weight dioxane-soluble polyamine as well as a dioxane-insoluble product. The dioxane-soluble polyamines have been characterized by the formation of phenylurea, phenylthiourea and *p*-bromophenylurea derivatives.

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[FROM THE DEPARTMENTS OF BIOCHEMISTRY AND ZOÖLOGY, COLUMBIA UNIVERSITY]

The Empirical Formula of β-Lactoglobulin[1]

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From the point of view of organic chemistry, the establishment of the empirical formula of proteins (*cf.* Mulder[2] in 1838), in terms of constituent amino acids, is fundamental to an understanding of their constitution and to the development of theories of protein structure.

An apparently complete analysis of a simple, crystalline, homogeneous protein of small molecular weight (42,000), *viz.*, β-lactoglobulin[3] is presented in this paper. This protein, which constitutes the greater part of the whey proteins, was isolated in crystalline form by Palmer[3,4] from the plasma of cow's milk. It is free from phosphorus and carbohydrate. Physico-chemical studies[5] indicate that β-lactoglobulin is a protein native to milk and that it is homogeneous as tested by the solubility method,[3] in the ultracentrifuge,[5] by electrophoresis[5] and with respect to dissociation.[6]

The results are given in Table I; the analytical values for the constituents are given in % in column 4, in % amino acid residue in column 5 and in atoms or moles per 10^5 g. of protein in column 7. Columns 6 and 8–14 in Table I are based upon the minimum molecular weight, the calculation of which is shown in Table III. The data will first be discussed without any reference to the molecular weight and subsequently on a molar basis.

The following assumptions have been made:

(1) On the basis of physico-chemical criteria quoted above, it is assumed that β-lactoglobulin is a chemical compound in which the constituent amino acids are present in integral molar quantities.

(2) It is assumed that the amino acids (RNH_2-COOH) are present in the molecule in peptide linkage. The yield is, therefore, given in per cent. of amino acid in column 4 and in per cent. of amino acid residue [R(NH—) (CO—)] in columns 5 and 6.

(3) It is assumed that the amide nitrogen (detn. 8) or amide NH_3 (detn. 8a) as obtained by our method indicates the amount of glutamine and asparagine present. Since it is not yet possible to differentiate between these two amides, all of the amide NH_3 has been arbitrarily assigned to glutamine in detn. 8b, and will subsequently be discussed as glutamine only. Consequently, all of the aspartic acid is considered as "free aspartic acid," while the "free glutamic acid" (detn. 30) is the difference between total glutamic acid and glutamine.

(4) In the interpretation of the data on the free amino nitrogen of the intact protein obtained in the manometric Van Slyke apparatus under our conditions[7] it is assumed (a) that no splitting with the liberation of reactive amino N occurs during the determination; (b) that all of the ε-amino N of lysine is free and reactive; (c) that all of the terminal free α-amino N of the peptide chain, or chains (detn. 12), is free and reactive and, therefore, can be obtained by deducting the ε-amino nitrogen (calculated from the lysine value (detn. 27a)) from the total free amino N (detn. 11); (d) that no proline or hydroxyproline is at the end of a peptide chain; (e) that one free terminal COOH group corresponds on the other end of the chain to each terminal α-amino group. In the accounting of the split products in per cent. of amino acid residue (detn. 12a) in columns 5 and 6, a value for "Terminal H_2O" corresponding to the terminal α-amino N has, therefore, been added (*i. e.*, one H atom in terminal α NH_2 and one OH group in terminal carboxyl).

(5) It is assumed that our determinations refer to the unhydrated molecule, since they were carried out on air dried material, corrected for moisture (*cf.* refs. 8, 9). We have previously contributed some evidence in favor of this contention in experiments in which H_2O containing the heavy oxygen isotope O^{18} was used as an indicator (*cf.* ref. 9, p. 374).

Two preparations of β-lactoglobulin were used for the experiments, for one we are indebted to Dr. R. K. Cannan, for the other to Dr. G. Haugaard. About 2.5 g. of protein was used for the determinations reported in Table I.

(1) Part of this work was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University. Much of the present data was obtained in the course of the development and standardization of methods for application to plasma proteins.

(2) Mulder, *Ann.*, **28**, 73 (1838).

(3) Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(4) *Cf.* Sörensen and Sörensen, *Compt. rend. trav. lab. Carlsberg*, Series C, **23**, 55 (1938).

(5) Pedersen, *Biochem. J.*, **30**, 948, 961 (1936).

(6) Sörensen and Palmer, *Compt. rend. trav. lab. Carlsberg*, Series C, **21**, 283 (1938).

(7) Brand and Diskant, unpublished work.

(8) Brand and Kassell, *J. Gen. Physiol.*, **25**, 167 (1941).

(9) Brand and Kassell, *J. Biol. Chem.*, **145**, 365 (1942).

TABLE I

COMPOSITION OF β-LACTOGLOBULIN (MOL. WT. = 42,020)

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
			Yield in %	of residue[c]			Per mole protein (42,020)						
							Atoms[c]					Moles (atoms)	
Detn.[a]	Constituent	Method of determination references[b]	Found, %	Found, %	Calcd. from integers in column, 14[d] %	Yield as moles, (atoms) per 10^5 g. protein	C	H	N	S	O	Found	Cor. to nearest integer[e]
1	Carbon	P	53.39			4445.5	1868					1868.0	1868
2	Hydrogen	P	7.22			7162.0		3010				3009.5	3010
3	Total Nitrogen	D	15.60			1113.6			468			468.0	468
4	Total Sulfur	P	1.604			50.0				21		21.0	21
5	Oxygen	Diff.	22.186			1386.6					583	582.7	583
21a + 22a	Cysteine S + Cystine S		0.905			28.3						11.9	12
23a	Methionine S		0.692			21.6						9.1	9
6 = 21a + 22a + 23a	Protein S		1.597			49.8						21.0	21
7	Sulfate S		0							0			
8	Amide N	MD	1.076			76.8			32			32.3	32
24a	Indole N		0.133			9.5			4			4.0	4
25a	Guanidino N		0.695			49.6			21			20.8	21
26a	Imidazole N		0.285			20.4			8			8.6	8
27a	ε-Amino N		1.092			78.0			33			32.8	33
9 = 8 + 24a + 25a + 26a + 27a	Non α-N		3.28			234.2			98			98.4	98
10 = 3 − 9	α-N		12.319			879.4			370			369.5	370
11	Free Amino N	VS	1.247			89.0			37			37.4	37
12 = 11 − 27a	Free α-Amino N	VS	0.155			11.1			4			4.6	4
13 = 10 − 12	Peptide N		12.164			868.3			366			365.0	366
14	Glycine	LM	1.4	1.06	1.09	18.7	16	24	8		8	7.8	8
15	Alanine	SF	6.2	4.95	4.91	69.6	87	145	29		29	29.1	29
16	Valine	LA	5.83	4.93	4.95	49.8	105	189	21		21	20.9	21
17	Leucine	NL-ID	15.6	13.46	13.46	118.9	300	550	50		50	50.0	50
18	Isoleucine	LA	8.4	7.25	7.27	64.0	162	297	27		27	26.9	27
19	Proline	NP	4.1	3.46	3.47	35.6	75	105	15		15	15.0	15
20	Phenylalanine	LA	3.54	3.15	3.15	21.4	81	81	9		9	9.0	9
21	Cysteine	Phot. C	1.11	0.94	0.98	9.2	12	20	4	4	4	3.9	4
22	Half-cystine	Phot. C	2.29	1.95	1.94	19.1	24	32	8	8	8	8.0	8
23	Methionine	Iod.	3.22	2.83	2.81	21.6	45	81	9	9	9	9.1	9
24	Tryptophan	UV	1.94	1.77	1.77	9.5	44	40	8		4	4.0	4
25	Arginine	Phot. A-I	2.88	2.58	2.60	16.5	42	84	28		7	7.0	7
26	Histidine	Phot. H	1.58	1.40	1.31	10.2	24	28	12		4	4.3	4
27	Lysine*	B.C; ID; LM	11.4	10.0	10.07	78.0	198	396	66		33	32.8	33
28	Aspartic Acid	LM-ID	11.4	9.86	9.86	85.6	144	180	36		108	36.0	36
29	Total Glutamic Acid	LA	19.5										
8a	Amide NH_3	MD	1.31										
8b	Glutamine[f]	MD		9.84	9.76	76.8	160	256	64		64	32.3	32
30 = 29−8b	Free Glutamic Acid			7.19	7.37	55.7	120	168	24		72	23.4	24
31	Serine[g]	Ox.	5.0	4.14	4.14	47.6	60	100	20		40	20.0	20
32	Threonine	Ox.	5.85	4.97	5.05	49.1	84	147	21		42	20.6	21
33	Tyrosine	Phot. T	3.78	3.40	3.49	20.9	81	81	9		18	8.8	9
34	Hydroxyproline	Col.	0										
35	Hydroxylysine[h]		0										
12a	Terminal H_2O[i]	VS		0.20	0.17			8			4		
36	Total		116.33	99.33	99.62	877.8	1864	3012	468	21	576	368.9	370
37	Total, cor. to nearest integ.		116.73										
38 = 36, col. 4 − 36, col. 5	Water taken up during hydrol.		17.00			943.6						396.5	397
39 = 37 − 36, col. 6	Same, cor. to nearest integ.		17.11			949.7						399.2	399
40 = 39 + 100%	Total yield, calcd.		117.11										

TABLE I

TERMS AND FOOTNOTES

[a] Values derived by calculation are designated by a or b (*e. g.*, tryptophan is no. 24, indole N no. 24a). [b] The abbreviations have the following significance, with references to the methods in brackets:

B.C. = Lysine decarboxylase of *B. cadaveris*[11,12,13,14]
Col. = Colorimetric[47]
D = Micro Dumas (*cf.* 9)
Diff. = Difference
I = Isolation[36]
ID = Isotope dilution[10]
Iod. = Iodometric[27]
LA = Bioassay with *Lactobacillus arabinosus 17-5*[15]
LM = Bioassay with *Leuconostoc mesenteroides P-60*[16]
MD = Microdiffusion in Conway vessels[41]
NL = Bioassay with "leucineless" mutant of *Neurospora*[15]
NP = Bioassay with "prolineless" mutant of *Neurospora*[25]
Ox. = Oxidation with periodic acid[30,31,32,33,34,35]

P = Pregl[45,46]
Phot. A = Photometric determination of arginine[37]
Phot. C = Photometric determination of cysteine and cystine[26]
Phot. H = Photometric determination of histidine[39]
Phot. T = Photometric determination of tyrosine[29]
SF = Bioassay with *Streptococcus faecalis R*[16]
UV = Ultraviolet absorption of tryptophan mercury complex[38]
VS = Manometric Van Slyke apparatus[7]

[c] Percentages in columns 5 and 6 and atoms in columns 8 to 12 are calculated on the basis of amino acid residues (R·NH·CO) for determinations nos. 14–33. [d] On the basis of a molecular weight of 42,020. [e] Except in determinations obtained by difference (nos. 13 and 30). [f] All of the amide N is arbitrarily assigned to glutamine. [g] Corrected 10% for serine destruction during hydrolysis. [h] *Cf.* ref. 36. [i] "Terminal H_2O" corresponding to the free (terminal) α-amino N (*cf.* text, assumption (4), p. 1524).

* The microbiological determination of lysine was worked out since the paper went to press (*cf.* addendum).

Methods of Analysis

All the values in Table I are our own results. Some of our methods have been described in detail, others reported in outline and some are as yet unpublished. We are greatly indebted to Professor G. L. Foster for making his results[10] for some constituents obtained by the isotope dilution method available to us in advance of publication. One of the preparations of β-lactoglobulin used by Foster was the same as one of ours (Haugaard).

In some cases (detns. 8, 11, 14, 16, 17, 25, 26, 27, 28, 29) the results are identical or in close agreement with those of other authors, who analyzed different preparations by fundamentally different methods. In some instances, average values are given (detns. 17, 27, 28).

The value for lysine was obtained with the specific decarboxylase of *B. cadaveris*,[11] (*cf.* refs. 12, 13, 14) and is identical with the lysine content determined by isotope dilution by Foster.[10] Through the coöperation of Dr. George M. Guest of the Committee on Medical Research of the Office of Scientific Research and Development we obtained a culture of *B. cadaveris* and acetone-dried bacteria from Dr. E. F. Gale in Oxford. We are also indebted to Dr. L. D. Wright of the Research Laboratories of Sharp and Dohme for preparing for us repeatedly acetone-dried cells of *B. cadaveris* (*cf.* addendum and Table I, footnote *).

The leucine value is the average of Foster's results (15.7%) by the isotope dilution method[10] and of those of Ryan and Brand (15.4%) obtained by bioassay with the aid of the "leucineless" mutant of *Neurospora*.[15] The values for valine, isoleucine, phenylalanine and glutamic acid are the results of bioassays with *Lactobacillus arabinosus 17-5*, that for glycine and aspartic acid with *Leuconostoc mesenteroides P-60*, and that for alanine with *Streptococcus faecalis R*. The details of these methods will be published elsewhere.[16] The development of these procedures was made possible through the recent pioneer work of Hutchings and Peterson,[17] Dunn[18,19] and Shankmann,[20] Kuiken, *et al.*,[21] Lyman, *et al.*,[22] McMahan and Snell,[23] Lewis and Olcott,[24] and others. We have used both lactic acid titrations and turbidity measurements (with the Beckmann Spectrophotometer), but have come to prefer the latter. We are indebted to Dr. J. J. Pfiffner of Parke, Davis and Co. for Vitamin B_c, to Dr. R. T. Major of Merck & Co. for pyridoxamine, to Dr. W. Baumgarten of Hiram Walker and Sons for a culture of *S. faecalis R* and to Dr. E. E. Snell for one of *Leuconostoc mesenteroides P-60*. Our result for valine (Table I, detn. 16) is in good agreement with that of McMahan and Snell (5.8%[23]). The value for glycine (1.4%, detn. 14) is in good agreement with Foster's value (1.5%) by the isotope dilution method.[10] For aspartic acid (detn. 28) we obtained 11.4%, practically identical with the isotope dilution value[10] of 11.3%. Our value for total glutamic acid (Table I, detn. 29) is about 2% higher than Foster's result obtained by the isotope dilution method (19.1%[10]).

The value for proline was obtained by a microbiological method, developed by Ryan and Brand,[25] using a "prolineless" strain of *Neurospora*, for which we are indebted to Dr. George W. Beadle of Stanford University.

The values in Table I for cysteine,[26] half-cystine,[26] methionine,[27] sulfate sulfur,[8,27,28] tyrosine,[29] threonine,[30,31,32,33] and serine[30,34,35] have been reported by Brand and Kassell[9]; references to our methods are given in the footnotes. The serine value is corrected for 10% destruction during acid hydrolysis (*cf.* ref. 34).[30,35]

The value for arginine in Table I is the average of Chibnall's results (2.89%) by his isolation procedure[36] and of the photometric value (2.87%) of Brand and Kassell.[37]

(10) Foster, *J. Biol. Chem.*, **159**, 431 (1945).
(11) Brand and Goldwater, unpublished work.
(12) Gale, *Biochem. J.*, **38**, 232 (1944).
(13) Neuberger and Sanger, *ibid.*, **38**, 119 (1944).
(14) Zittle, *J. Biol. Chem.*, **156**, 401 (1944).
(15) Ryan and Brand, *ibid.*, **154**, 161 (1944).
(16) Brand, Ryan, Saidel and Goldwater, unpublished work.
(17) Hutchings and Peterson, *Proc. Soc. Exp. Biol. Med.*, **52**, 36 (1943).
(18) Dunn, Camien, Rockland, Shankman and Goldberg, *J. Biol. Chem.*, **155**, 591 (1944).
(19) Dunn, Shankman, Camien, Frankl and Rockland, *ibid.*, **156**, 703 (1944).
(20) Shankman, *ibid.*, **150**, 305 (1943).
(21) Kuiken, Norman, Lyman, Hale and Blotter, *ibid.*, **151**, 615 (1943).
(22) Lyman, Kuiken, Blotter and Hale, *ibid.*, **157**, 395 (1945).
(23) McMahan and Snell, *ibid.*, **152**, 83 (1944).
(24) Lewis and Olcott, *ibid.*, **157**, 265 (1945).
(25) Ryan and Brand, unpublished work.
(26) Kassell and Brand, *J. Biol. Chem.*, **125**, 115, 131 (1938).
(27) Kassell and Brand, *ibid.*, **125**, 145 (1938).
(28) Kassell and Brand, *ibid.*, **125**, 435 (1938).
(29) Brand and Kassell, *ibid.*, **131**, 489 (1939).
(30) Brand and Kassell, unpublished work.
(31) Nicolet and Shinn, *J. Biol. Chem.*, **139**, 687 (1941); **142**, 139 (1942).
(32) Shinn and Nicolet, *ibid.*, **138**, 91 (1941).
(33) Winnick, *ibid.*, **142**, 461 (1942).
(34) Boyd and Logan, *ibid.*, **146**, 279 (1942).
(35) Brand, Kassell and Saidel, unpublished work.
(36) Chibnall, *Proc. Royal Soc.* (London), **B131**, 136 (1942).
(37) Brand and Kassell, *J. Biol. Chem.*, **145**, 359 (1942).

The figure for tryptophan was obtained by a method involving its isolation as the insoluble mercury complex and quantitative determination by ultraviolet absorption in the Beckmann Spectrophotometer.[38] The value is identical with the photometric figure,[29] previously reported by Brand and Kassell.[9]

Histidine was determined by a new micromethod, which has been reported in outline.[39] Chibnall's[36] value, obtained by an isolation procedure is slightly lower (1.54%).

The determination of amide NH_3 was carried out in Conway vessels,[40] following up a suggestion of Warner and Cannan.[41] The result in Table I is in good agreement with the value reported by these authors and by Chibnall.[36]

The free amino nitrogen[42] was determined in the manometric Van Slyke apparatus at 23.0°. The temperature was accurately maintained by letting water of the desired temperature flow at a rapid rate through the chamber of the apparatus.[7] The details of the technique will be published elsewhere. The results are slightly lower than Cannan's.[44]

The figures for the elementary composition[45] were obtained by Pregl methods (for the sulfur determination *cf.* refs. 8, 9, 28, 46). The total nitrogen (micro Dumas) content has been discussed previously (*cf.* ref. 9, footnote 6). The oxygen content was obtained by difference.

Hydroxyproline was not detected, when the colorimetric test of McFarlane and Guest[47] was applied. According to Chibnall[36] hydroxylysine is not present in β-lactoglobulin.

The amount of humin formed during acid hydrolysis is negligible (the protein contains no carbohydrate).

(38) Brand and Saidel, Am. Chem. Soc., Pittsburgh meeting, Sept., 1943, Abstracts, p. B-22.

(39) Saidel and Brand, Am. Chem. Soc., New York meeting, Sept., 1944, Abstracts, p. B-26.

(40) Saidel and Brand, unpublished work.

(41) Warner and Cannan, *J. Biol. Chem.*, **142**, 725 (1942).

(42) The accessibility of the lysine side chains to deamination by nitrous acid is not the same in different proteins, since we have recently found that the rate of liberation of nitrogen from the ε-amino groups varies markedly from protein to protein. It is, therefore, not possible to differentiate in the manometric Van Slyke apparatus between nitrogen contributed by free α-amino and ε-amino groups of proteins. Computations for these two types of nitrogen on the basis of the reactivity of the ε-amino nitrogen of free lysine, such as presented by Chibnall (ref. 36, Tables 8 and 9), do not seem to be permissible.

It appears that we have grossly underestimated the amount of ε-amino N as compared to free α-amino N in the plasma proteins. Preliminary results with *B. cadaveris* indicate a lysine content of about 12% for human and bovine serum albumin instead of about 6.0% as previously reported.[43] A similar high content of lysine in bovine serum albumin (12.4%) has recently been found by Dr. David Shemin (personal communication) by the isotope dilution method. In the plasma proteins the amount of free α-amino N seems to be quite small, corresponding to only a few sub-units per mole (for lysine determinations with *L. mesenteroides cf.* addendum).

(43) Brand, Kassell and Saidel, *J. Clin. Invest.*, **23**, 437 (1944).

(44) Cannan, Palmer and Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(45) Saschek and Brand, unpublished work.

(46) Saschek, *Ind. Eng. Chem., Anal. Ed.*, **9**, 491 (1937).

(47) W. D. McFarlane and G. H. Guest, *Canad. J. Res.*, **17**, 139 (1939).

Sulfur-partition.—Methionine together with cysteine and cystine account for the total sulfur determined by elementary analysis, sulfate being absent. This concordance constitutes a check on the accuracy of the values for the sulfur amino acids (*cf.* discussion, refs. 8, 9.)

Nitrogen-partition.—All amino acids contain one nitrogen atom in the α position, which we designate the α-N of proteins; this term includes proline and hydroxyproline. Additional N atoms are present in glutamine (amide N), tryptophan (indole N), arginine (guanidino N), histidine (imidazole N) and lysine (ε-amino N). If these amino acids have been determined, their non-α N (detn. 9) can be deducted from the total nitrogen to yield the α-N (detn. 10). This value, when expressed as atoms of nitrogen per 10^5 g. of protein (879.4, detn. 10, column 7) should be identical with the sum of the number of moles per 10^5 g. of all of the constituent amino acids (877.8, detn. 36, col. 7), as it has been found to be. In other words, the nitrogen partition derived from the determination of only six constituents agrees closely with the total amino acid composition based on the data for twenty-four constituents. This agreement is further emphasized by a consideration of the amount of water taken up on hydrolysis—17.00 g. per 100 g. of protein (detn. 38). This value, when expressed as moles of H_2O per 10^5 g. of protein (943.6, detn. 38, col. 7) should be identical with the sum of the atoms per 10^5 g. of the peptide N and of the amide N, if during hydrolysis only peptide and amide linkages are split. The calculation 868.3 + 76.8 = 945.1 atoms (col. 7, detns. 13 and 8) again shows this to be the case.

The reciprocal of the number of atoms of α-N (detn. 10, col. 7) and of the sum of the moles of the amino acids per 10^5 g. (detn. 36, col. 7) is the average residue weight (ARW), which is thus found as 113.7 and 113.9, respectively.

The average peptide weight (APW) we find as 114.8 (reciprocal of detn. 13, col. 7) corresponding to 366 peptide bonds per mole. Hotchkiss[48] determined the APW by titration of the increase of amino and carboxyl groups when β-lactoglobulin is completely hydrolyzed by hydrochloric acid. If his data are recalculated on the basis of a total N of 15.60% (*cf.* ref. 9, footnote 6) and of a molecular weight of 42,020, a value of 113.0 for APW corresponding to 372 peptide bonds per mole is obtained, within 2% of our value.

Elementary Composition of β-Lactoglobulin.—In order to compare the elementary analysis with the yield of amino acid residues in Table I, col. 5, the percentages of C, H, N, S and O corresponding to each constituent (detns. 8b, 12a, 14–35) were calculated. The individual percentages thus obtained are omitted from Table I, but their sum is given in Table II, together with the elementary analysis. The results of the amino

(48) Hotchkiss, *J. Biol. Chem.*, **131**, 387 (1939).

acid analysis are somewhat low for oxygen and carbon, the over-all recovery in terms of elements being 99.27%. If the yield of amino acid residues is corrected to the nearest integers on the basis of a molecular weight of 42,020 (*cf.* Table I, col. 6) the over-all yield in terms of elements is increased to 99.61% (*cf.* discussion below).

TABLE II

ELEMENTARY COMPOSITION OF β-LACTOGLOBULIN

Method	% of C	H	N	S	O	Total
1. Elementary Analysis						
Cf. Table I, Column (4), detns. 1–5, oxygen obtained by diff.	53.39	7.22	15.60	1.604	22.186	100
2. Amino Acid Analysis						
From the C, H, N, S, O content of the individual amino acid residues. *Cf.* Table I, detns. 8b, 12a, 14–35						
A, as found in Table I, column 5	53.09	7.17	15.53	1.597	21.86	99.37
B, as cor. to the nearest integers in Table I, col. 6, on the basis of $M_{min.}$ = 42,020	53.27	7.23	15.60	1.60	21.93	99.61

Minimum Molecular Weight ($M_{min.}$).—The minimum molecular weight of a protein is defined by the equation

$$M_{min.} = \frac{R_i \times M_i \times 100}{(\%)_i} \qquad (1)$$

where M_i, $(\%)_i$ and R_i are the molecular weight, the content in % and the number of residues of an individual constituent (i). R_i is always an integer. If C_i designates the concentration of the individual constituent in moles per g., equation (1) can also be written as

$$M_{min.} = R_i/C_i \qquad (2)$$

If a number of amino acids (1, 2, 3, . . ., n) have been determined, minimum, integral values for R_1, R_2, R_3, . . ., R_n are obtained by setting up simultaneous equations for $M_{min.}$, yielding a series of equations such as (3)

$$R_2 = R_1 \times C_2/C_1;\ R_3 = R_1 \times C_3/C_1;\ \ldots;\ R_n = R_1 \times C_n/C_1 \qquad (3)$$

In these equations C_1; C_2; C_3; . . . , C_n are known and for R_1 a value of 1, 2, 3, 4, etc., is taken successively until integers are found for R_2, R_3, . . ., R_n (or at least for most of them). In the elementary analysis of organic compounds the atomic concentrations (C) are usually divided by the smallest individual concentration. With proteins it is advisable to use as basis a constituent present in small concentration, which has been determined with great accuracy, and to make the adjustment to integers within 3% of a whole number. This tolerance, imposed by the fact that few amino acids can be determined at the present time with an accuracy greater than 3%, is satisfactory on account of the larger size of the amino acid molecules as compared to the atomic weights (C, H, N, S, O). With an accuracy of about 3% for the determination of some of the constituent amino acids (present in molar concentrations of 9–35 per 10^5 g. of protein), satisfactory minimum molecular weights can be obtained in this way for proteins with molecular weights up to about 50,000 (*cf.* refs. 8, 9). For larger minimum molecular weights a greater accuracy is required. An accuracy of at least 1% for the determination of some constituents would be necessary to obtain significant values for minimum molecular weights of about 175,000. The calculation of minimum molecular weights of proteins from analytical data has been extensively made by Cohn.[49,50,51]

In Table III, the calculation of the minimum molecular weight is presented. It can be seen that the two constituents present in smallest concentrations are cysteine and tryptophan (9.2 and 9.5 moles per 10^5 g., respectively). The molecular ratio of these two constituents (within 3%) is 1:1. We have chosen tryptophan as the basis for calculation because we believe that our tryptophan determination by ultraviolet absorption of the tryptophan mercury complex[38] is particularly accurate. On the other hand, the value for cysteine—although fairly accurate as indicated from the sulfur partition—is apt to be on the low side owing to some unavoidable oxidation; moreover, there is still some controversy about the status of the cysteine residues in the native protein molecule.

Serine has been omitted from the calculation because the value reported in Table I (detn. 31) includes a somewhat arbitrary correction of 10% for serine destruction during hydrolysis[30,35] (*cf.* ref. 34). In columns 3 and 4 of Table III, the yield in % and in moles per 10^5 g. are given. In column 5, the molar ratios on the basis of one tryptophan molecule are obtained by dividing by the number of moles of tryptophan (9.5). In columns 6, 7 and 8, these molar ratios multiplied by 2, 3 and 4 are shown. All figures in columns 5–8 are rounded out to the nearest integer within 3%. It can be seen that integral ratios on the basis of 1 or 2 tryptophans are obtained with all amino acids that are present in larger concentrations than 50 moles per 10^5 g. These constituents, therefore, are not very useful for the establishment of minimum molecular weights. Only 8, 11 and 15 constituents yield integral molar ratios for 1, 2 and 3 tryptophan molecules, respectively, but for 4 tryptophans there are 19 integral ratios out of a total of 20. The only amino acid that does not yield an integral molar ratio, even for 4 tryptophan molecules is histidine.

(49) Cohn, Hendry and Prentiss, *J. Biol. Chem.*, **63**, 721 (1925).
(50) Cohn, *Chem. Rev.*, **24**, 203 (1939).
(51) Cohn, *Bull. N. Y. Ac. of Med.*, sec. ser., **15**, 639 (1939).

TABLE III

CALCULATION OF MINIMUM MOLECULAR WEIGHT ($M_{min.}$) OF β-LACTOGLOBULIN

(1) Table I, detn.	(2) Constituent	(3) Yield, %	(4) Moles per 10^5 g., protein	(5)	(6)	(7)	(8)	(9) $M_{min.}$
				Molar ratios, adjusted to integers within 3% Tryptophan =				
24	Tryptophan	1.94	9.5	1	2	3	4	42,110
21	Cysteine	1.11	9.2	1	2	3	4	43,670[a]
25	Arginine	2.88	16.5	1.74	3.48	5.22	7	42,340
22	Half-Cystine	2.29	19.1	2	4	6	8	41,970
33	Tyrosine	3.78	20.9	2.20	4.40	6.60	9	43,140[a]
23	Methionine	3.22	21.6	2.27	4.54	7	9	41,700
4	Total S	1.604	50.0	5.26	10.52	16	21	41,970
					Average I (5 determinations)			42,020[b] ± 105
14	Glycine	1.4	18.7	2	4	6	8	42,900
20	Phenylalanine	3.54	21.4	2.25	4.50	6.75	9	42,000
19	Proline	4.1	35.6	3.75	7.50	11	15	42,100
32	Threonine	5.85	49.1	5.16	10.32	15.48	21	42,800
16	Valine	5.83	49.8	5.24	10.48	16	21	42,200
30	Glutamic acid	8.2	55.7	6	12	18	24	43,100
18	Isoleucine	8.4	64.0	6.74	13	20	27	42,200
15	Alanine	6.2	69.6	7.33	15	22	29	41,700
8b	Glutamine	11.2	76.8	8	16	24	32	41,700
27	Lysine	11.4	78.0	8	16	25	33	42,300
28	Aspartic acid	11.4	85.6	9	18	27	36	42,100
17	Leucine	15.6	118.9	12.5	25	38	50	42,100
					Average II (19 determinations)			42,300[b] ± 115
26	Histidine	1.58	10.2	1.07	2.14	3.21	4.28	
No. of Integers (out of 20)				8	11	15	19	

[a] Values marked are omitted from Average I since they deviate by more than three times the standard deviation. [b] Standard deviation for Average I = 230 and for Average II = 500.

It is present in small amount and may not have been determined accurately enough. Histidine is, therefore, omitted from the calculation of the minimum molecular weight (col. 9), which calculation was carried out using equation 1 and the minimum residue numbers in column 8.

Two average values for $M_{min.}$ are given in Table III, 42,020 ± 105 and 42,300 ± 115, the first based on the five most accurately determined constituents present in small concentrations, the second on all 19. These two values are not significantly different statistically (*cf.* Table III, footnote). We shall use the first one (42,020) throughout the paper.

Since Pedersen[5] found a molecular weight of β-lactoglobulin in the ultracentrifuge of 41,600, it can be concluded that the minimum molecular weight and the molecular weight are identical and that β-lactoglobulin is monomolecular in solution.

A comparison of values given in the literature for the molecular weight of β-lactoglobulin obtained by different methods is shown in Table IV.

Comments on Table I.—It can be seen (detn. 36) that from the hydrolysis of 100 g. of protein 116.33 g. of split products (col. 4) or 99.33 g. on a residue basis (col. 5) have been obtained. The difference between these two values or 17.00 g. is the amount of H_2O taken up during hydrolysis (detn. 38). On a molar basis this corresponds to 397 moles of H_2O (column 14), practically identical with the 398 moles of the H_2O expected to be taken up in the hydrolysis of 32 amide and 366 peptide linkages (col. 14, detns. 8 and 13). In column 6 the yield is calculated as residues of the various constituents from the molecular weight and from the integers in column 14. This correction raises the total accounting to 99.62% as

TABLE IV

MOLECULAR WEIGHT OF β-LACTOGLOBULIN*

(1) Minimum by analysis, $M_{min.}$		42,020 ± 105
Ultracentrifuge[a]	(2) Sed. vel. and diff., M_S	41,600
	(3) Sed. equilibrium, M_E	38,000
X-Ray, crystal form[b]	(4) Tabular, M_X	[40,000]
	(5) Needle, M_X	[40,100]
(6) Monomolecular film,[c] M_F		44,000

[a] Pedersen, ref. 5. [b] Quoted from Cohn and Edsall,[52] (p. 328); these values are given in brackets since they refer to "dry" crystals, not corrected for residual water. McMeekin and Warner[53] have assumed that the water content of the air dried crystals used in obtaining the X-ray data was the same as their own dried crystals. On this basis and using their own density measurements they have calculated from the X-ray data an anhydrous molecular weight of 35,800. [c] Bull, ref. 54. * Since this paper went to press we have noticed that Gutfreund[55] has obtained by osmotic pressure measurements a molecular weight, M_O = 38,000.

(52) Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Co., New York, N. Y., 1943.

(53) McMeekin and Warner, THIS JOURNAL, **64**, 2393 (1942).

(54) Bull, *ibid.*, **67**, 8 (1945).

(55) Gutfreund, *Nature*, **155**, 237 (1945).

residues or to 116.73% as split products (detn. 36, col. 6 and detn. 37, col. 4, respectively). We can, therefore, calculate that 100 g. of the protein should yield on hydrolysis 117.11 g. of split products (detn. 40). The elementary composition (in atoms per mole, detns. 1–5, cols. 8–12) is accounted for by the sum of the various atoms in the individual constituents (detn. 36, cols. 8–12), except for oxygen (about 1% too low in detn. 36, col. 12).

The data indicate that β-lactoglobulin consists of 370 amino acids (col. 14, detns. 10 and 36), arranged in 4 sub-units or chains (col. 14, detn. 12) with 366 peptide bonds. There are 4 S–S linkages per mole.

It will become imperative in the future to find a compact way of presenting the empirical formula of a protein in terms of its amino acid residues. We propose to use as symbols, whenever feasible, the first 3 letters of the amino acids: *e. g.*, glycine residue, Gly; arginine residue, Arg; isoleucine residue, Ileu; etc.; the residues of cysteine, half-cystine, asparagine, and glutamine we propose to designate by CySH, (Cy S-), (Asp-NH_2) and (Glu-NH_2), respectively. Such a formula for β-lactoglobulin is given in the summary. We hope that a satisfactory convention will be gradually developed.

Nature of Side Chain Groups.—A tabulation of the various side chain groups in lactoglobulin is presented in Table V (*cf.* ref. 50). The total number of basic and acid groups found by analysis is in excellent agreement with the titration data of Cannan, *et al.*[44] The sulfhydryl and phenolic hydroxyl groups are listed among the non-ionic polar groups, because we have found (ref. 9, footnote 5; *cf.* refs. 8, 56) that these groups are highly unreactive in the native protein.

The total number of polar side chain groups (*i. e.*, groups giving rise to dipole moments) is 219 per mole, of which 112 are ionic and 107 non-ionic. In column 4 are listed the polar side chain groups that are capable of forming hydrogen bonds (*cf.* ref. 57).

Pauling[57] has recently suggested that the peptide carbonyl and imido groups usually do not bind water, because of their mutual interaction by hydrogen bond formation, but that water is bound by carboxyl groups which are not so coupled (*i. e.*, one water molecule is bound by carboxyl groups per proline and hydroxyproline residue). According to Pauling, the data published by Bull[58] on the adsorption of water by proteins can be in considerable degree interpreted on the assumption that the initial process is the attachment of one water molecule to each polar side chain. Adding (*cf.* ref. 57) 15 proline residues (Table I, detn. 19) to the polar side chain groups listed in column 4 of Table V, we have 213 potential water binding groups. This number is much larger than the initial adsorption of 156 moles of water per mole of β-lactoglobulin indicated by Bull's data[58] (*cf.* ref. 57). However, the indole nitrogen has probably a very weak attraction for water; the phenolic hydroxyl groups are undetectable by many reagents and should, therefore, be incapable of taking up water; the aliphatic hydroxyl groups may contribute to the cohesion of the molecule by hydrogen bonding, in which case they also may be incapable of binding water. Thus there is some evidence that the number of water binding side chain groups is probably smaller than the 213 groups enumerated above. But additional experimental data are needed before Pauling's stimulating theory can be properly evaluated in the case of β-lactoglobulin.

TABLE V

NATURE AND NUMBER OF SIDE CHAIN GROUPS IN β-LACTOGLOBULIN (370 AMINO ACIDS PER MOLE OF 42,020 WITH 4 SUB-UNITS)

Type of Group (Table I, detn.)	Number of groups per mole by anal.	Number of groups per mole by titration Cannan, *et al.*[44]	Number of groups per mole Hydrogen bond forming
Cationic groups			
Guanidine (25)	7	6 (5–7)	7
Ammonium (12 + 27)	37	36 (35–37)	37
Imidazole (26)	4	6	4
Total cationic groups	48	48	48
Anionic groups			
Carboxyl (12 + 28 + 30)	64	60–63	64
Total ionic groups	112	108–111	112
Non-ionic polar groups[a]			
Sulfhydryl (21)	4		
½ Disulfide (22)	8		
Methionyl (23)	9		
Indole (24)	4		4
Phenolic hydroxyl (33)	9		9
Aliphatic hydroxyl (31 + 32)	41		41
Amide (8)	32		32
Total non-ionic polar groups	107		86
Total polar groups	219		198
Non-polar groups			
Hydrogen (14)	8		
Paraffin (15 + 16 + 17 + 18)	127		
Benzene (20)	9		
Pyrrolidine (19)	15		
Total non-polar groups	159		
Total number of CH_2 (or CH_3) groups in paraffin side chains[b]	430		

Total number of atoms in paraffin side chains	Atoms per mole atoms	Per cent. of total C or H atoms, %
Carbon	430	23
Hydrogen	1094	36

[a] Giving rise to dipole moments. [b] Alanine, methionine have 1, valine 3, the leucines 4 CH_2 (or CH_3) groups each.

In the paraffin side chains (*cf.* Table V, footnote *b*) there is a total of 430 of CH_2 (or CH_3) groups which account for 23% of the carbon and for 36% of the hydrogen in the molecule.

Discussion

Although practically all of the β-lactoglobulin molecule (Table I, detn. 36, col. 6) has been accounted for, we have to consider seriously the possibility that this is the result of numerous compensating errors. There is little doubt that the minimum molecular weight and a consider-

(56) Brand and Kassell, *J. Biol. Chem.*, **133**, 437 (1940).
(57) Pauling, THIS JOURNAL, **67**, 555 (1945).
(58) Bull, *ibid.*, **66**, 1499 (1944).

able number of constituents have been determined with an accuracy that will be subject to little, if any, future change. But is our correction of 10% for destruction of serine during hydrolysis justified? Is our value of 19.5% for glutamic acid, or is Foster's[10] of 19.1% the correct one? Although our value for valine is in excellent agreement with that reported by McMahan and Snell,[23] we have no *independent* evidence that the microbioassay for this amino acid is completely reliable. Our methods for the determination of proline and of alanine are certainly subject to further improvements. Even in the case of leucine, where there is no doubt that both the microbioassay with the "leucineless" strain of *Neurospora* and the isotope dilution method give accurate results, there remains the question whether there are 49, 50, or 51 residues of leucine in the molecule. The negative test for hydroxyproline does not exclude the possible presence of a small amount of this amino acid, etc. These difficulties will be resolved in the future, we hope, by further improvements in the methods of amino acid determinations.

Some conclusions regarding the structure of β-lactoglobulin can be drawn from the data—most of them rather obvious and not particularly startling. We can conclude with some certainty that in this protein and perhaps in many others, the constituent amino acids are primarily linked by typical peptide bonds; other linkages, such as atypical peptide bonds (as in glutathione), esters, anhydrides, or imides, if they are present at all, can be present only in small numbers. There is a possibility that the molecule may contain a small amount of bound water, or of water that we have not determined in our usual moisture determination. This is not excluded by the analytical data, since we have not fully accounted for the protein (only 99.62%), nor for all of the oxygen atoms (576 instead of 583). On the other hand, these discrepancies are well within experimental error.

We have interpreted the data for the free α-amino nitrogen as indicating the presence of 4 sub-units or polypeptide chains in the molecule of β-lactoglobulin (based on assumptions no. 4(a) to 4(e). The only definite conclusion that we can draw with respect to these sub-units is that they cannot all be the same, since the minimum molecular weight is identical with the molecular weight. We have no information with respect to the size and composition of the four sub-units, nor how they are linked chemically in the molecule. To be sure, there are 4 S–S groups in the molecule which could be imagined to link the sub-units together, but there is as yet no evidence that this is actually the case.

The apparently complete analysis of β-lactoglobulin is based upon 28 independent determinations (*cf.* Table I). Of these 18 were obtained by chemical micro methods, 1 (*lysine*) by a specific enzyme and 9 (*glycine*, alanine, valine, *leucine*, isoleucine, proline, phenylalanine, *aspartic* and *glutamic* acid) by microbiological procedures (*italics* indicate close agreement with results obtained by the isotope dilution method[10]). Similar data on the composition of other proteins are in preparation[59] such as the plasma proteins (*cf.* ref. 43) hemoglobins of various species, insulin, chymotrypsinogen, pepsin and others.

Addendum (August 1, 1945).—Since this paper went to press, we have worked out a bioassay for lysine with *Leuconostoc mesenteroides P–60* (*cf.* ref. 19). The results obtained by the microbiological method are in close agreement with the lysine values obtained by the isotope dilution method on identical preparations (for β-lactoglobulin *cf.* Table I, detn. 27 and footnote *, for bovine serum albumin we obtained 12.4% of lysine, practically identical with the isotope dilution value obtained by Dr. D. Shemin[60]).

The time for the microbioassay for lysine and for other amino acids with *L. mesenteroides* has been shortened to about twenty-two hours. We now prefer this bioassay for lysine to the determination of this amino acid with the specific decarboxylase of *B. cadaveris*,[11,12,13,14] particularly since Gale's newly published results[61] seem to us to be frequently somewhat on the low side.

Summary

The elementary and amino acid composition of crystalline β-lactoglobulin is presented. From the hydrolysis of 100 g. of protein 116.73 g. of split products or 99.62 g. on a residue basis have been obtained; 17.11 g. of water is taken up during hydrolysis.

From the data, a minimum molecular weight ($M_{min.}$) of 42,020 ± 105 was calculated along the lines of orthodox organic chemistry, almost identical with the molecular weight (M_S = 41,600). β-Lactoglobulin is, therefore, monomolecular in solution.

The atomic empirical formula is: $C_{1864}H_{3012}N_{468}S_{21}O_{576}$.

The empirical formula in terms of amino acid residues is (using the first 3 letters of each as the symbol for the amino acid residues): $Gly_8Ala_{29}Val_{21}Leu_{50}Ileu_{27}Pro_{15}Phe_9CySH_4(CyS-)_8Met_9Try_4Arg_7His_4Lys_{33}Asp_{36}Glu_{24}(Glu\text{-}NH_2)_{32}Ser_{20}Thr_{21}Tyr_9(H_2O)_4$.

β-Lactoglobulin consists of 370 amino acid residues with 366 peptide bonds, arranged in 4 sub-units (polypeptide chains). The average residue weight (ARW) is found as 113.7–113.9.

It can be concluded that the constituent amino acids are primarily linked by typical peptide bonds; other linkages, such as atypical peptide bonds (as in glutathione), esters, anhydrides, or imides, if they are present at all, can be present only in extremely small numbers.

(59) Brand, *et al.*, unpublished work.

(60) Shemin, personal communication, *J. Biol. Chem.*, **159**, 439 (1945)

(61) Gale, *Biochem. J.*, **39**, 46 (1945).

It is concluded that the 4 sub-units cannot all be the same, since the minimum molecular weight is identical with the molecular weight.

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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND ZOÖLOGY, COLUMBIA UNIVERSITY]

Leucine Content of Proteins and Foodstuffs[1,2]

BY ERWIN BRAND, FRANCIS J. RYAN AND EUGENE M. DISKANT

In a recent publication[3] we have described a simple microbiological method for the accurate determination of leucine with the aid of the "leucineless" strain of *Neurospora crassa* developed by Regnery[4] in Beadle's laboratory. The reliability of the microbiological method was clearly established by a comparison with the methods involving solubility product and isotope dilution. The proportions of leucine found in crystalline egg albumin, gelatin and crystalline horse hemoglobin were practically the same by these fundamentally different methods, identical preparations having been analyzed in each case. For β-lactoglobulin we reported[3] a leucine content of 15.4%. Since then another sample of β-lactoglobulin (obtained from Dr. G. Haugaard) has yielded in the hands of Dr. G. L. Foster[5] 15.7% by the isotope dilution method. This increases our confidence in the results by the *Neurospora* method. We have previously[3] pointed out that the bioassay for leucine with *Lactobacillus arabinosus*, as carried out by Kuiken, *et al.*,[6] gave results for gelatin and casein only slightly lower than those reported by us.[3] Schweigert, *et al.*,[7] have since obtained a leucine value of 9.6% for casein by the *L. arabinosus* method in good agreement with our value of 9.8%.[3]

Reliable values for the leucine content of proteins obtained with chemical methods are available only for silk fibroin, gelatin and egg albumin which were obtained by the solubility product method[8] and for horse hemoglobin and β-lactoglobulin by the isotope dilution method.[5] Most of the older values for leucine in the literature are of questionable reliability. Moreover, they usually refer to the sum of the leucine and isoleucine, although this is not always explicitly stated nor recognized. Since the *Neurospora* method yields results which confirm the chemical methods in the case of the above proteins, it was thought desirable to extend our knowledge of protein composition by determining the leucine content of a number of common proteins and especially of certain crystalline preparations.

The data are presented in Table I. The leucine values previously reported[3,9] are included. Except as especially noted, the values are on a moisture-, ash- and sulfate-free basis.

Protein Preparations

We are indebted to many workers in the field of protein chemistry for samples of their preparations, as indicated below for the individual proteins.

Blood Proteins.—Most of the proteins were prepared[10] by the Department of Physical Chemistry, Harvard Medical School. Human serum albumin (no. 42) and bovine serum albumin (no. 456) were crystalline preparations (*cf.* ref. 9) and were practically homogeneous by electrophoretic and ultracentrifuge criteria (*cf.* ref. 11). Horse serum albumin A (the carbohydrate containing fraction, so designated by Kekwick)[12] was prepared by Dr. Hans Neurath, while horse serum albumin B (the carbohydrate free fraction, (*cf.* ref. 12)) was prepared by Dr. Manfred Mayer of the Department of Medicine, College of Physicians and Surgeons, according to the method of Adair and Robinson.[13]

Human serum γ-globulin (no. 36, II-1, *cf.* refs. 9, 11) was electrophoretically uniform, but was not homogeneous with respect to size (*cf.* ref. 9, Table II, footnote 2 and ref. 14). The human β-globulin (no. III-2, Prep. GL291, refs. 9, 11) was a complex mixture of human serum globulins about two-thirds of which had the mobility associated with β-globulin.

Human fibrinogen (no. 81, RI, *cf.* refs. 9) was clottable to the extent of about 87% of its protein content; the value reported is calculated on the assumption that the content of total nitrogen is the same in human fibrinogen as in human fibrin

(1) Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

(2) Some of the data in this paper were presented before the Division of Biological Chemistry at the 106th meeting of the American Chemical Society, Pittsburgh, Pa., Sept., 1943.

(3) F. J. Ryan and E. Brand, *J. Biol. Chem.*, **154**, 161 (1944).

(4) D. C. Regnery, *ibid.*, **154**, 151 (1944).

(5) G. L. Foster, *ibid.*, in press.

(6) K. A. Kuiken, W. H. Norman, C. M. Lyman, F. Hale and L. Blotter, *ibid.*, **151**, 615 (1943).

(7) B. S. Schweigert, J. M. McGuire, C. A. Elvehjem and F. M. Strong, *ibid.*, **155**, 183 (1944).

(8) S. Moore and W. H. Stein, *ibid.*, **150**, 113 (1943).

(9) E. Brand, B. Kassell and L. J. Saidel, *J. Clin. Invest.*, **23**, 437 (1944).

(10) These proteins were prepared from blood collected by the American Red Cross, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(11) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr. and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 417 (1944).

(12) R. A. Kekwick, *Biochem. J.*, **32**, 552 (1938).

(13) G. S. Adair and M. E. Robinson, *ibid.*, **24**, 993 (1930).

(14) J. W. Williams, M. L. Peterman, G. C. Colovos, M. B. Goodloe, J. L. Oncley and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 433 (1944).