrochloric acid removed by distilling *in vacuo*. The contents of the flask was then transferred to a 50-cc. volumetric flask and diluted to volume. For determining the acidity of this solution, 1 cc. was removed and titrated with standard sodium hydroxide and the calculated hydrochloric acid or sodium hydroxide required to bring the remainder of the cystine solution to a concentration of 2% hydrochloric acid was added. The cystine was now reduced to cysteine by the addition of 0.5 g. of zinc dust, reacting overnight at room temperature. The solution was filtered and the zinc and filter paper washed with 50 cc. of 2% hydrochloric acid and to the filtrate 5 cc. of 5% potassium iodide solution and an equal volume of 4% hydrochloric acid were added, the temperature adjusted to 18.5° and the titration carried out at once with $M/300$ potassium iodate, using starch as indicator.

The results of these experiments are recorded in Table II. It is to be noted that the recovery of cystine is quantitative in solutions which have not been heated strongly, but that in solutions which have been boiled even for twenty-four hours, considerable

TABLE II

DESTRUCTION OF CYSTINE BY 20% HYDROCHLORIC ACID

No.	Cystine in expt., mg.	Period of boiling, hours	Vol. KIO_3 soln. reqd.,[a] cc.	Cystine recov., mg.	Cystine recov., %
1[b]	35.00	0	15.70	34.94	99.8
2[c]	35.00	0	15.74	35.01	100.0
3	35.00	24	14.42	32.09	91.6
4	35.00	48	13.90	30.93	88.4
5	35.00	72	13.54	30.11	86.1

[a] 1 Cc. of KIO_3 soln. = 2.225 mg. of cystine. [b] Sample 1 not boiled with 20% hydrochloric acid and not vacuum distilled. [c] Samples 2, 3, 4 and 5 with varying periods of boiling; vacuum distilled.

[20] Folin, *J. Biol. Chem.*, **8**, 9 (1910).

cystine has been lost. The rate of loss appears to be greatest during the first twenty-four hours but additional boiling produces additional losses. These results parallel those obtained by Hoffman and Gortner[16] but the losses in cystine are somewhat greater in magnitude than they record.

That cystine analyses on proteins must be corrected for the losses incurred during the period of boiling with 20% hydrochloric acid is evident. That the same amount of destruction of cystine would occur in the hydrolysis of a protein or in a solution where other amino acids are present, from the foregoing data we are unable to say. This matter will be taken up later in this paper.

Recovery of Cystine.—It is entirely possible that what has often been reported in the literature as cystine in proteins, existed in the protein not as cystine, but in the reduced form as cysteine. It is almost useless to try to distinguish between cystine and cysteine quantitatively when both occur together, without the presence of an invert gaseous phase during the entire period of hydrolysis of the protein. Cysteine is very easily oxidized to cystine by oxygen of the air, particularly if the solution is warm, and strong reducing agents are required to carry out the reduction of cystine to cysteine.[21]

Experimental Part

A solution of purified cystine in hydrochloric acid was reduced overnight with tin,[21] treated with hydrogen sulfide to remove tin, filtered in an atmosphere of hydrogen, and hydrogen sulfide removed from the filtrate by the rapid passage of hydrogen gas through the solution; 10-cc. samples of this solution (39.00 mg. of cysteine) were pipetted into Kjeldahl flasks and the calculated amounts of concd. hydrochloric acid and water were added to make 100 cc. of 20% hydrochloric acid. Test-tube condensers were lowered into the necks of the flasks and the flasks heated in an air-bath at the boiling temperature in the same way as the cystine experiments just described. Number 1 was made up to volume in a 100-cc. volumetric flask with 2% hydrochloric acid and was not boiled or distilled *in vacuo*. Numbers 2, 3, 4 and 5 were boiled for various intervals, transferred to distilling flasks and the bulk of the hydrochloric acid distilled off *in vacuo*, the residue transferred to 50-cc. volumetric flasks and made up to volume; 1-cc. samples of these were titrated with standard sodium hydroxide and the calculated amount of hydrochloric acid or sodium hydroxide added to the remainder of the solution to bring the hydrochloric

TABLE III

DESTRUCTION OF CYSTEINE BY 20% HYDROCHLORIC ACID

No.	Cysteine in expt., mg.	Period of boiling, hours	Vol. KIO_3 soln. reqd.,[a] cc.	Cysteine recov., mg.	Cysteine recov., %
1[b]	39.00	0	17.64	39.00	100
2[c]	39.00	0.5	15.98	35.33	90.6
3	39.00	24	15.34	33.90	87.0
4	39.00	48	15.40	34.05	87.3
5	39.00	72	15.36	33.98	87.1

[a] 1 Cc. of KIO_3 soln. = 2.211 mg. of cysteine. [b] Sample 1 not boiled with 20% hydrochloric acid and not vacuum distilled. [c] Samples 2, 3, 4 and 5 with varying periods of boiling; vacuum distilled.

[21] Andrews, *J. Biol. Chem.*, **69**, 209–217 (1926).

acid concentration to 2%. Each of these solutions was then reduced with 0.5 g. of powdered zinc overnight at room temperature, the solutions filtered and the zinc residue and paper washed with 50 cc. of 2% hydrochloric acid; then 5 cc. each of 5% potassium iodide and 4% hydrochloric acid solutions were added and the solutions titrated with $M/300$ potassium iodate without delay in the presence of starch indicator. The results of these experiments are given in Table III, and show that considerable cysteine is lost even by a short period of boiling with 20% hydrochloric acid. No further loss seems to occur after twenty-four hours.

Recovery of Cystine and Cysteine in Protein Hydrolysis.—While the foregoing data show that considerable cystine or cysteine is lost during a heating with 20% hydrochloric acid, they do not necessarily represent the losses encountered in the presence of other amino acids, especially in the presence of the quantities of the latter present in the native protein under investigation. We have, therefore, determined the recovery of known amounts of cystine and cysteine when added to gelatin and also to casein when hydrolyzed with 20% hydrochloric acid for seventy-two hours, following the same procedure for handling the hydrolysate and estimating cystine and cysteine as outlined before. These data are given in Tables IV and V and show that in both gelatin and casein there is considerably less cystine or cysteine recovered when the boiling with 20% hydrochloric acid is done under conditions approximating those of the actual protein hydrolysis. There is, therefore, no "sparing action" on the decomposition of the cystine or cysteine through the presence of other amino acids. The

TABLE IV

RECOVERY OF ADDED CYSTINE AND CYSTEINE FROM HYDROLYSIS OF GELATIN BY 20% HYDROCHLORIC ACID FOR SEVENTY-TWO HOURS

Expt.	Gelatin, g.	Added cystine, mg.	Added cysteine, mg.	Vol. KIO_3 soln. reqd.,[a] cc.	Cystine or cysteine recov., mg.	Cystine recov., %	Cysteine recov., %
1	1.000	0	0	0.00			
2	1.000	4.00	0	12.49	2.54	63.5	
3	1.000	0	4.00	14.03	2.82		70.5

[a] By actual standardization 1 cc. of KIO_3 soln. = 0.2033 mg. of cystine and 0.201 mg. of cysteine.

TABLE V

RECOVERY OF ADDED CYSTINE FROM HYDROLYSIS OF CASEIN BY 20% HYDROCHLORIC ACID FOR SEVENTY-TWO HOURS

Expt.	Casein hydrolyzed, g.	Added cystine, mg.	Vol. KIO_3 soln. reqd.,[a] cc.	Cystine recov., mg.	Cystine recov., %
1	1.068	0	14.66	3.32	
2	1.068	0	14.60	3.30	
3	1.068	0	14.71	3.33	
4	1.068	4.00	25.87	5.85	63.2
5	1.068	4.00	26.00	5.87	63.9
Mean					63.5

[a] By actual standardization 1 cc. of KIO_3 soln. = 0.226 mg. of cystine.

above data show that of added amounts of cystine only about 63% is reclaimed under the conditions of hydrolysis stated, with either protein. In the case of cysteine about 70% is recovered when gelatin is hydrolyzed. That account must be taken of these losses before an accurate estimation of the cystine or cysteine in a protein can be made is entirely obvious.

Cystine Content of Casein.—In Table VI are recorded data of four separate casein hydrolyses in which the cystine has been determined as outlined before. In calculating the actual cystine content of the protein we have assumed that the added amino acid behaves the same in the hydrolysis process as the original amino acid contained in the protein and for cystine we have used the recovery factor as obtained from Tables IV and V. These data show that the cystine content of casein is close to 0.488%. Calculated as cysteine this is 0.44%, but as will be shown later in this paper, it is probable that there is no cysteine in casein, it being there in the oxidized form as cystine.

TABLE VI

CYSTINE CONTENT OF CASEIN

Expt.	Casein hydrolyzed, g.	Vol. KIO_3 soln. reqd., cc. (*a*)	Cystine, mg.	Corr. cystine, mg. (*b*)	Cystine, %
1	1.336	18.41	4.16	6.55	0.490
2	1.336	18.25	4.12	6.49	.485
3	1.068	14.60	3.30	5.20	.487
4	1.068	14.71	3.33	5.24	.490
Mean					.488
Calculated as cysteine					.440

(*a*) 1 Cc. of KIO_3 soln. equals 0.226 mg. of cystine at 18.5°. (*b*) Using recovery factor of 63.5% for cystine and 70.5% for cysteine.

Tryptophane and Tyrosine.—In the hydrolysis of protein to be used for tryptophane analyses we are limited to alkaline reagents and to splitting by enzymes. Even with these, appreciable amounts of the tryptophane are converted into indole.[22] The majority of methods for the estimation of tryptophane are colorimetric and in practically all of these the presence of indole or its derivatives so alters the color of the solution to be compared that anything but a rough match is impossible. For instance the method of Folin and Ciocalteu[23] gives excellent results on pure tryptophane solutions but is of little use on hydrolysates, due to the red color developed by indole derivatives on the phenol reagent. Kraus[24] finds that the indole products may be removed quantitatively by shaking out with toluene and meets with considerable success in using the vanillin–hydrochloric reaction in estimating tryptophane after an enzymatic hydrolysis of the protein.

[22] Herzfeld, *Biochem. Z.*, **56**, 82 (1913).

[23] Folin and Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

[24] Kraus, *ibid.*, **63**, 157 (1925); **80**, 543 (1928).

Homer[25] hydrolyzes the protein with barium hydroxide and after the customary separating by mercuric sulfate, brominates the tryptophane with nascent bromine and titrates the excess bromine as iodine with sodium thiosulfate.

Experimental

The method which we have adopted and used in this work is essentially the hydrolysis method of Folin and Ciocalteu[23] using sodium hydroxide, followed by Homer's method of bromination[25] at a low temperature.

One gram of protein was introduced into a new clean 250-cc. Kjeldahl flask, 2 cc. of butyl alcohol added to prevent foaming, and also a short spiral of silver wire and 4 g. of sodium hydroxide (as a 20% soln.). A test-tube condenser was placed in the neck of the flask, the flask mounted over a hot air-bath and boiled for twenty-four hours. At the end of the boiling period the condenser was removed, washed off with 10 cc. of water, and the boiling continued for ten minutes to remove the alcohol; the flask was then removed from the bath and while still hot 10 cc. of 14 *N* sulfuric acid added dropwise from a pipet, shaking well to precipitate silica; 5 cc. more of 14 *N* sulfuric acid was added, the flask cooled, the contents rinsed into a 100-cc. volumetric flask and diluted to 100 cc. The flask was well shaken and the solution filtered through a dry filter. The filtration is slow and the funnel should be covered with a watch glass to prevent evaporation.

To ascertain whether tryptophane was removed from a solution containing freshly precipitated silica we have conducted the experiments given in Table VII, adding various amounts of sodium silicate to change the amount of silica present. After silica removal these solutions were compared by the colorimetric method of Folin and Ciocalteu using

TABLE VII

RECOVERY OF TRYPTOPHANE

No.	Tryptophane in expt., mg.	SiO_2, g.	Mean height compared color, mm. (*a*)	Tryptophane recov., mg.	Tryptophane recov., %
1	8.70	0	29.15	8.70	100
2	8.70	0.445	30.00	8.45	97.1
3	8.70	0.890	30.05	8.43	96.9
4	8.70	1.335	30.00	8.45	97.1
Mean					97.1

(*a*) Color standard contained 0.60 mg. of tyrosine (0.676 mg. of tryptophane) per 100 cc.; height compared, 30 mm.

TABLE VIII

RECOVERY OF TYROSINE

No.	Tyrosine in expt., mg.	SiO_2, g.	Mean height compared color, mm. (*a*)	Tyrosine recov., mg.	Tyrosine recov., %
1	60.0	0	26.88	58.05	96.8
2	60.0	0.445	26.70	58.5	97.5
3	60.0	0.890	27.03	57.7	96.2
4	60.0	1.335	26.14	59.8	99.7
Mean					97.5

(*a*) Color standard contained 5.00 mg. of tyrosine per 100 cc.; height compared, 25 mm.

[25] Homer, *J. Biol. Chem.*, **22**, 369 (1915).

carefully prepared tyrosine as the color standard. As there was no indole in these solutions the color comparison was entirely satisfactory. Approximately 97% of the total tryptophane content appeared in the filtrate no matter how much silica was present as adsorbent. This seems to indicate that whatever loss of tryptophane occurs is due to adsorption by the filter paper rather than by silica. About the same recovery is shown for tyrosine in the experiments reported in Table VIII. In subsequent experiments the first 10 cc. of filtrate from the removal of silica was discarded, the remainder of the filtrate reserved for analysis and stored in the dark at 0°.

For the analysis of the hydrolysate, 8 cc. of hydrolysate was transferred to a 15-cc. centrifuge tube and 4 cc. of 15% mercuric sulfate in 6 *N* sulfuric acid added. The mixture was allowed to stand overnight in the ice box and then centrifuged for five minutes. The liquid was decanted into a 100-cc. volumetric flask, draining well and rinsing the tube edge with 0.1 *N* sulfuric acid. The sediment in the centrifuge tube was washed with 10 cc. of 1.5% mercuric sulfate in 2*N* sulfuric acid, stirring with a fine glass rod. The tube was centrifuged again and the liquid decanted as before into the 100-cc. flask, draining and washing again as before. The sediment in the centrifuge tube was reserved for the tryptophane determination.

Tyrosine.—Tyrosine in the mother liquid was determined as recommended by Folin and Ciocalteu, except that we have found that diluting the red-orange solution after development of the color was best done with *N* sulfuric acid instead of with water. If the dilution to standard volume is made with water, an opacity develops within a few moments that renders color comparisons inaccurate. No trouble has been experienced when *N* sulfuric acid has been used as the diluent.

Tryptophane.—The sediment in the centrifuge tube was suspended in 10 cc. of *N* hydrochloric acid, warmed on the steam-bath, decomposed with hydrogen sulfide and centrifuged. The solution containing the tryptophane was then decanted into a beaker and the precipitated mercuric sulfide washed twice with 0.1 *N* hydrochloric acid, which after centrifuging was added to the main tryptophane solution. The solution was heated over the steam-bath until freed from hydrogen sulfide and treated with phosphotungstic acid solution to remove polypeptides, avoiding an excess. The solution was placed in the ice box overnight and the slight precipitate of polypeptides filtered off. The excess phosphotungstic acid was removed by barium hydroxide and the excess barium removed by sulfuric acid, avoiding an excess of the latter. The solution was filtered into a stoppered Erlenmeyer flask and cooled to 0°.

For the bromination of the tryptophane, 0.5 cc. of concd. hydrochloric acid, 5 cc. of 10% potassium bromide and 10 cc. of 0.01 *N* potassium bromate were added to the solution and the flask placed at 0° overnight (eighteen hours) for the reaction to complete itself. The excess bromine in the reaction flask was estimated by adding 5 cc. of 10% potassium iodide and titrating the liberated iodine with 0.01 *N* sodium thiosulfate solution, adding 1 cc. of starch solution as indicator.

The potassium bromate and sodium thiosulfate solutions are, of course, standardized against each other and the potassium bromate in turn against purified tryptophane in known amounts. Conducting the bromination under the conditions we have used, 1 cc. of 0.01 *N* potassium bromate will brominate 0.2296 mg. of tryptophane. This is practically identical with the value 0.2304 mg. found by Homer[25] for the bromination at 17° after six to eight hours, each tryptophane molecule requiring 8.86 moles of nascent bromine.

Recovery of Tryptophane and Tyrosine from Casein Hydrolysates and the Tryptophane and Tyrosine Contents of Casein.—In Tables IX and X are recorded our results on the tryptophane and tyrosine content of

casein and the percentage recovered of known amounts of each amino acid added before hydrolysis of the protein. The data show that 81.3% of the added tryptophane and 99.4% of the added tyrosine were reclaimed quantitatively under the conditions of hydrolysis. The correct contents of tryptophane and tyrosine in the simplest casein are therefore 1.237 and 5.55%, respectively.

TABLE IX

TRYPTOPHANE CONTENT OF CASEIN

Expt.	Casein, g.	Added tryptophane, mg.	Added tyrosine, mg.	N/100 $KBrO_3$ soln. reqd., cc.[a]	Tryptophane found, mg.	Tryptophane recov., %	Tryptophane in casein %
1	0.948	0.00	0.00	41.60	9.55		1.235
2	0.948	8.70	0.00	72.55	16.60	81.3	
3	0.948	0.00	60.00	41.50	9.52		1.239
Mean							1.237

[a] Standardization of $N/100$ potassium bromate solution against weighed amounts of pure tryptophane gave 1 cc. of $N/100$ $KBrO_3$ = 0.2295 mg. of tryptophane.

TABLE X

TYROSINE CONTENT OF CASEIN

Expt.	Casein, mg.	Added tryptophane, mg.	Added tyrosine, mg.	Mean heights in mm. of column compared Stand.[a]	Unknown	Tyrosine found, mg.	Tyrosine recov., %	Tyrosine in casein, %
1	94.8	0.00	0.00	30.00	34.23	5.205		5.52
2	94.8	0.87	0.00	30.00	34.56	5.255		5.58
3	94.8	0.00	6.00	25.00	26.84	11.160	99.4	
Mean								5.55

[a] Standard for Expts. 1 and 2 contained 6.00 mg. of tyrosine per 100 cc. Standard for Expt. 3 contained 12.00 mg. of tyrosine per 100 cc.

Homer[25] has shown that the bromination of indoleacetic and indolepropionic acid takes very nearly the same amount of bromine as required by tryptophane and hence the presence of these substances would not interfere materially in the above determination, as each of them no doubt originally occurred in the protein as tryptophane. Indole and skatole, however, require only about two-thirds as much bromine as tryptophane in the bromination reaction. Homer believes that as the solubility of both of these substances is very low, very little error will be introduced into the final titration by their presence. At any rate the tendency would be to give a calculated tryptophane content slightly too low and a calculated molecular weight of the protein too high on this account.

Our data show that the presence of added tryptophane and tyrosine has no influence on the recovery of each other from the hydrolysate.

Histidine.—For the determination of the basic amino acids in proteins, Vickery and Leavenworth[26] have worked out a reliable method. The

[26] Vickery and Leavenworth, *J. Biol. Chem.*, **76**, 707 (1928).

method is, however, only applicable to large quantities of protein. As we have not had sufficient material for carrying out their method, we have used the method described by Hanke[27] and Koessler and Hanke[28] in our work, as the latter is a colorimetric method in which only small amounts of protein are required. The method in brief after separation of histidine and arginine from the hydrolysate consists in coupling histidine with *p*-diazobenzenesulfonic acid in the presence of sodium carbonate to form a reddish-orange color. The principal drawback to the determination is the fleeting character of the color developed, which usually makes more than one reading on a given trial impossible. Hanke states that no other substances likely to be present except histamine and tyramine affect the color to be compared, and that these would not be expected to be present except in putrescent decomposition. The reader is referred to Hanke's paper[27] for details of the method. We have used Hanke's method throughout with no changes, except that we have hydrolyzed the casein with sodium hydroxide in the same way as in the foregoing tryptophane determinations. In fact we have used the identical hydrolysate solutions which were prepared for the tryptophane and tyrosine determinations.

Through lack of a larger supply of the pure casein species under examination, we have been unable as yet to carry out experiments to ascertain the recovery of known added amounts of histidine from casein digests under the conditions of the alkaline hydrolysis used, and we will report the histidine content as we have found it, without reference to any possible corrections for histidine destroyed during the hydrolysis.

Histidine Content of Casein.—In Table XI are recorded data of three separate casein hydrolyses, in which the histidine has been determined as

TABLE XI

HISTIDINE CONTENT OF CASEIN

Expt.	1	2	3
Casein hydrolyzed, g.	0.948	0.948	0.948
Tryptophane added, mg.	None	8.70	None
Tyrosine added, mg.	None	None	60.00
Casein in color soln. compd., mg.	1.896	1.896	1.896
	18.10	18.20	18.20
Standard color (a) depth (mm.) matched against 20 mm.	18.15	18.20	18.30
depth of unknown.	18.35	18.40	18.30
	18.40	18.10	....
Mean.	18.25	18.225	18.26
Histidine found, mg.	0.0337	0.0336	0.0337
Histidine, %.	1.777	1.774	1.778

[a] 21.67 mm. standard color = 0.04 mg. of histidine.

[27] Hanke, *J. Biol. Chem.*, 66, 475 (1925).

[28] Koessler and Hanke, *ibid.*, 66, 505 (1925).

given above. Assuming that no histidine has been lost during the hydrolysis, the histidine content appears to be 1.776%. As the same amount of histidine was recovered from hydrolyzing casein to which known amounts of tryptophane and tyrosine had been purposely added, it appears that either these amino acids interfere to a like degree or else not at all, in the recovery and determination of histidine.

Part II. Estimation of the Molecular Weight.—It is obvious that provided we have a pure protein and have made no mistakes in the analyses for the various constituents, each constituent will occur in the molecular weight of the protein a whole number of times its own molecular (or atomic) weight. In the instance at hand we may also be guided by the centrifuging results of Svedberg, Carpenter and Carpenter,[2] who showed that the molecular species in question had a molecular weight between 75,000 and 100,000.

Calculated from the Sulfur and Phosphorus Content.—On the basis of 0.7855% sulfur in the protein we calculate that the minimal molecular weight possible for the protein is 4081.4 and from the value 0.8559% for phosphorus, that the corresponding value is 3626.5. The smallest number which will contain each of these numbers a whole number of times (least common multiple) is obviously

Sulfur.....4081.4 × 8 = 32,651.2
Phosphorus.....3626.5 × 9 = 32,598.5

with a mean value for the least common multiple (S_8P_9) of 32,624.8. The actual molecular weight of the protein is some whole multiple of this last number. The values possible are

1×	2×	3×	4×
32,625	65,250	97,875	130,500

Out of these values the only one which satisfies the centrifuging results is the number 97,875, which shows that the least common multiple number is multiplied by 3 and that S_{24} and P_{27} represent the respective proportions of these elements in the protein. Calculating the molecular weight directly from the content of each of these elements separately, we obtain

From sulfur determination (S_{24}), molecular weight = 97,954
From phosphorous determination (P_{27}), molecular weight = 97,796

Calculated from Cystine Content.—From the cystine value 0.488% we calculate that the minimum molecular weight of the protein is 49,234. The molecular weight is some whole multiple of this number

1×	2×	3×
49,234	98,468	147,702

from which 98,468 is selected as the *only* value possible to reconcile with the centrifuging results. This result agrees well with that calculated from the sulfur and phosphorus analyses.

Assuming that the cystine occurs as cysteine in the amount 0.44%, we calculate that the minimum molecular weight must be 27,536 and the possible molecular weight

1×	2×	3×	4×
27,536	55,072	82,608	110,144

The value 82,608 is the only possibility from this series and this is impossible to reconcile with the values obtained from the sulfur and phosphorus analyses. In other words the assumption that cysteine occurs in the protein instead of cystine does not appear to be in accord with the facts.

Calculated from Tryptophane Content.—From the tryptophane content of 1.23% we calculate the minimum molecular weight of the protein to be 16,498. The molecular weight is, of course, a whole number multiple of this minimum number

1×	2×	3×	4×	5×	6×
16,498	32,996	49,494	65,992	82,490	98,988

Of this series the values 82,490 and 98,988 both satisfy the centrifuging results, but only the latter, 98,988, is in harmony with the sulfur and phosphorus results. It is concluded, therefore, that the latter is the only possible choice.

Calculated from Tyrosine Content.—As there is so large a quantity of tyrosine present in casein, 5.55%, we cannot estimate the molecular weight of the protein with any certainty, for the minimal molecular weight is calculated to be 3266. Thirty times this value, however, gives a possible molecular weight of 97,980, which is in harmony with all the results we have obtained thus far with this protein.

Calculated from Histidine Content.—Assuming that the histidine figure, 1.776%, requires no correction, we calculate that the minimal molecular weight is 8953. The only possible multiples of this value that are in harmony with the centrifuging results are the multiples 9, 10 and 11, giving molecular weight values, respectively, of 80,577, 89,530 and 98,480. As the latter value 98,480 is in harmony with our results with other constituents, it is considered to be the best choice of the three values.

TABLE XII

SUMMARY OF RESULTS AND CALCULATION OF MOLECULAR WEIGHT

	Found, %	Minimum mol. wt.	Multiple	Probable mol. wt. of casein
Sulfur	0.7855	4,081	24	97,954
Phosphorus	0.8559	3,626	27	97,796
Cystine	0.488	49,234	2	98,468
Tryptophane	1.237	16,498	6	98,988
Tyrosine	5.55	3,266	30	97,980
Histidine	1.776	8,953	11	98,480
			Mean	98,279

Results and Conclusions

We have summarized our analytical results and calculations of the molecular weight of the simplest casein species in Table XII and conclude that the value 98,000 for the molecular weight agrees best with all our different analyses and also with our previous results with the ultracentrifuge.

Summary

1. The protein contained in crude casein, which has been shown to have a molecular weight between 75,000 and 100,000 by the ultracentrifuge, has been subjected to analysis.

2. The sulfur, phosphorus, cystine, tryptophane, tyrosine and histidine contents have been found to be 0.785, 0.856, 0.488, 1.237, 5.55 and 1.776%, respectively.

3. The most probable value for the molecular weight of this protein, consistent between the above analyses and the results obtained with the ultracentrifuge, was shown to be 98,000.

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[CONTRIBUTION FROM THE DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF CALIFORNIA]

THE ABSORPTION SPECTRA OF ORTHO-CHLOROPHENOL INDOPHENOL AND OF ORTHO-CRESOL INDOPHENOL

BY MATILDA MOLDENHAUER BROOKS

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Spectrophotometric determinations of the absorption spectra of *o*-chlorophenol indophenol and of *o*-cresol indophenol[1] between wave lengths of 480 and 680 mμ are presented in this paper. Two samples of each dye were used: a very pure sample[2] obtained from Dr. W. Mansfield Clark, to whom the writer is indebted for its presentation, and a commercial sample from the LaMotte Chemical Products Co.

The dyes were dissolved in various buffer solutions in concentrations of 0.00005, 0.0001 and 0.0002 *M*. These concentration values were based upon the assumption that the samples used consisted of the designated dyestuffs exclusively. Since in the case of one sample, this was apparently not true, the actual concentrations in this case were probably lower than

[1] Cohen, Gibbs and Clark, *Public Health Rpts.*, **39**, 381 (1924).

[2] Dr. Clark writes: "Each of the dyes was sufficiently free from components of an oxidation–reduction system other than the dye system to permit fairly accurate characterizations of the oxidation–reduction potential of the true dye system, but no claim is made that the material consists of dye alone. Indeed, it was found preferable to salt-out the sodium salts of the indophenols rather than to attempt methods of purification which were found to produce appreciable decomposition of the dye with the introduction of other oxidation–reduction systems. In consequence, many of the preparations in this group of dyes contain large quantities of sodium chloride."