LXXXV. THE AMINO-ACID COMPOSITION OF RABBIT MYOSIN

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THE studies of Weber [1933] and others have proved that the intracellular protein of muscle consists of at least three distinct fractions, the globulin myosin, the albumin myogen and globulin X. To these must be added the myoalbumin fraction recently isolated by Smith [1937], although this fraction has not yet been obtained in its native state. According to Smith, the distribution of the intracellular protein of rabbit's muscle is: 67.5 % myosin, 9 % myogen, 22.5 % globulin X and 1 % myoalbumin.

Of these fractions myosin has been studied more thoroughly than the others. It is characterized by an isoelectric point at pH 5.5, has typical globulin solubilities and in presence of salts is extremely susceptible to denaturation. It is distinguished from the other muscle proteins by exhibiting double refraction of flow, a property associated with the presence of long rod-shaped anisotropic particles, and on this account can be certainly identified with the anisotropic substance of the muscle fibril.

Bailey [1937] has summarized the few available data on the chemical constitution of this protein and has made a comparative study of myosins from various sources with regard to their contents of nitrogen, amide-nitrogen, sulphur, methionine, cystine, tyrosine and tryptophan. His results indicate the existence of a fundamental amino-acid plan for the elaboration of myosin in the skeletal muscle of mammals, birds and fish.

In the present analysis the original scheme consisted of three stages:

- (1) The separation of the monoamino-acids from the dicarboxylic acids and the bases by butyl alcohol extraction.
- (2) The isolation of the dicarboxylic acids and the bases in the unextracted aqueous solution.
- (3) The determination of the monoamino-acids by fractional distillation of the esters and subsequent identification of the ester fractions.

This plan had to be revised when it was found that portions of both the bases and dicarboxylic acids were extracted by the butyl alcohol and since these two groups of amino-acids are the more important from the viewpoint of structure they were determined without previous extraction of the hydrolysates with butyl alcohol.

By incorporation of Bailey's [1937] values, 76 % of the N of the protein has been identified, as shown in Table I, while evidence has been obtained suggesting that the remaining 24 % is largely associated with the monoamino-acid group.

The relations between the chemical constitution and the physical properties of myosin will be discussed fully in a separate communication and will only be mentioned briefly here. Astbury & Dickinson [1935] have shown that in the denatured state myosin exhibits the $\alpha\beta$ transformation, permanent set and supercontraction, phenomena which are associated with the keratin group of

Table I. *Amino-acid analysis of rabbit myosin*

Amino-acid	N % protein-N	Wt. % protein
Glycine	2.1	1.9
Alanine	3.5	3.7
Valine	2.2	3.0
Leucines	7.1	11.1
Proline	0.4	0.5
Phenylalanine	2.0	4.0
Threonine	0.9	1.2
*Methionine	1.9	3.4
*Cystine	0.56	0.77
*Tyrosine	1.6	3.4
*Tryptophan	0.66	0.82
Arginine	13.4	7.0
Histidine	2.8	1.7
Lysine	11.3	9.9
Glutamic acid	12.5	22.1
Aspartic acid	5.6	8.9
Ammonia	7.9	1.6
Total accounted for	76.4	85.0

* From Bailey [1937].

proteins. In fibre form, therefore, myosin has the same fundamental folded chain structure which is characteristic of the keratins. It seems that myosin in living muscle is in the form of highly organized systems of peptide chains capable of shortening when appropriately stimulated and mainly determining the optical and elastic properties of the tissue. The changes associated with activity must be closely related to the specific chemical configuration of the constituent molecules.

Myosin is found to contain relatively high proportions of glutamic and aspartic acids, lysine and leucine. The dicarboxylic acids, together with the bases, arginine, histidine and lysine, in constituting half the protein molecule, provide a large number of polar side-chains. The cystine plus cysteine content is low, being only 0.77–0.79 % of the protein [Bailey, 1937] and of this 0.67 % is cysteine [Mirsky, 1936]. The presence of a large number of polar side-chains, together with a large number of long non-polar side-chains and an unusually small number of rigid disulphide links, would constitute, according to present theories of protein structure, a relatively open system of polypeptide chains with many centres for the free co-ordination of water molecules.

Of the intracellular proteins myosin has by far the greatest power of hydration, since it possesses an affinity for water of the same order as gelatin. The usual preparation of the native protein precipitated by dilution from salt solution at pH 6.8 centrifuges to a jelly-like mass with a water content of 98–99 %. It can, indeed, form a gel with a protein content of only 0.2 % by weight [von Muralt & Edsall, 1930], while a salt-free preparation after drying in a chilled desiccator for a week still contains 1 g. H₂O/g. protein [Mirsky, 1937]. Amongst the fibrous proteins its hydration is exceeded only by the mucins, which contain upwards of 30 % of polysaccharide.

The chemical constitution, therefore, fully supports the theories of the relationship between structure and hydration which have been developed by Jordan Lloyd & Phillips [1933]. Furthermore, if an attempt be made to depict the process of muscular contraction as being due to the action of certain products of metabolism on specific groupings present in the peptide chains, it is clear that an open structure, such as that which can be postulated from the chemical data, will

favour the rapid diffusion of these substances and their secondary products into and away from the sites of action. Such ideas emphasize the importance of the present work in the interpretation of muscular contraction.

EXPERIMENTAL

Preparation of myosin. The protein was prepared by extraction of rabbit's muscle with buffered KCl solution and dilution of the filtered solution with 20 vol. distilled water [Edsall, 1930]. This material was redissolved in salt solution and reprecipitated by dilution to give a product free from traces of the other protein fractions of muscle. The various preparations were thoroughly washed with distilled water and finally brought to the state of a fine flour by treatment with alcohol and ether. On drying *in vacuo* they contained 0.3–0.8 % of ash and 2–3 % of moisture. The ash-free N content was 16.8 %.

Determination of the basic amino-acids

Block's [1934] procedure was followed in detail, using 2–3 g. of protein for each analysis. The values given in Table II are the mean of closely agreeing duplicates and are calculated from the weights of the isolated crystalline derivatives, namely, arginine flavianate, histidine diflavianate and lysine picrate. The experimental values are corrected for the solubilities of arginine silver and lysine phosphotungstate and also for the small overall losses suggested by Tristram (unpublished data).

Table II. *Basic amino-acids of rabbit myosin and myogen*

Amino-acid	Myosin				Myogen			
	Experimental		Corrected*		Experimental		Corrected*	
	Wt. % protein	N % protein-N	Wt. % protein	N % protein-N	Wt. % protein	N % protein-N	Wt. % protein	N % protein-N
Histidine	1.69	2.70	1.74	2.80	2.90	4.67	2.95	4.75
Arginine	6.7	12.7	7.0	13.4	6.0	11.4	6.3	12.1
Lysine	9.5	10.9	9.9	11.3	7.8	8.9	8.0	9.2
Total bases	17.9	26.3	18.6	27.5	16.7	25.0	17.3	26.1

* See text.

The lysine picrate after recrystallization exploded at 265–266° and the arginine flavianate decomposed at 258–260°. The histidine diflavianate, when first separated, gave a low decomposition point and a high sulphur value. The recrystallized product gave a satisfactory decomposition point at 246° but the sulphur content was still high owing to the presence of free flavianic acid. (Found 8.46 % S. Theory 8.17 % S.) The small amount of material available prohibited further purification of the salt.

Rabbit muscle myogen, kindly provided by Dr E. C. Bate Smith, has also been analysed (Table II). The distributions of bases in the two proteins are very different, the calculated molecular ratios of histidine, arginine and lysine being 2 : 7 : 12 and 2 : 4 : 6 in myosin and myogen respectively.

Precipitation of the basic N under the Hausmann conditions gave values of 29.4 and 29.1 % of the total N of myosin and myogen respectively. These values are as usual 2–3 % higher than those obtained by calculation from the amounts of isolated bases.

Separation of the monoamino-acids by extraction with butyl alcohol

Two samples of myosin, weighing 72 g. and 114 g. respectively, were hydrolysed with 10 times their weight of 6N HCl for 30 hr. Aliquots were then withdrawn for analysis (Table III). In each case the ammonia-N was 7.9 % and the

Table III. *Analysis of myosin hydrolysates*

Hydrolysate	Wt. of protein g	Total N g.	Amino-N g.	Amide-N g.
1	72	12.1	9.8	0.96
2	114	19.1	15.4	1.51
Total	186	31.2	25.2	2.47

amino-N (after removal of ammonia) was 81 % of the total N respectively. Bailey's [1937] value for the amide-N (7.2 %) obtained after hydrolysis with 2N HCl for only 3 hr. is a truer estimate of the N originally bound in amide form, since in the present case the action of the relatively strong acid and longer period of hydrolysis has undoubtedly brought about a small amount of deamination.

Each hydrolysate was then freed from the greater part of the excess HCl by repeated alternate concentration *in vacuo* and dilution, the remaining HCl being removed with Ag₂O and H₂SO₄ in the usual way. The H₂SO₄ was then removed as BaSO₄ and in each case the filtrates and washings were collected, concentrated *in vacuo* to incipient crystallization, kept at 0° for 12 hr. and the separated material filtered off (Table IV: fractions 1-1 and 2-1). Additional

Table IV. *Crystalline fractions removed before butyl alcohol extraction*

Hydrolysate	Fraction	Wt. g.	Total N g.	N %
1	1-1	1.41	0.11	7.8
	1-2	2.50	0.25	10.0
	1-3	5.67	0.63	11.1
2	2-1	2.26	0.17	7.5
	2-2	12.7	1.30	10.2

crops of crystalline material were obtained on further concentration of the filtrates (Table IV: fractions 1-2; 1-3; 2-2). All these fractions consisted in very large part of tyrosine and leucine.

The remainder of hydrolysate 1 and one half of that of hydrolysate 2 (referred to henceforth as 2A) were separately extracted *in vacuo* with aqueous saturated butyl alcohol in a continuous extraction apparatus similar to that used by Dakin [1920], whose procedure in the separation and drying of the extracted monoamino-acids was followed throughout. The longer period of extraction required in the case of hydrolysate 2A was due to the lowering of the bath temperature from 40-45° (employed with hydrolysate 1) to 35-40°.

In each case the butyl alcohol mother liquors were concentrated to a small volume *in vacuo* and the material which separated at 0° was centrifuged off before further concentration to remove all solvent and final solution of the residual syrup in water. Traces of brown tar which remained insoluble in water were found to contain only negligible amounts of N. Moreover, the relatively larger amount of N which remained in the butyl alcohol mother liquors of 2A was due to the difficulty of separating the sticky insoluble material which was obtained in the later period of the prolonged extraction.

The summarized results of the butyl alcohol extractions are given in Table V. The loss of total N was due to the escape of ammonia. Considered from the point of view of effectiveness of the method, the figures show that under the different conditions employed the separations were very similar in each case, and as will

Table V. *Fractionation obtained by butyl alcohol extraction*

	Total N		Amino-N
	g.	As % of total protein-N	g.
Hydrolysate 1. Period of extraction 156 hr.			
Butyl alcohol extract			
(1a) Solid amino-acids (22.6 g.)	2.72	22.5	2.53
(1b) Soluble in water	1.04	8.6	0.76
Insoluble in water	0.02	—	—
(1c) Unextracted aqueous residue	6.33	52.0	4.79
Losses (by difference)	0.49	4.1	0.08
Total	10.60		8.16
Hydrolysate 2A. Period of extraction 247 hr.			
Butyl alcohol extract			
(2Aa) Solid amino-acids (15.0 g.)	1.72	18.0	1.68
(2Ab) Soluble in water	1.66	17.4	1.41
Insoluble in water	0.003	—	—
(2Ac) Unextracted aqueous residue	5.07	53.0	3.69
Losses (by difference)	0.35	3.7	0.12
Total	8.80		6.90

be shown later in Table VI, the unextracted residues contained almost identical proportions of dicarboxylic acids, N precipitable by phosphotungstic acid and residual amino-N. The latter, which represents 10 % of the total unextracted N, must be ascribed to monoamino-acids, the extraction of which was therefore incomplete. The total N precipitable by phosphotungstic acid was lower by 3 % than the Hausmann value on the initial hydrolysate, which indicates that part of the basic N may have been extracted by the butyl alcohol. Moreover, as is shown in a later section, dicarboxylic acids were undoubtedly extracted by the butyl alcohol under the conditions of these experiments. The studies of egg albumin by Calvery [1932] and of cocksfoot protein by Miller (private communication) have revealed similar partial extractions of both these groups of compounds by butyl alcohol, and in view of the efficient fractionation obtained with hydrolysates of casein, gelatin and zein by Dakin [1918; 1920; 1923], it appears that the success of the method depends on the acid-base relationships in the hydrolysate as prepared for extraction.

Isolation of the dicarboxylic acids after extraction of the monoamino-acids with butyl alcohol

Fractions 1c and 2Ac were of similar composition for they contained 52 and 53 % of the total protein-N of which 76 and 73 % were amino-N respectively. Each solution was made alkaline to phenolphthalein with baryta, concentrated *in vacuo* to remove ammonia and Ba removed completely with H_2SO_4 . The clarified solutions were then concentrated to a small volume *in vacuo*, saturated at 0° with HCl and two crops of glutamic acid hydrochloride removed in each case. At this stage opportunity was taken to compare the efficiencies of the two routine procedures for the separation of the dicarboxylic acids by precipitation of the Ca salts [Foreman, 1914] and of the Ba salts [Dakin, 1920] with alcohol.

The mother liquors from the separation of glutamic acid hydrochloride were freed as far as possible from HCl by concentration *in vacuo*, the residual syrups dissolved in water and saturated with either $\text{Ba}(\text{OH})_2$ or $\text{Ca}(\text{OH})_2$ at a concentration of 2.5–3.0 g. N in 100 ml. aqueous solution; 9 vol. 95 % ethyl alcohol were then added slowly with rapid stirring and after 15–18 hr. at room temperature the insoluble salts were separated and washed with alcohol. The salts were redissolved in water and freed from Ba or Ca as sulphate or oxalate respectively. The filtrates and washings in each case were collected, concentrated and the copper salts formed by addition of $\text{Cu}(\text{OH})_2$ at 100° . The hot solutions were filtered and on standing overnight at 0° the filtrates yielded large crops of copper aspartate. These were filtered off and Cu removed from the filtrates as sulphide. The solutions at this stage contained a large proportion of non-amino-N as shown by the following analyses: 1c, 2.19 g. N, 1.8 g. amino-N; 2Ac, 0.82 g. N, 0.72 g. amino-N. Basic N was obviously present and it was removed by precipitation with phosphotungstic acid in HCl solution under the usual conditions [Van Slyke, 1911]. The filtrates from the insoluble phosphotungstate precipitates were freed from phosphotungstic acid in the usual way and resaturated with gaseous HCl to give a further crop of glutamic acid hydrochloride. Following this, from 2Ac in which lime had been employed as initial precipitant, two additional crops of copper aspartate and a final crop of glutamic acid hydrochloride were separated. The remaining unidentified residue contained only 0.16 g. N. Fraction 1c, however, in which baryta had initially been employed, contained at this stage such a high proportion of monoamino-acids that, in view of the relatively clean separation obtained from fraction 2Ac, it was decided to reprecipitate the residual dicarboxylic acids as their Ca salts. Following this further crops of glutamic acid hydrochloride and copper aspartate were isolated, leaving a final unidentified residue containing 0.19 g. N.

The analyses of fractions 1c and 2Ac at this stage are summarized in Table VI and the final distribution of the N in the salts insoluble in alcohol is given in

Table VI. *Analysis of fractions 1c and 2Ac and hydrolysate 2B*

Fraction	1c (Using baryta and lime)		2Ac (Using lime)		2B (Using lime)	
	Total N g.	N as % of protein-N	Total N g.	N as % of protein-N	Total N g.	N as % of protein-N
Glutamic acid hydrochloride	1.1	9.1	0.87	9.1	1.19	12.5
Copper aspartate	0.51	4.2	0.41	4.3	0.53	5.6
Precipitated by phospho- tungstic acid	3.15	26.0	2.44	25.6	3.01	31.6
Residual amino-N	0.71	5.9	0.49	5.14	2.91	30.4
Ammonia	0.47	3.9	0.41	4.3	0.76	7.9
Losses on precipitates, etc.	0.39	3.2	0.45	4.7	0.40	4.2
Total	6.33	52.3	5.07	53.14	8.80	92.2

Table VII. Although equivalent amounts of the dicarboxylic acids were eventually isolated from the two fractions, the greater selectivity of Ca is apparent, for both fractions, after removal of approximately 80 % of the total glutamic acid present before Ca or Ba salt precipitation, were of similar composition and the data show clearly the larger proportions of basic N and of N apparently belonging to monoamino-acids which are precipitated as Ba salts.

Table VII. *Comparison of the N precipitated as Ba and Ca salts by means of alcohol*

(Values are % total protein-N)

	1c (Using baryta)	2Ac (Using lime)	2B (Using lime)	
			1st ppt.	2nd ppt.
Total N in salts insoluble in alcohol	21.1	12.0	26.2	21.0
Containing:				
Total isolated dicarboxylic acid N	6.3	5.9	18.0	18.0
N in insoluble phosphotungstate	10.1	4.4	3.0	0.0
Residual amino-N	4.1	1.7	5.2	3.0

Isolation of the dicarboxylic acids without previous extraction of the monoamino-acids with butyl alcohol

The remaining aliquot of the second hydrolysate, referred to henceforth as 2B, contained 8.8 g. N, 6.9 g. amino-N and 0.76 g. ammonia-N. Excess $\text{Ca}(\text{OH})_2$ was added, free ammonia removed by concentration *in vacuo* and the insoluble Ca salts precipitated by addition of 9 vol. 95 % ethyl alcohol at a concentration of 2.0 g. N in 100 ml. aqueous solution. The mixture was kept for 15–18 hr. at room temperature, the precipitate filtered off, redissolved in water and reprecipitated at a concentration of 5.0 g. N in 100 ml. aqueous solution. The reprecipitated salts contained no non-amino-N and were found later to give no precipitate with phosphotungstic acid. Four crops of glutamic acid hydrochloride and two crops of copper aspartate were subsequently obtained by the usual procedure. The final residue, which appeared to be free from dicarboxylic acids, contained 0.28 g. N, all in the amino form.

The results of the complete fractionation are given in Table VI and the efficiency of the Ca salt precipitation of the dicarboxylic acids is given in Table VII. At no stage in any of the three separate analyses were products resembling the hydroxyglutamic acid of Dakin [1918] encountered. If the values for glutamic and aspartic acids and the N removed in the phosphotungstate precipitates from hydrolysate 2B are taken as maximum values for the protein, it is clear that large proportions of the dicarboxylic acids and N precipitable by phosphotungstic acid were extracted by butyl alcohol.

It appears from the results given in Tables VI and VII that the higher concentration of N precipitable by phosphotungstic acid present in fractions 1c and 2Ac led to serious contamination of the insoluble Ca and Ba salts to the extent of 48 % and 37 % respectively of the total N present therein, whereas the corresponding value for hydrolysate 2B was only 11 % of the total N, an amount which was completely removed on reprecipitation of the Ca salts. The procedure adopted for hydrolysate 2B was very efficient, glutamic acid hydrochloride and copper aspartate having been isolated in a pure state with relative ease to account for 86 % of the total N recovered from the Ca salts.

Preparation of the ethyl esters of the monoamino-acids

All monoamino-acid residues from the separation of the dicarboxylic acids and the bases from hydrolysates 1, 2A and 2B, all fractions extracted by butyl alcohol (1a, 1b, 2Aa and 2Ab) and fractions 1–2, 1–3 and 2–2 were collected. The

position at this stage is summarized in Table VIII. Discounting the N removed as ammonia the total losses on the various inorganic precipitates were equal to 6.8 % of the protein-N. The material for esterification, although equivalent to

Table VIII. *Position of analysis before esterification*

Fraction	N g.	Amino-N g.	N % protein-N
Glutamic and aspartic acids	4.60	4.60	14.8
Removed as insoluble phosphotungstates	8.64	5.60	27.7
Tyrosine fractions 1-1 and 2-1	0.28	0.29	0.9
Ammonia plus total losses on precipitates etc.	4.57	2.48	14.7
Residues from dicarboxylic acid separations	0.35	0.35	1.12
Material for esterification	12.77	11.88	41.0
Original values in hydrolysates	31.2	25.2	100.2

41 % of the protein-N, contained, by calculation, 1.05 g. dicarboxylic acid-N and 1.21 g. of N precipitable by phosphotungstic acid. Deduction of these values leaves 33.7 % of the protein-N which can be ascribed to the monoamino-acids.

The summation of the N of the identified amino-acid constituents of the protein—glutamic and aspartic acids = 18.1 %; histidine, arginine and lysine = 27.5 % and the amide-N = 7.9 %—gives a total of 53.5 % of the protein-N and leaves 46.5 % to be accounted for as monoamino-acids. The difference of 12.8 % between this estimate and the above value of 33.7 % is explained by the losses on the various precipitates (6.8 %); the additional N which was precipitated with the base phosphotungstates (31.6 less 27.5 = 4.1 %); the small unidentified residues from the separation of the dicarboxylic acids (1.12 %), and finally the tyrosine fractions 1-1 and 2-1 (0.87 %). These four factors represent essentially monoamino-acid-N equivalent to 12.9 % of the protein-N and equivalent to 28 % of the estimated total monoamino-acid-N of the protein. This was rather a large handicap with which to commence the most difficult part of the analysis, but since very little was known regarding the monoamino-acid constitution of myosin, it was decided to obtain by fractional distillation of the ethyl esters as much information as possible about the relative proportions of the monoamino-acids present.

The dry finely powdered amino-acid fractions were suspended in absolute alcohol and the mixture saturated with dry gaseous HCl until complete solution of the amino-acids had been obtained. The solution was then refluxed gently for 30 min., during which time a slow current of the gas was passed continuously. After being cooled to room temperature the solution was freed as much as possible from the water of esterification by alternate concentration *in vacuo* and dilution with absolute alcohol. This complete process was repeated twice. The final concentrate of the ethyl ester hydrochlorides was dissolved in absolute alcohol and adjusted to approximately pH 4 by addition of dry alcoholic ammonia as recommended by Foreman [1919] to remove most of the excess HCl. The NH_4Cl which crystallized from solution at 0° was filtered off. Alcohol was removed from the filtrate by alternate concentration *in vacuo* and dilution with dry chloroform, and the final syrupy residue taken up in dry chloroform to permit the preparation of the free esters from their hydrochlorides by treatment with anhydrous $\text{Ba}(\text{OH})_2$.

It was found impossible to obtain a Cl-free solution of the esters by simply shaking the chloroform solution in contact with excess $\text{Ba}(\text{OH})_2$, and it was necessary to transfer the mixture to a "runner mill".¹

The finely divided solid residue of material insoluble in chloroform was removed and washed thoroughly with dry chloroform. Ba was removed quantitatively, the amino-acids in solution re-esterified and a second chloroform solution of free esters obtained.

After the first esterification 79 % of the N present was obtained in the form of free esters in chloroform solution. The re-esterification of the Ba residues brought an additional 13 % of the total N into chloroform solution. 7 % of the N remained in the Ba residues from the second esterification and 1.4 % of the N was lost in the removal of Ba from the residues of the first esterification. The necessary data are given in Table IX.

Table IX. *Distribution of N after liberation of esters*

Fraction	N g.	Amino-N g.
Free esters in chloroform solution	11.70	11.13
In Ba residue after removal of Ba	0.89	0.69
Losses on BaCl_2 and BaSO_4 precipitates	0.18	0.08
Total	12.77	11.90

The chloroform solution of the free esters was concentrated at a bath temperature of 30° and pressure of 100 mm. During this and subsequent concentrations, all vapours which escaped condensation were trapped in HCl to ensure that no loss of volatile esters occurred. On addition of anhydrous ether to the residue, a flocculent precipitate formed. Ether was added until no further precipitation took place. The precipitated esters were filtered off and the ethereal solution after standing overnight at 0° in contact with anhydrous Na_2SO_4 was filtered and concentrated to 150 ml. at 100 mm. pressure and a bath temperature of 30°. The concentrated solution of the esters was transferred to the fractional distillation apparatus and the bulk of the remaining ether removed at a bath temperature of 40° and a pressure of 60 mm. Traps cooled by liquid air were used to prevent loss of volatile ester and a slow current of CO_2 was passed continuously through the ester mixture to promote even boiling.

The ester fraction soluble in chloroform but insoluble in ether contained 0.64 g. total N and 0.39 g. amino-N. The ether solution of the esters contained 11.06 g. total N and 10.74 g. amino-N, these values being equivalent to 86 % of the original N for esterification and to 35 % of the total protein-N.

On completion of the distillation, the small amounts of ester in the liquid air traps were added to their respective main fractions and with the exception of the first fraction the distilled esters were immediately hydrolysed in 6-8 vol. of water at boiling point. The resulting solutions of the free amino-acids were concentrated to dryness *in vacuo* for N analysis and subsequent fractional crystallization.

¹ Owing to the difficulty of obtaining easily a small test sample free from suspended BaCl_2 , the determination of the presence or absence of Cl^- in the chloroform solution of the esters has by all published accounts been rather a difficult step in the procedure. A method for determining the completion of the reaction has been devised by Dr J. W. H. Lugg and is worthy of particular mention. A clean sample can be obtained by touching the surface of the chloroform mixture with a narrow strip of filter paper. After a few seconds the wetted strip is withdrawn and the lower part which contains traces of BaCl_2 is immediately cut away. The upper part wetted by capillary action contains a clean sample of the chloroform solution which can be washed out and tested for Cl^- .

Table X. *Fractions obtained after distillation of the esters*

Fraction	Pressure mm.	Bath temp. °C	Vapour temp. °C	Vapour temp. Bulk of fraction °C	Wt. of ester g.	Recovered acids				
						N g.	Amino-N g.	N %	N %	protein-N
1 + trap A	10	40-92	20-49	45-46	36.6	1.89	1.95	—	—	6.1
2	"	92-98	49-70	60-65	8.7	0.89	0.86	13.3	13.3	2.9
3 + trap B	10	98-102	70-73	70-71	8.9	0.74	0.69	11.7	11.7	2.4
	5	88-90	57-61	60-61						
4	5	90-100	61-63	62-63	18.2	1.69	1.46	11.6	11.6	5.4
5 + trap C	1.5	80-96	40-41	40	9.6	0.65	0.58	11.5	11.5	2.1
6	"	96-110	41-48	44	4.9	0.42	0.39	10.8	10.8	1.4
7	"	110-113	51-60	54	2.8	0.22	0.22	10.5	10.5	0.7
8	"	113-130	60-81	79	5.4	0.47	0.46	11.0	11.0	1.5
9	"	130-146	81-85	84	5.8	0.49	0.46	10.4	10.4	1.6
10	Distillation residue { Ether phase Aqueous phase					0.80	0.70	—	—	2.6
11						1.98	1.63	—	—	6.4
12	Esters soluble in chloroform, insoluble in ether					0.64	0.39	—	—	2.1
13	Trapped during removal of chloroform and ether					0.32	0.27	—	—	1.1
14	Ba residues from 2nd esterification					0.89	0.69	—	—	2.8
	Loss on 1st BaSO ₄ precipitate					0.18	0.08	—	—	0.6
	Loss in distillation					0.50	1.07	—	—	1.6
Original values						12.77	11.90	—	—	41.3

The details of the distillation and preliminary analysis of the various fractions are presented in Table X.

Separation and identification of the monoamino-acids

Fraction 1. This fraction was expected to consist mainly of glycine ester and was treated by saturation with gaseous HCl in alcoholic solution; 3.18 g. glycine ester hydrochloride were obtained containing 10.1 % N (theory 10.0 % N). To the mother liquor and washings were added the re-esterified amino-acids of fraction 13 and from the mixed alcohol solution, after concentration and saturation with gaseous HCl at 0°, a second crop of ester hydrochloride was obtained. This material weighed 1.49 g. and the N content of 12.3 % indicated the presence of glycylglycine ester. The fraction was accordingly refluxed in 20 % HCl; glycine was precipitated from the hydrolysate as the nitranilate [Town, 1936]. The precipitated glycine was equivalent to 90 % of the total N of the fraction, which therefore consisted essentially of a mixture of glycine and glycylglycine esters and corresponded to a glycine content of 0.98 g.

No further crop of ester hydrochloride could be separated from the main mother liquor and, after hydrolysis of the esters and removal of HCl, three crops of free acids were obtained from aqueous solution. These crops were apparently mixtures of glycine, alanine and valine and were dealt with individually.

Crop 1 was further fractionated by precipitation with nitranilic acid. 0.45 g. of pure glycine nitranilate was isolated equivalent to 0.18 g. of glycine, and, after removal of reagents, 4.42 g. of alanine (15.8 % N; $[\alpha]_D^{20}$ in 10 % HCl = +10.4°) were crystallized from solution. The final mother liquor contained N equivalent to only 0.5 % of the protein-N and was neglected.

Crop 2 contained 15.1 % N. A test nitranilate precipitation proved the presence of 0.34 g. of glycine and therefore, by calculation from the N content, the remainder of the fraction consisted of 2.8 g. alanine and 1.08 g. valine.

Without previous removal of the glycine present, the main bulk of the fraction was treated for the separation of alanine and valine by phosphotungstic acid precipitation according to Levene & Van Slyke [1913]; 2.5 g. alanine (16.0 % N; $[\alpha]_D^{20}$ in 10 % HCl = +10.4°) and 0.22 g. valine (12.1 % N) were isolated. The residue, weighing 1.23 g. and apparently consisting of all the glycine present with a large proportion of valine and traces of alanine, was not investigated further.

Crop 3 weighed 1.73 g. and contained 14.7 % N. By nitranilic acid precipitation the equivalent of 0.37 g. of glycine was isolated as nitranilate, leaving a residue of 1.36 g. (13.6 % N) which remained unidentified. Since no evidence of the presence of any acid other than glycine, alanine and valine was found in the whole of fraction 1, the probable composition of this end-fraction was 0.59 g. alanine and 0.77 g. valine.

From fractions 1 and 13 therefore the amino-acids actually isolated and identified either as free acids or as derivatives were 3.59 g. glycine, 6.92 g. alanine and 0.22 g. valine. The unidentified fractions by calculation from the N values very probably consisted of 0.89 g. alanine and 1.63 g. valine.

Fraction 2. By fractional crystallization from aqueous solution no single amino-acid could be separated. The several crops of crystalline material were evidently mixtures of valine and leucine with traces of alanine. By the lead salt separation of Van Slyke & Levene [1909] 2.01 g. of pure valine (N = 11.96 %; $[\alpha]_D^{20}$ in 20 % HCl = +26.3°) were isolated.

Fraction 3. 2.05 g. of leucine (10.7 % N) were separated by crystallization from aqueous solution, and 1.69 g. of valine (12.0 % N; $[\alpha]_D^{20}$ in 20 % HCl = +25.1°) were isolated by precipitation in acetone solution.

Fraction 4. By fractional crystallization from aqueous solution and additional separation of the lead salts 10.7 g. of leucine (10.7 % N; $[\alpha]_D^{20}$ in 20 % HCl = +20.8°; in water = -3.54°) were isolated. The aqueous mother liquors were collected (0.38 g. N; 0.2 g. amino-N), taken to dryness and the residue extracted with absolute ethyl alcohol to give a fraction insoluble in alcohol (0.15 g. N, all in the amino form), and a fraction soluble in alcohol (0.224 g. N; 0.038 g. amino-N). From the latter 3.02 g. of proline picrate (melting point 150°) equivalent to 1.01 g. of proline were isolated. By difference only 0.062 g. of the non-amino-N in fraction 4 remained unidentified.

At this stage the residual leucine-valine mixtures from fractions 2, 3 and 4 were mixed and partly separated by means of the lead salts. 1.71 g. of valine (11.9 % N; $[\alpha]_D^{20}$ in 20 % HCl = +25.4°) were isolated, leaving a residue of 1.73 g. which was further treated as described below.

Fraction 5. By crystallization from aqueous solution 3.70 g. of leucine (10.7 % N) were isolated. The final mother liquor contained 0.25 g. N. By acetone precipitation 0.6 g. of leucine (10.7 % N) was isolated, leaving a final residue which contained 13.4 % N and which was treated subsequently with the residual fractions of 2, 3 and 4. These were apparently mixtures of leucine, valine, alanine and proline. A small fraction of insoluble lead salts was removed to leave a final solid residue of 7.62 g. containing 1.04 g. N and 0.87 g. amino-N. This mixture was not resolved into its individual components, but assuming that it consisted of proline, valine and alanine its composition can be calculated on amino-N and total N values to be: 1.4 g. proline, 3.36 g. alanine and 2.86 g. valine.

Fraction 6. 3.07 g. of leucine (10.7 % N) were isolated by fractional crystallization from aqueous solution. The mother liquors gave a residue containing 12.8 % N which was not identified.

Fraction 7. The acids present in this fraction were very soluble in water and had to be recrystallized from increasing concentrations of alcohol. 0.55 g. of leucine (10.7 % N) was isolated in this way, the remaining material being unidentified.

No attempt was made to separate the individual isomerides in the various leucine fractions isolated. The specific rotations in 20 % HCl of the different crops varied between +19.4° and +24.4° at a temperature of 21° and showed evidence of increasing proportions of *d*-isoleucine and/or *nor*leucine with increasing vapour temperature of distillation of the ethyl ester fractions.

Fractions 8 and 9. Preliminary titration showed that 7 % and 10 % respectively of the total N of these fractions were dicarboxylic acid-N. Fraction 9 was not investigated further. The mixed acids of fraction 8, however, contained 11.0 % N, were very soluble in water and gave the positive biuret reaction characteristic of threonine and serine, suggesting the presence of the former. The dicarboxylic acids were removed by precipitation of the Ba salts in alcohol, the soluble salts were freed from Ba and the final aqueous solution concentrated to small volume *in vacuo*. Unfortunately at this stage a large aliquot of the solution was lost through breakage but from the remaining aliquot two solid fractions were separated. Fraction A crystallized from 80 % alcohol solution and the mother liquor on being taken to dryness provided a small residue (10.6 % N) which probably consisted of leucine residues.

Fraction A on analysis gave 40.25 % C,¹ 7.73 % H, 11.75 % N and $[\alpha]_D^{20}$ in 4 % aqueous solution = -20.2°. The theoretical values for *d*-threonine are

¹ All C, H and S determinations were carried out at the Micro-analytical Laboratory, Oxford.

40.31 % C, 7.62 % H, 11.76 % N and $[\alpha]_D^{25}$ in aqueous solution = -28.3° [West & Carter, 1937]. The material gave a strong positive biuret reaction, although all the N was present in amino form, and on recrystallization from alcohol gave an increased specific rotation, $[\alpha]_D^{25}$ in 4 % aqueous solution = -23° with no significant change in elementary analysis. On heating, the product turned slightly brown at 220° and melted with decomposition at 240° which is $11-13^\circ$ lower than the melting point of the synthetic *d*-threonine prepared by West & Carter [1937]. As a result of the loss of a large part of the original solution, there was insufficient material for more complete identification, but the elementary composition, positive biuret reaction, specific rotation, melting point and solubilities all indicate that the fraction may be accepted as slightly racemized *d*-threonine. After making allowance for the aliquot lost, the estimated total threonine was equivalent to 1.24 % of the protein and 0.87 % of the protein-N.

d-Threonine has been isolated so far from the hydrolysate of only one other protein, namely fibrin, from which it was originally separated and identified to the extent of 0.08 % of the protein by McCoy *et al.* [1935], who estimated that there was possibly a loss of 80–90 % during the course of their procedure for separation of the pure substance. The threonine isolated from myosin was equivalent to 54 % of the mixed acids of fraction 8 and was isolated without difficulty from the dicarboxylic acids and the apparent leucine isomerides present. With previous removal of the dicarboxylic acids and more efficient fractionation of the ethyl esters, a relatively pure threonine fraction could probably be obtained without the large experimental losses involved in the procedure of McCoy and his associates.

The residue in the distillation flask (fractions 10 and 11). The dark brown viscous residue was partitioned between ether and water to obtain separation of phenylalanine ester from the remaining material. The ether solution (fraction 10) contained 0.80 g. N and 0.70 g. amino-N, while the ether-soluble aliquot (fraction 11) contained 1.98 g. N and 1.63 g. amino-N. Four crops of phenylalanine hydrochloride with a total weight of 6.93 g. containing 0.48 g. N were separated from fraction 10. The free phenylalanine contained 8.57 % N and gave $[\alpha]_D^{25}$ in water = -16° , showing that some racemisation had occurred at the temperature of distillation. The final mother liquor and washings were combined with fraction 11, the Ca salts insoluble in alcohol were removed and the insoluble phosphotungstates separated under the usual conditions. The insoluble Ca salts contained by difference 0.87 g. N and 0.50 g. N was removed in the form of insoluble phosphotungstates. From the remaining solution of monoamino-acids, 1.83 g. of phenylalanine (8.57 % N) were isolated, leaving an unidentified residue which contained 0.77 g. N.

Fraction 12. This fraction consisted of the esters which were soluble in chloroform but insoluble in ether. Of the original 0.63 g. N, 0.37 g. was precipitable by phosphotungstic acid, leaving a residue which contained 0.04 g. tyrosine and an unidentified mixture equivalent to 0.8 % of the total protein-N.

Fraction 14. The Ba residue from the second esterification was freed from Ba and the N precipitable by phosphotungstic acid removed. Of the original 0.87 g. N in the fraction 0.34 g. was removed by phosphotungstic acid precipitation and 0.91 g. of crude tyrosine containing 0.07 g. N was separated, leaving a final unidentified residue containing 0.42 g. N.

The results of the analyses of the ester fractions are summarized in Table XI. Of the original 12.8 g. N in the material which was esterified, 5.74 g. were associated with the isolated free amino-acids or their derivatives, 0.96 g. was associated with the dicarboxylic acids and 1.21 g. were associated with N precipitable by

Table XI. *Summary of the fractionation of the monoamino-acids*

Ester fractions	Isolated fractions	N identified g.	N unidentified g.
1 + 13	Glycine	0.67	0.42
	Alanine	1.09	
	Valine	0.026	
2, 3, 4	Valine	0.65	1.19
	Leucines	1.36	
	Proline	0.12	
5	Leucines	0.46	0.19
6	Leucines	0.33	0.09
7	Leucines	0.059	0.16
7, 8, 9	Dicarboxylic acids by titration	0.086	0.60
8	Threonine	0.27	
10 + 11	Phenylalanine	0.64	0.77
10 + 11	Basic N (insoluble phosphotungstates)	0.50	
10 + 11	Dicarboxylic acids (insoluble Ca salts)	0.87	
12	Tyrosine (colorimetric)	0.003	0.27
12	Basic N (insoluble phosphotungstates)	0.37	
14	Tyrosine (isolation and colorimetric)	0.065	0.46
14	Basic N (insoluble phosphotungstates)	0.34	
	Losses in esterification	—	0.68
Total		7.91	4.83

phosphotungstic acid. The remaining 4.83 g. represented incidental losses and the residual fractions of unidentified material in the ester fractions. Although only 18.4 % of the N of the protein was identified as monoamino-acid-N, important information was gained regarding the efficiency of the esterification procedure and the distribution of the amino-acids in the main ester fractions. Attention may be drawn to the following points.

(1) On the basis of the values obtained from hydrolysate 2B, it was estimated that the dicarboxylic acid-N in the material to be esterified was 1.05 g. The total dicarboxylic acid-N traced by titration in fractions 7, 8 and 9 and by separation of the insoluble calcium salts from fractions 10 and 11 was equal to 0.96 g. This would indicate that the values quoted for the dicarboxylic acids are maximum values for the protein.

(2) The total basic N present in the material for esterification was estimated to be 1.21 g. A total of 1.21 g. N was removed subsequently by precipitation with phosphotungstic acid and this N was distributed in approximately equal proportions amongst the residue from distillation of the esters, the esters which were insoluble in ether and the Ba residue remaining after liberation of the esters from their hydrochlorides.

(3) According to Bailey's [1937] determination the total tyrosine-N is equal to 1.57 % of the protein-N. The free tyrosine which separated during concentration of the Cl-free hydrolysate (see Table IV) contained the equivalent of 0.9 % of the protein-N. The greater part of the remainder, equivalent to 0.22 % of the protein-N, was present in the Ba residues (fraction 14).

(4) The total monoamino-acid-N in the material which was esterified was 10.51 g. ($12.77 - (1.05 + 1.21)$). The N remaining in the Ba residue from the second esterification was 1.07 g. and of this 0.34 g. was subsequently proved to be essentially basic N. The actual monoamino-acid-N which remained insoluble in chloroform was equal to 0.73 g. and therefore 93 % of the monoamino-acid-N was obtained in the form of free esters in chloroform solution. The procedures for esterification and liberation of the esters of the monoamino-acids may be accepted without alteration as highly efficient if account is taken of the known difficulties in the esterification of the hydroxy-acids, tyrosine and serine, which have usually been found in the Ba residue after liberation of the esters.

SUMMARY

Investigation of the amino-acid composition of myosin from rabbit's muscle has led to the identification of 72 % of the N of the protein, equivalent to 77 % of the protein by weight. By incorporation of the values obtained by Bailey [1937] for tyrosine, tryptophan, methionine and cystine, the total identified material is raised to 76 % of the N of the protein, equivalent to 85 % of the protein by weight.

The basic amino-acids were determined by the Block [1934] procedure, the dicarboxylic acids by separation of the calcium salts and the monoamino-acids by fractional distillation of the ethyl esters.

In the course of the analysis the following observations relating to analytical procedure were made:

(a) Large proportions of both the basic amino-acids and the dicarboxylic acids were extracted from an acid-free protein hydrolysate by butyl alcohol.

(b) Under the conditions of experiment, the calcium salts were more selective than the barium salts for the separation of the dicarboxylic acids.

(c) Foreman's procedure for esterification and liberation of the free esters of the monoamino-acids was effective in obtaining 93 % of the N in the form of free esters in chloroform solution.

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