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End-Group Assay in some Proteins of the Keratin-Myosin Group

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The fluorodinitrobenzene (FDNB) method for the detection and estimation of amino groups in proteins (Sanger, 1945) has been applied for the most part to proteins of the soluble corpuscular type. The present study extends the technique to the soluble asymmetric proteins tropomyosin, myosin and fibrinogen, which possess a common intramolecular structure similar to that of α-keratin. For reasons which will be discussed later, the results for fibrinogen are still tentative, and most interest centres at present upon tropomyosin and myosin, which are being studied collaterally from a physico-chemical standpoint (Tsao, Bailey & Adair, 1951). The interpretation of the chemical data is indeed very dependent upon other information, the purity of the protein, its particle weight, and especially upon independent evidence for the existence of sub-units. The difficulty of interpretation arises from the fact that tropomyosin and myosin appear to be built of cyclic polypeptide chains, and the small amounts of terminal amino-acids discovered when dinitrophenyl-protein (DNP-protein) is hydrolysed may be due to very tenacious impurities. Sanger's method in fact, if properly applied, is likely to become the most searching of all tests in the determination of protein purity, and its application has so far been too limited to decide the degree of chemical heterogeneity or contamination in other 'pure' proteins.

MATERIALS AND METHODS

Protein preparations

Ox fibrinogen. The fibrinogen was precipitated from fresh citrated ox plasma by the addition of an equal volume of 2M-potassium phosphate buffer, pH 6·6 (Jaques, 1943). It was dissolved and reprecipitated four times, and stored frozen as a solution in 0·25M-phosphate buffer. Fibrin was prepared by diluting with 2 vol. of water and incubating with a trace of purified thrombin for 2 hr. at 25°. The clot was squeezed, dried and ground in ethanol. The amount of non-clottable material was only 5%.

Rabbit myosin. Preparations *SG*1 and *SG*2 (Tables 1 and 3) were the myosin A of Szent-Györgyi (1947) four times precipitated. Preparations *D*1–*D*3 (Table 4) represent a fraction of myosin A precipitating between 40 and 50% saturation with $(NH_4)_2SO_4$ (Dubuisson, 1946, modified by Szent-Györgyi, 1947). Osmotically, this fraction behaves differently from myosin A itself and is thought to be free of actin (Tsao, 1950).

Rabbit tropomyosin. The original method of Bailey (1948) has been only slightly modified. The chief impurity appears to be a colloidal denatured material. Its removal is facilitated by precipitating the tropomyosin from neutral solution with ethanol and drying in ethanol and ether. The protein is then dissolved in water, dialysed against 0·1M-KCl, and the aggregated protein spun down. The ethanol treatment and dialysis are repeated once more, when a smaller amount of impurity is separated, leaving a clear supernatant liquid.

Treatment with fluorodinitrobenzene

Fibrinogen. In some cases the soluble native protein was treated with FDNB under the conditions which Sanger (1945) recommends; in other cases, the protein was precipitated with ethanol, dried and ground before treatment with the reagent. Such preparations are comparable in physical state with those of fibrin.

Myosin and tropomyosin. To conserve reagent, dilute solutions of these proteins were first treated with M-sodium acetate buffer (pH 5). The precipitated protein was spun down, and brought into solution by addition of solid $NaHCO_3$.

Separation of DNP-amino-acids

General. The DNP-proteins were hydrolysed for various times and under varying conditions to meet the requirements for the detection of the more acid-labile DNP-amino-acids as well as those which are relatively stable, as described by Porter & Sanger (1948). The ether-soluble DNP-acids were first investigated on silica gel-$CHCl_3$ columns, which separate the fast-moving artifacts such as 2:4-dinitroaniline. Characterization of discrete bands on these chromatograms was carried out on other silica columns using a variety of solvents as ambient phase (Sanger, 1945).

ϵ-*N*-DNP-lysine, which remains in the aqueous phase after shaking out the ether-soluble DNP-acids, was quantitatively estimated by evaporating down the solution, extracting with methyl ethyl ketone (66% by vol.)-ether, and passing through a column of an acid gel (1 part silica to 1 part N-HCl), on which the compound is more stable than on neutral gel (Porter, 1950). It was estimated spectrophotometrically at 390 mμ. after dissolving in N-HCl. Ether-soluble DNP-amino-acids, on the other hand, were first dissolved in 1% (w/v) aqueous $NaHCO_3$ and read at 350 mμ. (see Sanger, 1949).

Identification of α-N-*DNP-arginine in the water-soluble fraction.* DNP-arginine and bis-DNP-histidine, if present, would remain with ϵ-*N*-DNP-lysine in the aqueous phase. With methyl ethyl ketone-ether, the band rates of the lysine and arginine derivatives are rather close, so that the latter is likely to be masked by the former which is usually present in large amounts. It was thought that a better separation of these two components might be achieved by suppressing the dissociation of the lysine α-amino group. This was achieved by the incorporation of formaldehyde into the silica gel: 100 g. silica (9% moisture) were mixed with 40 ml. of 1:9 (by vol.) B.P. formaldehyde-water, first neutralized to pH 7 with 0·5M-Na_2HPO_4. ϵ-*N*-DNP-lysine, dissolved in methyl ethyl ketone-ether, at first penetrates the gel slowly, but as it reacts with formaldehyde, proceeds to run fast: *R* of ϵ-*N*-DNP-lysine and bis-DNP-histidine >1; DNP-arginine, 0·3. An amount of α-DNP-arginine just visible by itself on a column can be recovered after mixing with 50 mol. of ϵ-*N*-DNP-lysine.

Amide determinations

Air-equilibrated DNP-proteins generally contain about 70% of protein, when allowance is made both for water and DNP content. When the absolute protein weight had to be known accurately, as in the estimation of ϵ-*N*-DNP-lysine, the amide N of the sample was determined and compared with that of the pure protein. The general procedure was similar to that of Bailey (1937).

RESULTS

The reactivity of lysine ϵ-N-*amino groups*

Porter (1948) has reported that a fraction of the lysine side chains in some proteins is unreactive unless the protein is first denatured. On the other hand, the reactivity of terminal amino groups is the same in native proteins as in the denatured. The amounts of ϵ-*N*-DNP-lysine found in fibrinogen, fibrin, tropomyosin and myosin are listed in Table 1. In the case of fibrinogen and fibrin, whatever the pretreatment of the protein before reaction with FDNB, the amount of lysine determined is less than that found on complete hydrolysis with acid. The interesting feature is that the reactive side chains are consistently fewer in fibrin than in fibrinogen, but when the fibrin clot is redissolved in urea and treated with FDNB, the reactivity matches that of fibrinogen. Mihalyi & Lorand (1948) have suggested that amino groups are important in the clotting process, and the present results support the view that the unreactive lysine side chains may play some part in the aggregation of fibrin particles.

Table 1. ϵ-N-*Dinitrophenyllysine from various dinitrophenyl-proteins*

			Lysine N as % of protein N	
Protein	Sample	Conditions before treatment with fluorodinitrobenzene	As ϵ-*N*-DNP-lysine	Other methods of analysis*
Ox fibrinogen	*KB*	Precipitated with ethanol	8·4	9·6–10·3
	KB	8 hr. at 0° in N-HCl	8·4	
Ox fibrin	*KB*	Dried in ethanol	7·3	
Ox fibrinogen	*KB-IMG*	Precipitated with ethanol	8·6, 8·9†	
Ox fibrin	*KB-IMG*	Dried in ethanol	7·7, 7·8†	
	KB-IMG	Dissolved in conc. urea	8·7	
Myosin	*SG*1	Precipitated at pH 5	13·2	13·7
	*SG*1	8 hr. at 0° in N-HCl	14·0	
Tropomyosin		Precipitated at pH 5	17·1, 16·4†	18·0
		Dissolved in guanidine hydrochloride	19·5, 18·6†	

* See Bailey (1944, 1948).
† Each value is derived from the analysis of separately prepared hydrolysates.

The ε-*N*-DNP-lysine found in DNP-myosin, whether this latter is prepared from native or denatured material, agrees with the value obtained by conventional analysis. In tropomyosin, however, full reactivity is attained only in solvents such as guanidine hydrochloride, and some 15 % of the total lysine appears to be unreactive in the native form.

Because of destructive loss on hydrolysis of DNP-protein, and for other reasons, the absolute accuracy of the DNP-method is not very high. Duplicate analyses on the same hydrolysate, using the same gel, will agree within 5 %. In assessing differences of reactivity such as those reported above, it is important to hydrolyse the proteins side by side and to perform all other operations in parallel. Differences of 15 % are then significant.

Water-soluble DNP-amino-acids other than ε-N-DNP-lysine

In general, the water-soluble DNP-acids were dissolved in methyl ethyl ketone-ether and run on two types of column: (*a*) the acid silica column on which bis-DNP-histidine travels fast, and (*b*) on the silica-formaldehyde column upon which only DNP-arginine runs slowly. Neither histidine nor arginine has been detected as terminal group in myosin and tropomyosin; the investigation of fibrinogen has been deferred.

Ether-soluble DNP-amino-acids

Fibrinogen. Making an approximate correction for hydrolytic destruction, fibrinogen contains one terminal residue of tyrosine/200,000 g. of protein and one of glutamic acid in about 300,000 g. of protein. It is not known whether the fibrinogen molecule, which has a molecular weight of about 500,000 (Adair, see Bailey, 1944; Edsall, Foster & Scheinberg, 1947), contains sub-units to which these terminal residues could be assigned. Moreover, Morrison, Edsall & Miller (1948) have shown that fibrinogen may be contaminated with the so-called 'cold-insoluble globulin', whose relation to fibrinogen remains somewhat obscure. The studies of Abrams, Cohen & Meyer (1949) strongly suggest that the cryoglobulin component arises from pathological plasma which may fortuitously have been admitted into the pooled samples of normal plasma. In the light of these considerations, it would be unwise to interpret the results at the present time.

Tropomyosin. The physico-chemical evidence presented in the accompanying paper (Tsao *et al.* 1951) suggests that the limiting value of the particle weight of tropomyosin, whether native or in depolymerizing solvents, is 53,000; it suggests moreover, almost unambiguously, that the molecule consists of two chains lying side by side in the α-configuration. The results of end-group assay, shown in Table 2, support the idea that the double chain is in fact part of one single continuous polypeptide containing no end groups, i.e. the unit is a cyclic polypeptide.

In impure specimens of tropomyosin, containing visible colloidal material (see p. 23) at least four types of terminal residue occur: one gives an uncharacterized band which is stationary on chloroform gel; there is another band consisting almost entirely of bis-DNP-lysine; finally, there is a faster band consisting mostly of DNP-valine and a little DNP-alanine. In tropomyosin preparations, which have been considered pure by physical tests, the first two bands almost disappear and the valine band remains the strongest. The total colour, however, represents only one terminal residue in $40-50 \times 10^4$ g. of protein, or one residue in 9 mol. of tropomyosin. Appropriate tests for acid-labile DNP-acids such as glycine and proline have not revealed bands which are different, qualitatively or quantitatively, from those in Table 2. It seems probable, therefore, that the ether-soluble DNP-amino-acids arise from the impurities common to the obviously impure and the 'pure' preparations. If these impurities contained terminal residues to the extent found in insulin, they would amount to a total impurity of less than 1 %.

Table 2 also shows that no peptide bonds are split when tropomyosin is kept at pH 12 and 0° for 14 days, and the same is true with acid conditions at pH 2.

Table 2. *Ether-soluble dinitrophenyl-amino-acids from tropomyosin*

Band character on $CHCl_3$ column	Estimated breakdown (18 hr. hydrolysis)* (%)	Estimated weight of protein containing 1 mol. α-DNP-amino-acid after correction for hydrolytic breakdown			
		Impure tropomyosin 1	Impure tropomyosin 2	Pure tropomyosin	Tropomyosin after 14 days at 0°, pH 12
Stationary	40	320,000	350,000	1,710,000	1,630,000
R 0·2 (bis-DNP-lysine)	10	1,070,000	1,160,000	2,110,000	1,730,000
R 0·5 (DNP-valine, DNP-alanine)	30	290,000	290,000	700,000	1,070,000
Total		112,000†	139,000	403,000	470,000

* Cf. Porter & Sanger (1948).

† Identical recovery on the $CHCl_3$-buffer *B* column of Blackburn (1949).

Table 3. *Ether-soluble dinitrophenyl-amino-acids from myosin*

(Figures in parenthesis are the estimated percentage breakdown appropriate to the conditions of hydrolysis (see Porter & Sanger, 1948).)

	Estimated weight of protein containing 1 mol. DNP-amino-acid after correction for hydrolytic breakdown		
Band character on $CHCl_3$ column	Myosin *SG*1 (16 hr. hydrolysis)	Myosin *SG*1 (4 hr. hydrolysis)	Myosin *SG*1 (hydrolysis in sealed tube)
Stationary and slow (*R* 0·08)	1,120,000 (40)	3,570,000 (nil)	960,000 (30)
R 0·2 (mainly bis-DNP-lysine)	4,900,000 (10)	6,270,000 (nil)	2,400,000 (30)
R 0·4 (DNP-alanine, DNP-valine)	4,100,000 (30)	2,610,000 (nil)	2,400,000 (20)
Total	740,000	1,200,000	535,000

Myosin. Like tropomyosin, DNP-myosin gives only traces of ether-soluble DNP-acids (Table 3). Again there is an unidentified band stationary on chloroform columns, a band of bis-DNP-lysine and one consisting mainly of DNP-alanine with a little DNP-valine.

Portzehl & Weber (see Weber, 1950) have recently reported that the particle weight of myosin from sedimentation-diffusion and also from osmotic pressure measurements is $8{\cdot}3 \times 10^5$. The number of end groups discovered, supposing them to be homogeneous, amount to no more than one residue/mol. For our present purpose, however, the particle weight is less significant than that of the sub-units of which myosin is composed. Weber & Stöver (1933) were the first to investigate the depolymerization of myosin (actually actomyosin) in urea. More recently, Snellman & Erdös (1948) found that when myosin was first dissolved in urea, the solution remained monodisperse although the molecules became more symmetrical. On standing for 7 days, material of lower sedimentation constant appeared and the system became polydisperse. This fragmentation process has been re-investigated recently in our laboratory by Mr T.-C. Tsao, not only in urea but in acid (pH 2) and alkali (pH 9–13). Although we have no information on the polydispersity of the system under these varying conditions, it is assumed, by analogy with the experiments of Snellman & Erdös, that material of high molecular weight is still present. On this basis, the sub-unit weight must be less than the number-average value of 160,000 which Tsao has obtained in alkali at pH 10·5–11. At this pH, no hydrolysis of peptide bonds occurs; at pH 13, the average particle weight falls to quite low values, but here peptide cleavage is readily detectable (Table 4). Amongst the residues with free amino groups liberated under these conditions are threonine, serine and the dicarboxylic acids.

Table 4. *Ether-soluble dinitrophenyl-amino-acids on myosins subjected to alkaline conditions before treatment with fluorodinitrobenzene*

Sample		Estimated weight of protein containing 1 mol. DNP-amino-acid after correction for hydrolytic breakdown
Myosin *D*1*	Treated at pH 13 for 26 days at 0°	156,000
Myosin *D*2	Native protein	420,000
	Treated pH 10·5–11 for 10 days at 0°	520,000
Myosin *D*3	Native protein	360,000
	Treated at pH 13 for 30 days at 0°	37,000

* No control on the native protein.

It is not impossible that the true sub-unit weight of myosin is commensurate with that of tropomyosin, and the arguments adduced for the existence of cyclic polypeptide chains in tropomyosin are relevant for myosin also. In both cases, the results are only interpretable on these lines.

Terminal residues of other proteins. The adequacy of the author's technique in relation to that of other workers was tested on two proteins, horse globin and ovalbumin. For the former, Porter & Sanger (1948) found six valine residues/mol. of 64,000, and Desnuelle, Rovery & Bonjour (1950) only five. In ovalbumin, Desnuelle & Casal (1948) found no terminal amino groups. In the author's hands, horse globin (one determination) gave 5·5 valine residues/mol. Twice recrystallized ovalbumin (American source) was found to contain several kinds of terminal residues, amounting in all to one in 260,000 g. of protein. A sample prepared by the author, four times recrystallized, gave a mere trace of colour on the chloroform column, amounting only to one residue in 5×10^6 g. of protein. No acid-labile DNP-amino-acids could be detected.

DISCUSSION

The absence of terminal residues except in traces in tropomyosin and myosin suggests that these proteins are built of cyclic polypeptide units. By virtue of these negative findings extra care has been taken to ensure that amino-acids which give rise to the

more labile DNP derivatives are not present. The possibility of cysteine as an end group was also considered, for this acid was not investigated in Sanger's original paper. *NS* bis-DNP-cysteine was prepared and found to be as stable to acid as DNP-alanine and to run with an *R* value of 0·1 on a chloroform column. This acid would thus have been readily detected.

The precedent for the existence of cyclic chains in proteins is to be found in gramicidin S (Sanger, 1946), which is much simpler in structure than anything investigated here. It is not known whether ovalbumin is composed of cyclopeptides, or whether the terminal amino groups are linked to the carbohydrate residues. Such an explanation cannot hold, however, for myosin and tropomyosin in which prosthetic groups are entirely absent. It is encouraging to find that in the case of tropomyosin at least, the concept of a cyclopeptide helps to explain features which would otherwise be puzzling. These aspects are considered in the following paper (Tsao *et al.* 1951).

SUMMARY

1. Sanger's (1945) fluorodinitrobenzene method has been applied to a study of the terminal amino groups in tropomyosin, myosin and fibrinogen.

2. Tropomyosin and myosin appear to consist of cyclopeptides. It is not yet possible to decide whether the terminal residues in fibrinogen arise from the protein itself or from impurities.

3. The lysine side chains in myosin are fully reactive. There is evidence that tropomyosin contains a small proportion of unreactive lysine side chains, and the same is true of fibrin but not of fibrinogen.

4. The end-group method has been used to investigate the stability of the peptide bonds of these proteins in acid and alkaline media. Myosin at 0° is stable to alkali at pH 11 but not at pH 13; tropomyosin is stable up to pH 12.

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The Size, Shape and Aggregation of Tropomyosin Particles

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Much of the recent work on the proteins of the myofibril has been devoted to the two major components, actin and myosin. The study of the interaction of these two proteins with each other and with adenosinetriphosphate has led to the belief that this system contains the essential components of the contractile mechanism. A knowledge of the properties of the proteins concerned must be of significance in the interpretation of the process of contraction and, for this reason, we have attempted to define the structure of one of the simpler asymmetric proteins of the myofibril, tropomyosin (Bailey, 1948).

Previous investigations (Bailey, 1948; Bailey, Gutfreund & Ogston, 1948; Astbury, Reed & Spark, 1948) have suggested that tropomyosin is a proto-