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Isolation and Partial Characterization of Methylated Arginines from the Encephalitogenic Basic Protein of Myelin

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Two methylated derivatives of arginine were isolated from the encephalitogenic protein of myelin from the central nervous system. Evidence is presented for the proposed structures, ω-*NN'*-dimethylarginine and ω-*N*-monomethylarginine. In the encephalitogenic protein from human brain the proportion 1:6:10 for arginine:monomethylarginine:dimethylarginine residues was found to occur at position 107. Possible roles for the methylated arginine in conformational changes and altered ion-exchange behaviour are discussed.

During sequence studies on the encephalitogenic protein from human brain, two unidentified amino acids were observed at residue 107 (Carnegie, 1971). Their elution volumes from a sulphonated polystyrene resin indicated that they might be methylated derivatives of a basic amino acid.

Several such derivatives have been isolated from proteins in the last decade. ϵ-*N*-Methyl-lysine was first found in bacterial flagellar protein (Ambler & Rees, 1959), ϵ-*NN*-dimethyl-lysine in calf thymus histones (Paik & Kim, 1967), ϵ-*NNN*-trimethyllysine in wheat-germ cytochrome *c* (DeLange, Glazer & Smith, 1969), and 3-methylhistidine in rabbit muscle actin (Asatoor & Armstrong, 1967). None of these methylated amino acids had the same elution volumes as the unidentified amino acids from the encephalitogenic protein. An enzyme that transfers methyl residues from *S*-adenosylmethionine to arginine in histones has been studied by Paik & Kim (1968, 1969, 1970). The methylated arginines had similar elution volumes to our unidentified amino acids. An enzyme has been shown to transfer methyl groups to only one of 19 arginine residues in the human encephalitogenic protein (Baldwin & Carnegie, 1971). Eylar (1970) is studying a modified arginine residue at position 107 in the bovine protein.

The present paper reports the isolation and some properties of methylated arginines from the encephalitogenic basic protein from bovine spinal cord and the proportion of these amino acids in the human protein. Bovine spinal cord was used for the isolation because it was more readily available in quantity.

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MATERIALS

Ion-exchange resins Amberlite IR120 (Mallinckrodt, St Louis, Mo., U.S.A.) and Bio-Rad AG 50W (X2; Bio-Rad, Richmond, Calif., U.S.A.) were used. Authentic samples of the following compounds were obtained as follows: methylamine and dimethylamine, British Drug Houses Ltd., Poole, Dorset, U.K.; monomethylguanidine, Sigma, St Louis, Mo., U.S.A.; ornithine, Calbiochem., Los Angeles, Calif., U.S.A.; guanidine, Mann Research Laboratories, New York, N.Y., U.S.A.; dimethylurea from Dr Casey, Department of Chemistry, University of Melbourne; monomethylurea, Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Preparation and hydrolysis of 'basic fraction' from bovine spinal cord. The preparation of 'basic fraction', consisting of encephalitogenic basic protein and polypeptides derived from it, was based on the method of Carnegie, Bencina & Lamoureux (1967).

Fresh bovine spinal cord (1.5kg) was stripped of meninges and homogenized in 3vol. of acetone at 4°C. The residue after filtration was resuspended in 3 litres of light petroleum (b.p. 60–80°C), and left for 1h. The solid obtained on centrifugation was left overnight at 4°C in 5 litres of acetone.

After separation by centrifugation the residue was suspended in 3.5 litres of 0.02M-HCl, the pH adjusted to 2.5 and the suspension was thoroughly mixed. The suspension was left for 10min before the pH was again adjusted to 2.5. After centrifugation the residue was re-extracted in the same way. The pH of the combined extracts was adjusted to 5 with NaOH, and $(NH_4)_2SO_4$ was added to 85% saturation. The precipitate of 'basic fraction' was refluxed in 3 litres of 8M-HCl for 24h at 110°C.

Isolation of basic amino acids X and Y. The solution was evaporated to dryness on a rotary evaporator. Solid was dissolved in 500ml of water and adjusted to pH1 with HCl and the solution was applied to a column (450g; 100cm×3.0cm diam.) of Amberlite IR120 (H^+ form). Amino acids were eluted with 2M-NH_3 at a flow rate

of 3ml/min, fractions being collected every 5min. Electrophoresis (Table 1) was used to localize the basic amino acids X and Y and the fractions containing them were pooled.

After evaporation to dryness the pooled fractions were re-dissolved in 0.2M-pyridine–acetate buffer, pH3.5, and applied to a column (90cm×1.5cm diam.) of Bio-Rad AG 50W (X2; H^+ form, 50g). A gradient of pyridine–acetate from pH3.5, 0.2M, to pH5.0, 2.0M was applied to the column via a constant-volume (100ml) mixing chamber. The flow rate was 0.8ml/min and fractions were collected every 5min. Regions containing X and Y were localized by electrophoresis. Appropriate fractions were pooled and purified by electrophoresis on Whatman 3MM paper in formic acid–acetic acid at pH2.1 (Table 1). The amino acids were eluted with 0.01M-NH_3 and evaporated to dryness with a rotary evaporator. Further purification was achieved by chromatography on Whatman 3MM paper with butan-1-ol–acetic acid–water (12:3:5, by vol.) for 40h.

Alkaline hydrolysis. A plastic tube containing the amino acid (50nmol) was inserted into a Pyrex test tube (6in×1in) containing 0.5ml of 1M-H_2SO_4; 0.2ml of 1M-NaOH was added to the inner plastic tube and the Pyrex tube immediately sealed. After heating at 105°C for 24 or 72h, the outer tube was opened. The acid and alkaline solutions were applied separately to a Technicon amino acid analyser, after addition of 100nmol of norleucine to each as an internal standard.

Amino acid analysis, digestion and isolation of peptides. The techniques used were described in the preceding paper (Carnegie, 1971) and in Table 2. Standard solutions of ornithine and methylamine were used to obtain colour factors for the reaction of these compounds with ninhydrin. Ion-exchange chromatography has been used in the analysis of methylamine by Hatano, Sumizu, Rokushika & Murakami (1970).

Electrophoresis of DNS derivatives. The DNS derivatives of X, Y and arginine were subjected to electrophoresis at pH12.7 (Gray, 1967) and pH10.6 (Martenson, Deibler & Kies, 1969) on Whatman 3MM paper on a cold-plate apparatus at 16V/cm and 2°C for 3h. *N*-2,4-Dinitrophenylethanolamine was used as a marker of electroendosmosis (Carnegie & Synge, 1961).

RESULTS AND DISCUSSION

Isolation and properties of basic amino acids X and Y. The acid hydrolysate of the 'basic fraction' (Carnegie *et al.* 1967) was fractionated on ion-exchange columns of sulphonated polystyrene resin to remove neutral and acidic amino acids. Spots for compounds X and Y were obtained in the zone between histidine and arginine and were further purified by electrophoresis at pH2.1 and by chromatography in butan-1-ol–acetic acid–water. The mobilities and R_F values of compounds X and Y are shown in Table 1. Compounds X and Y were eluted from a Technicon C2 resin by using the standard gradient, with elution volumes 0.92 and 0.97 respectively that of arginine (Table 2). In the calculation of the yields of compound X and Y the colour factor for their reaction with ninhydrin was assumed to be the same as for arginine. The final products were not contaminated by other amino acids. The yield of compound X was 0.35mg/kg of spinal cord and of compound Y 0.29mg/kg of spinal cord.

Evidence for structures of compounds X and Y. Yields of the products of alkaline hydrolysis of compounds X and Y are presented in Table 3. Methylamine and ornithine were identified by their

Table 1. *Chromatography of compounds X and Y in butan-1-ol–acetic acid–water and electrophoresis at* pH2.1

The solvent was butan-1-ol–acetic acid–water (12:3:5, by vol.) with Whatman no. 1 paper for 31h at 20°C. Electrophoresis was on Whatman no. 54 paper with acetic acid–formic acid–water (4:1:45, by vol.), pH2.1, at 3kV for 45min at 20°C. m_{Lys}, mobility from point of application relative to lysine.

Compound	R_F	R_M*	ΔR_M	m_{Lys}
Arginine	0.12	0.87		0.92
			0.08	
Y (Monomethylarginine)	0.14	0.79		0.87
			0.10	
X (Dimethylarginine)	0.17	0.69		0.83
Guanidine	0.30	0.37		—
			0.12	
Monomethylguanidine	0.36	0.25		—
Histidine	0.11	0.91		0.92
			0.08	
Monomethylhistidine	0.13	0.83		0.90
Lysine	0.10	0.95		1.00
			0.04	
Monomethyl-lysine	0.11	0.91		0.97

* $R_M = \log(1/R_F - 1)$.

Table 2. *Relative elution volumes of compounds X and Y from amino acid-analysers*

Conditions used for Technicon amino acid analyser were a column (50cm×0.6cm diam.) of C-2 resin, standard 40ml gradient, 60°C and for Beckman amino acid analyser were a column (10.5cm×0.9cm diam.) of Aminex A5 resin with 0.35M-citrate buffer, pH 5.28, at 51°C.

	Technicon analyser	Beckman analyser
X (Dimethylarginine)	0.92	0.90
Y (Monomethylarginine)	0.97	1.00
Arginine	1.00	1.00
Lysine	0.84	0.49
Histidine	0.88	0.56
Ornithine	0.80	—
NH_3	0.74	0.64
Monomethylamine	0.78	—

positions on the amino acid-analyser chromatogram (Table 2). Ornithine was further identified by co-electrophoresis (pH 2.1, 3kV, 45min) and co-chromatography (butan-1-ol–acetic acid–water, 22h) with an authentic sample.

The possible structure for arginine derivatives methylated on the guanidino groups are shown in Scheme 1. Since no trace of δ-*N*-methylornithine was observed by ion-exchange and paper chromatography of the hydrolysates of either compound, structures (II) and (V) may be eliminated.

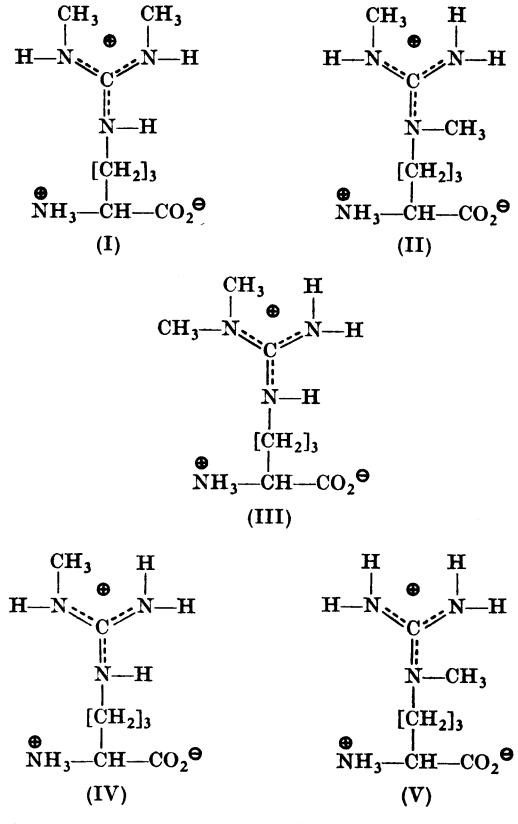
Table 3 shows that 1mol of compound Y yields 1mol of methylamine and 1mol of ornithine. The decrease in yield of the latter compound after 72h hydrolysis is caused by further degradation. Recovery of a standard sample of ornithine after this period was only 66%.

In the 24h hydrolysate of compound X a small peak of an additional ninhydrin-positive compound was observed on the analyser chromatogram with an elution volume 0.46 times that of norleucine. After 72h hydrolysis this peak had disappeared, with a concomitant increase in the yield of methylamine. Thus 1mol of compound X ultimately yields 2mol of methylamine and 1mol of ornithine, if allowance is made for instability of the latter.

The quantitative recovery of 2mol of methylamine from the hydrolysis of 1mol of compound X eliminates structure (III), which would yield 1mol of dimethylamine. It is therefore proposed that compound X is ω-*NN'*-dimethylarginine (structure I), and that compound Y is ω-*N*-monomethylarginine (structure IV).

Additional evidence for the proposed structure of dimethylarginine and monomethylarginine was obtained from the chromatographic behaviour in two solvents, as shown in Tables 1 and 4. R_M values were calculated from these by using the relationship $R_M = \log(1/R_F - 1)$ proposed by Bate-Smith & Westall (1950). For the repeated addition of a single substituent group to any compound, ΔR_M in a given solvent system is theoretically proportional to the number of groups added. The increments in R_M between arginine and monomethylarginine and between mono- and di-methylarginine, are similar in each solvent and are in reasonable agreement with those between other amino acids and their monomethyl derivatives. This suggests that mono- and di-methylarginine are formed by the sequential addition of methyl groups to arginine.

Additional evidence for the presence of methyl groups in compounds X and Y came from the demonstration of specific enzymic methylation of the human basic protein. Baldwin & Carnegie (1971) showed that an arginine methylase from a brain homogenate (Paik & Kim, 1969) transferred [^{14}C]methyl groups from *S*-adenosylmethionine specifically to Arg-107 in the protein. The labelled products in an acid hydrolysate of peptides T23 and P9 (Carnegie, 1971) had the same chromatographic properties as compounds X and Y.



Scheme 1. Possible structures for compounds X and Y.

Table 3. *Products of alkaline hydrolysis of compounds X and Y*

Compound	Amount taken (nmol)	Hydrolysis time (h)	Methylamine (nmol)	Methylamine Yield (%)	Ornithine (nmol)	Ornithine Yield (%)
X	59.8	24	92.1	154.2	51.8	86.6
X	59.8	72	121.7	203.6	40.0	67.0
Y	72.5	24	74.1	102.2	73.8	101.9
Y	72.5	72	69.2	95.4	59.2	81.6

Table 4. *Chromatography of compounds X and Y in phenol–m-cresol–borate*, pH 9.3

The solvent was 88% (w/v) phenol–*m*-cresol–borate, pH 9.3, (38:33:9, by vol.) with Whatman 3MM paper dipped in EDTA (DeLange *et al.* 1969) for 16 h at 20°C.

Compound	R_F	R_M*	ΔR_M
Arginine	0.46	0.07	
			0.59
Y (Monomethylarginine)	0.77	−0.52	
			0.48
X (Dimethylarginine)	0.91	−1.00	
Lysine	0.23	0.53	
			0.68
Monomethyl-lysine	0.59	−0.15	
Histidine	0.53	−0.05	
			1.05
Monomethylhistidine	0.93	−1.10	

* $R_M = \log(1/R_F - 1)$.

The occurrence of two methylated arginine derivatives has been reported (Paik & Kim, 1968, 1970; Kaye & Sheratzky, 1969). The former authors obtained a considerable amount of qualitative evidence on the structure of these compounds by alkaline hydrolysis of a minute amount of samples radioactively labelled in the methyl group.

Although monomethylarginine and dimethylarginine have chromatographic properties (Table 2) on the Beckman amino acid analyser identical with those of the derivatives isolated by Paik & Kim (1970), their assignment of structures differs from ours. The derivative that we postulate to be dimethylarginine they claimed to be ω-*N*-monomethylarginine from the occurrence of methylamine and 'monomethylurea' in its hydrolysate. The derivative that we postulate to be monomethylarginine was claimed by them to be α-*N*-ω-*N*-dimethylarginine from the occurrence of methylamine in its hydrolysate, its resistance to the action of snake venom L-amino acid oxidase and its apparent failure to form a dinitrophenyl derivative. However, we obtained dinitrophenyl and DNS-derivatives of both compounds and in any case it is unlikely that an α-*N*-methyl-amino acid would occur within a protein chain. Paik & Kim (1968, 1970) made no attempt to determine the absolute amounts of methylamine formed from either compound. In addition, because they were only able to study the labelled products, ornithine could not be identified.

During the present study no evidence was obtained for the formation of urea derivatives as intermediates in the alkaline hydrolysis of mono- and di-methylarginine. In the 24 h hydrolysate of dimethylarginine only, a peak was observed in the citrulline region of the analyser chromatogram. A minor pathway for the alkaline hydrolysis of arginine involves citrulline as an intermediate (Hellerman & Stock, 1938) and a similar situation exists for creatinine (Gaebler, 1926). In addition, authentic samples of monomethylurea and 1,3-dimethylurea gave no reaction with the ninhydrin reagent used. It therefore seems likely that the 'monomethylurea' observed by Paik & Kim (1970) may have been ω-*N*-monomethylcitrulline.

Conclusive identification of monomethylarginine and dimethylarginine must await their synthesis. Although the derivative corresponding to dimethylarginine has been isolated from the hydrolysate of poly-L-arginine treated with [^{14}C]methyl iodide (Paik & Kim, 1970), such a synthesis is not specific. A similar method to that described for δ-*N*-monomethylarginine by Thomas, Kapfhammer & Flaschenträger (1922) is required.

Electrophoresis of monomethylarginine and dimethylarginine. Table 1 shows that the addition of methyl groups caused the methylated amino acids to be retarded at pH 2.1. This is presumably due to their increased size (Offord, 1966). Since there was insufficient material for an accurate determination of the p*K* of the guanidino group of mono- and dimethylarginine, electrophoresis of their DNS derivatives was used to obtain an estimate of their charge relative to that of DNS-arginine. At pH 12.7 (Gray, 1967) all moved towards the anode. The mobilities of DNS-monomethylarginine and DNS-dimethylarginine with respect to DNS-arginine were 0.7 and 0.3 respectively. At pH 10.6 the mobilities of DNS-monomethylarginine and DNS-dimethylarginine were respectively 1.04 and 1.17 relative to DNS-arginine with movement towards the cathode. Because of the increased size due to the methyl groups the difference in mobility at pH 12.7 would possibly be greater, and that at pH 10.6 less, than expected from a consideration of the net charge. It would appear that the methylated guanidino group in monomethylarginine and dimethylarginine had a slightly higher p*K* than that of arginine.

Microheterogeneity in the basic proteins. Martenson *et al.* (1969) showed that although the encephalitogenic proteins from a number of species appeared homogeneous on electrophoresis at an acid or neutral pH, there was an unexplained heterogeneity at pH 10.6. The slight difference in electrophoretic mobility of DNS-dimethylarginine from DNS-monomethylarginine at this pH might offer an explanation for the observed heterogeneity, especially as the p*K* for a guanidino group in this region of the protein is not known. A definitive answer must await the separation of the components observed by Martenson *et al.* (1969).

Proportions of arginine:monomethylarginine: dimethylarginine in the basic protein from human

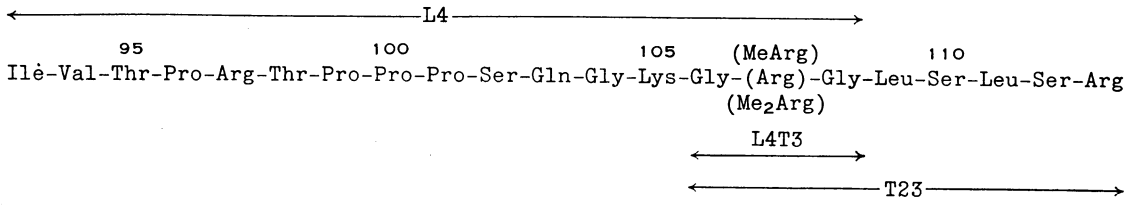


Fig. 1. Amino acid sequence of residues 93–113 in the human encephalitogenic protein. T, Tryptic digestion (monomethylarginine and dimethylarginine are trypsin-resistant). L, Thermolysin digestion.

Table 5. *Amino acid composition of peptides*

	T23	T23L3	T23L3a	T23L3b	L4T3
Gly	2.00	2.00	2.00	2.00	2.00
Arg	1.10	—	—	—	0.07
MeArg	0.24	0.41	0.85	0.13	0.41
Me_2Arg	0.47	0.62	—	0.85	0.65
Leu	1.77	—	—	—	—
Ser	1.97	—	—	—	—
% yield from T23	—	52.1	6.4	11.3	42.5

brain. The sequence of the region surrounding residue 107 in the encephalitogenic protein from human brain is shown in Fig. 1. The peptide T23 Gly-$\binom{\text{MeArg}}{\text{Me}_2\text{Arg}}$-Gly-Leu-Ser-Leu-Ser-Arg was isolated from a tryptic digest of the human encephalitogenic protein (Carnegie, 1971). No arginine was present at residue 107 because the arginine-containing peptide would have been digested by trypsin. Since the peaks for arginine and for monomethylarginine are not completely resolved by either the Technicon or the Beckman analysers, the mono-/di-methylarginine ratio could not be determined with sufficient accuracy by hydrolysis of this peptide. The *C*-terminal region was therefore removed by digestion with thermolysin and the resultant peptides separated by electrophoresis at pH 6.5. The mono-/di-methylarginine ratio in the band T23L3, which contained Gly-MeArg-Gly and Gly-Me_2Arg-Gly, was 1:1.5.

To determine the quantity of arginine at residue 107, peptide L4 (Carnegie, 1971) from a thermolysin digest of the whole protein, was digested with trypsin. The band L4T3, which contained Gly-Arg, Gly-MeArg-Gly and Gly-Me_2Arg-Gly, was isolated by electrophoresis at pH 6.5. By using the previously determined mono-/di-methylarginine ratio, the proportion of arginine to monomethylarginine to dimethylarginine at position 107 was then calculated to be approx. 1:6:10.

Monomethylarginine was shown to be actually present in the protein and not a product of acid hydrolysis of dimethylarginine by separating the peptides T23L3a (Gly-MeArg-Gly) and T23L3b (Gly-Me_2Arg-Gly) by chromatography in butan-1-ol–acetic acid–water for 30h. The compositions of all of the above peptides are given in Table 5. In addition, monomethylarginine and dimethylarginine were released from peptide T23 when it was digested with aminopeptidase O as described in the preceding paper (Carnegie, 1971).

Possible role of methylated arginines in myelin. Although it is clear that methylation of basic amino acids in proteins occurs by enzymic transfer of methyl groups to specific residues in proteins (reviewed by Baldwin & Carnegie, 1971), the function and metabolic significance of these methylated amino acids remains a puzzle. Baldwin & Carnegie (1971) have suggested that the methylation of arginine in the basic protein might aid in the transfer of this region of the protein into the hydrophobic environment in myelin and that the presence of dimethylated arginine is essential for the correct molecular structure of myelin. We have predicted (Baldwin & Carnegie, 1971) that in the disease of the central nervous system 'subacute-combined degeneration', which occurs as a result of deficiency of vitamin B_{12}, the characteristically 'spongy' myelin will be found to yield basic protein deficient in dimethylarginine.

The methylated arginine may also be involved in changing the electrostatic interaction with acidic groups of phospholipids. The elution volume of the methylated arginines from the sulphonated polystyrene resins is the opposite of that expected

from a comparison with other amino acids. Normally an increase in pK and addition of $-CH_2-$ groups causes amino acids to be retarded. However, instead of appearing after arginine, dimethylarginine and monomethylarginine were eluted before arginine. A possible explanation for this paradox could come from a consideration of ion-association complexes (Rice & Nagasawa, 1961). Bolto *et al.* (1968) have shown that if non-polar groups are placed in close proximity to a charged group the formation of ion-association complexes is promoted. Thus dimethylarginine would be expected to have a greater tendency than monomethylarginine to form complexes with the citrate counter ions, which would result in a decrease in the amount of ionized dimethylarginine available to exchange with the sulphonated polystyrene. If this were the case it would account for the observed order of elution dimethylarginine, monomethylarginine then arginine. Alternatively steric hindrance due to the methyl groups could disturb the ion-exchange equilibria with the resin. Since arginine methylase is found in various tissues (Paik & Kim, 1969), methylarginine may be present in other membrane proteins and be involved in alteration of conformation and ion-exchange properties.

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