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OX PERIPHERAL NERVE MYELIN MEMBRANE. PURIFICATION AND PARTIAL CHARACTERIZATION OF TWO BASIC PROTEINS

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SUMMARY

Two basic proteins were purified from the peripheral nervous system. The isolation was achieved by (1) delipidation with chloroform–butanol mixtures, dry acetone, and dry ether, (2) acid extraction at pH 2 and then (3) dialysis against distilled water, lyophilization, and solubilization in pH-10.7 buffer. Further purification steps included: (4) ion exchange chromatography on QAE-Sephadex G-25 and (5) gel filtration on Sephadex G-75. Each of the two basic proteins was shown to be homogeneous by disc electrophoresis at pH 2.0, 4.3, and 9.2.

The acid extractable proteins of an isolated fraction from peripheral nerve were studied by using gel filtration and disc electrophoresis techniques. Two basic proteins were also recovered in this case. These proteins gave the same elution volumes and the same electrophoretic mobility as the basic proteins recovered from peripheral nerve. Myelin was extracted with butanol and 72–86 % of the myelin proteins were recovered as soluble lipoproteins. They were compared to the acid extractable proteins.

Some properties of the proteins purified from peripheral nerve were determined. (1) Molecular weights of the two basic proteins were estimated by gel filtration and disc electrophoresis. The main basic protein was found to have a molecular weight of 12300 ± 600. The other basic protein was found to have a molecular weight of 14200 ± 600. (2) The amino acid composition of the two basic proteins were similar to each other but different from the amino acid composition reported of the basic proteins from central nervous system studied by Dawson[14]. No free N-terminal amino acids were detected in either of the two basic proteins.

INTRODUCTION

Differences between the myelin membrane of peripheral nervous system and the central nervous system have been found in the embryological origin and in staining properties[1]. Differences in lipid composition[2,3] protein composition[1,4–7], and solubility properties[2,3] have also been found. Extensive studies have dealt with the characterization and structure of the basic proteins of the central nervous system and their role in autoallergic encephalitis. The reports on peripheral nerve proteins are scarce[5,6,8]. This is mainly due to the difficulties in obtaining pure myelin from this tissue.

In this paper we describe the extraction, purification, and partial characterization of the basic proteins derived from ox peripheral nerve myelin. These proteins

have been found to be markedly different from the basic proteins isolated from central nerve myelin[14].

MATERIALS AND METHODS

Materials

Sephadex G-200, G-100 and G-75, SE-Sephadex A-25, QAE-Sephadex A-25, and Dextran blue 2000 were purchased from Pharmacia. Egg lysozyme (crystalline) and horse cytochrome *c* were purchased from Fluka. Bovine hemoglobin and bovine pancreatic ribonuclease were purchased from Koch-Light Laboratory, England. Sperm whale myoglobin (crystalline) came from Mann Research laboratory, trypsin (3 times crystallized) from Fluka, and dithiothreitol from Cal. Biochem. Phospholipase A from porcine pancreas was kindly provided by Dr. G. H. de Haas of this laboratory. The solvents and reagents were all of analytical grade.

Methods

Isolation of myelin. Fresh bovine spinal cords were obtained from mature animals at a local slaughter house. Intradural spinal roots were dissected from the caudal portions of each spinal cord less than one hour after the death of the animal. Usually the preparation of myelin was carried out immediately after the tissue was obtained. When the tissue was stored first, it was kept at a temperature of $-20°$ prior to use. Myelin was isolated by a modification of the procedure of AUTILIO *et al.*[23] as proposed by O'BRIEN *et al.*[2] and WOLFGRAM AND KOTORII[5]. All manipulations were carried out at a temperature of 0°.

The extraction of peripheral whole nerves. The peripheral nervous system tissue was delipidated according to MARTENSON *et al*[24] with chloroform–methanol (2:1, v/v) or according to VERGER *et* AL.[25] with chloroform–butanol mixtures, acetone, and ether. The dry powder was homogenized and extracted for 20 min with ice-cold water and the pellet was then recovered after centrifugation for 10 min at 40000 × *g* at 0°. The water soluble proteins were discarded. The pellet was reextracted four times, each time for one hour with ice cold solutions of 0.06 M HCl containing NaCl (0.2 M). Routinely 5 g of powder were extracted each time with 50 ml of the acidic solution. The supernatants were isolated by centrifugation and pooled. Part of the acidic extract was concentrated by microfiltration and applied to Sephadex columns. The main part of the acidic extract was dialysed against water at 4° in preswollen dialysis tubes and lyophilized. Samples from the various fractions were run in disc electrophoresis.

The extraction of peripheral nerve myelin proteins. The peripheral nerve myelin preparation was delipidated according to VERGER *et al.*[15] and the dry powder was extracted at acidic pH by the same method used for peripheral nerves. The extracted proteins were applied to a Sephadex G-200 column and samples from the different fractions were run in disc electrophoresis.

Butanol extraction. The isolated myelin fraction was washed several times with 0.02 M hypotonic phosphate buffer, pH 7.4, 0.1 mM EDTA. The resultant pellet was suspended in the same buffer and was frozen overnight at $-15°$. The next day the myelin suspension was submitted to butanol extraction[26]. The water phase was recovered and dialysed against three changes of cold 1 mM carbonate–bicarbonate buffer,

pH 10.7. The water soluble proteins were studied by disc electrophoresis. Protein concentrations were determined according to LOWRY *et al.*[27] and total phosphate concentrations according to BÖTTCHER *et al.*[28].

Column chromatography. All column material was swelled in the elution buffer and the columns were eluted before use with at least 10 vol. of buffer. The chromatography was performed at 4° and the eluted fractions were monitored at 280 nm and disc electrophoresis. Sephadex G-200 columns were employed to study the total acid extractable proteins. Sephadex G-100 and G-75 columns were utilized for molecular weight estimations of the basic proteins and for final purification of these proteins. QAE-Sephadex A-25 and SE-Sephadex A-25 ion exchanges were utilized for purification of the basic proteins. The SE-Sephadex A-25 was equilibrated with 0.05 M sodium acetate buffers, pH 4.6. The proteins were eluted with a linear gradient of NaCl ranging from 0 to 1.0 M NaCl. The total volume of the gradient was 800 ml.

Disc electrophoresis. Acrylamide electrophoresis was carried out as described by TAKAYAMA[29] at pH 2.0. Disc electrophoresis was also carried out at pH 4.3 with β-alanine buffer towards the anode and at pH 9.2 using Tris–glycine buffer towards the anode and cathode. The gels were fixed with 7 % acetic acid, stained with 1 % solutions of amido black in 7 % acetic acid. Gels were destained electrically or by diffusion.

Molecular weight estimations. Molecular weights of the basic proteins were estimated from their elution volumes (V_e) on Sephadex G-100 columns calibrated with Dextran blue 2000(V_0) and with reduced proteins of known molecular weights, according to DEIBLER *et al.*[30]. 10 mg of each protein was applied to the column and eluted with 0.01 M HCl at 4°. Molecular weights were estimated by acrylamide electrophoresis according to PARISH AND MARCHALONIS[31]. Disc electrophoresis was done according to the procedure of TAKAYAMA[29].

Amino acid analysis. Determinations of the amino acid composition of the purified proteins was carried out on a Spinco Model 120B amino acid analyzer. 5 mg of protein was hydrolyzed with 6 M HCl in sealed evacuated tubes for 24, 48, and 72 h at 110°, according to SPACKMAN *et al.*[32]. Corrections were made for losses of threonine and serine. Trytophan was determined according to GAITONDE AND DOVEY[33].

N-terminal determinations. Determinations of N-terminal amino acids were carried out according to the dansylation method of GROS AND LUBOUESSE[34] and with the dinitrophenyl method of PORTER AND SANGER[35]. Phospholipase A from porcine pancreas and cytochrome *c* (horse heart, Type III) were used as controls.

RESULTS

The acid extractable proteins recovered from peripheral nerve

The recovery of proteins from delipidated nerves extracted with 0.06 M HCl, 0.2 M NaCl was usually 12 mg/g of fresh tissue. When the total acidic extract was subjected to gel filtration on Sephadex G-200 in 0.01 M HCl (Fig. 1), part of it was excluded by the gel. This fraction contained lipoproteins and was opalescent (Fig. 2a). Fractions 2 and 3 (Figs. 2b and 2c) contained proteins which were later found not to be present in the myelin preparation. Fraction 4 was found to contain basic proteins (Fig. 2e) which came together with a compound containing non-phospholipid phosphorus and sugar (Fraction 6) which was not analysed further. When Fraction 4,

which was recovered from Sephadex G-200, was applied to Sephadex G-100, the basic proteins were eluted in an unsymmetrical peak. It was divided into two portions, P_1 and P_2 (Fig. 3). P_1 contained a basic protein which moved differently on disc-eletrophoresis than the one found in P_2. These two proteins could be purified by rechromatography on Sephadex G-75.

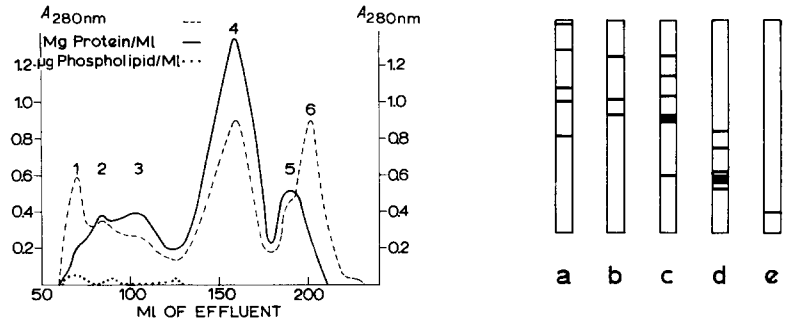


Fig. 1. Gel filtration of the acid extractable proteins of peripheral nerves. 50 mg of protein were applied to a 1.5 cm × 100 cm column of Sephadex G-200 in 0.01 M HCl at 4°. The flow rate was 20 ml/h. 4-ml fractions were collected.

Fig. 2. Disc electrophoresis patterns of the different fractions of the acid extractable proteins of the peripheral nerves recovered after gel filtration on Sephadex G-200 (Fig. 1). a Fraction 1; b Fraction 2; c Fraction 3; d Fraction 4; e Fraction 5.

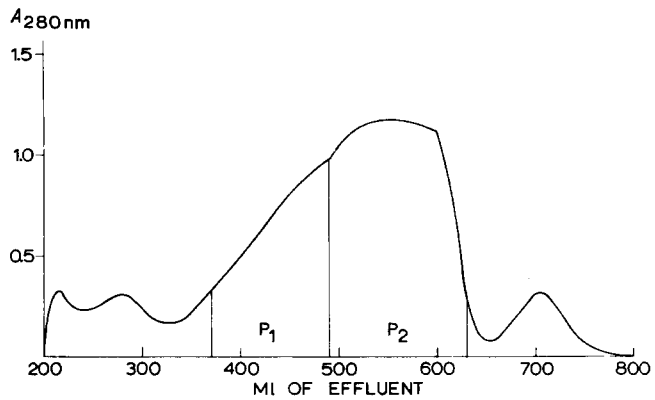


Fig. 3. Preparative gel filtration on Sephadex G-100 of the basic proteins recovered after Sephadex G-100 gel filtration (Peak 4, Fig. 1). 400 mg of protein were applied to a 2.5 cm × 135 cm column of Sephadex G-100 in 0.01 M HCl at 4°. The flow rate was 15 ml/h and 4-ml fractions were collected.

Purification of the two basic proteins from peripheral nerve. The acid extractable proteins were dissolved in 0.04 M carbonate–bicarbonate buffer pH 10.7 and applied to QAE-Sephadex A-25 column (see METHODS). One basic protein remained unbound tot the ion exchange under the experimental conditions and was eluted in the "dead" volume (Fig. 4). The second protein was eluted with approx. 0.05 M NaCl. Because of cross-contaminations of the two basic proteins, Sephadex G-75 was used for the final purification step. The first protein (P_2) to be eluted from the ion exchanger gave a higher elution volume on Sephadex G-75 than the one which was eluted with the

gradient (P_1). They showed also different electrophoretic mobility (Fig. 5). When the two proteins were chromatographed on a negatively charged ion exchange, SE-Sephadex A-25, they were eluted under the conditions of the experiment (pH 4.6 and a linear gradient of 0–1.0 M NaCl) in an unsymmetrical peak at approximately 0.30 M NaCl.

The acid-extractable proteins recovered from peripheral nerve myelin. When 400

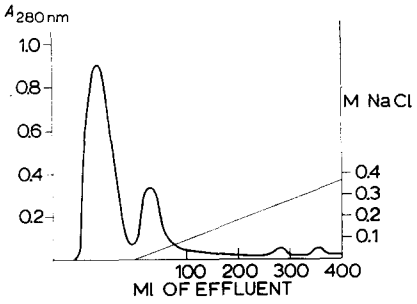


Fig. 4. QAE-Sephadex A-25 ion exchange chromatography of the pH 10.7 soluble proteins of the peripheral nerve acidic extract. 50 mg of protein were applied to a 1.5 cm × 25 cm column of ion exchange and eluted at 4° with a linear gradient of NaCl ranging from 0 to 0.4 M NaCl in 0.04 M carbonate–bicarbonate buffer, pH 10.7. The flow rate was 60 ml/h and the total volume of the gradient was 400 ml. 6-ml fractions were collected.

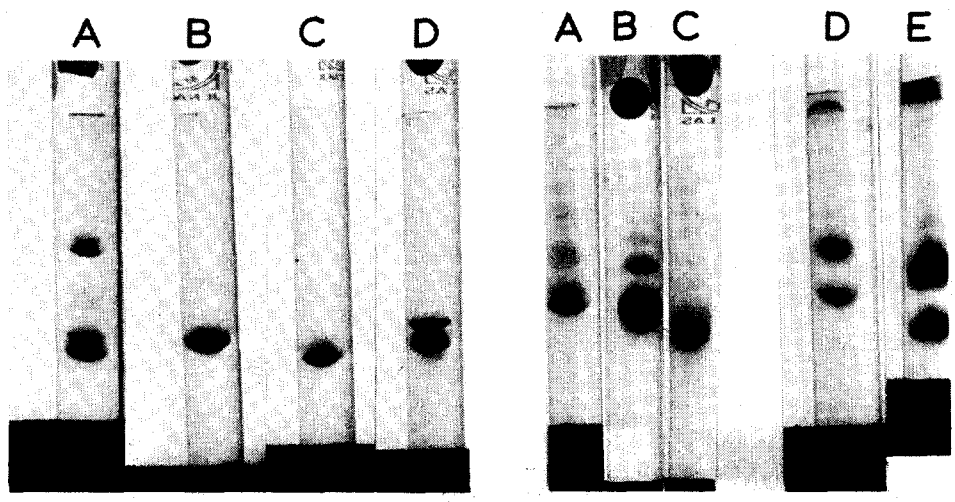


Fig. 5. Disc electrophoresis of the purified basic proteins from bovine peripheral nervous system (system of TAKAYAMA[29]). A, total acid extractable proteins of peripheral nerve (200 μg); B, P_1 basic protein (100 μg) after final purification on Sephadex G-75; C, P_2 basic protein (100 μg) after final purification on Sephadex G-75; D, a cross-contaminated fraction from QAE-Sephadex A-25 (75 μg).

Fig. 6. Disc electrophoresis patterns of the acid extractable proteins of peripheral nerve myelin and of the soluble proteins recovered after butanol extraction of peripheral nerve myelin. Disc electrophoresis was performed according to TAKAYAMA[29] in 7.5% gels (see METHODS). A, the total acid extractable proteins from peripheral nerve myelin; B, part I of the protein peak recovered from Sephadex G-200 gel filtration of the acid extractable proteins of peripheral nerve myelin; C, part II of the peak; D, peripheral nerve myelin proteins; E, The water phase-soluble proteins after butanol extraction of peripheral nerve myelin (see METHODS).

mg of lyophilized peripheral nerve myelin preparate was delipidated[25] and extracted with 0.06 M HCl containing 0.2 M NaCl, the total recovery was 12 mg of soluble proteins of which the main proteins were the basic proteins (Fig. 6c). The acidic extract gave a non-symmetrical peak on Sephadex G-200 gel filtration which was divided into two and showed two basic proteins with different electrophoretic mobilities (Figs. 6A and 6B). These two basic proteins gave the same elution volume on Sephadex G-75 as the basic proteins recovered from peripheral nerve.

Butanol extraction of myelin[20]. The recovery of soluble proteins in the water phase after centrifugation was 72–86 % of the total proteins in the myelin suspension. The proteins remained soluble when the butanol-saturated water phase was dialysed against buffer of pH 10.2–10.7 and pH 3. The recovery of total P in the aqueous phase was constantly 15–16 % and was mainly contributed by phospholipids. The soluble proteins were run in disc electrophoresis (Fig. 6F). The soluble lipoproteins were fractionated on Sepharose 2B without satisfactory resolution.

Properties of proteins

Molecular weight estimations. The molecular weights of the two proteins isolated from the peripheral nervous system were estimated by two independent methods. The first system was gel filtration on Sephadex G-100 column in 0.01 M HCl calibrated with a number of reduced proteins and the R_V values of the proteins were calculated from the elution volume V_e, the void column V_0, and the total volume V_t[30] (Table I). The R_V values were plotted against log molecular weight and the molecular weights of the two basic proteins were found (Fig. 7). A molecular weight of 14100 $\pm$ 600 for P_1 and 12300 $\pm$ 600 for P_2. A number of reduced proteins were subjected to disc electrophoresis on acrylamide gels with gel concentrations varying from 6.5–13.5 %[31]. The values calculated are given in Table II. Frictional ratio values were

TABLE I

RELATIONSHIP BETWEEN R_V VALUE AND MOLECULAR WEIGHT

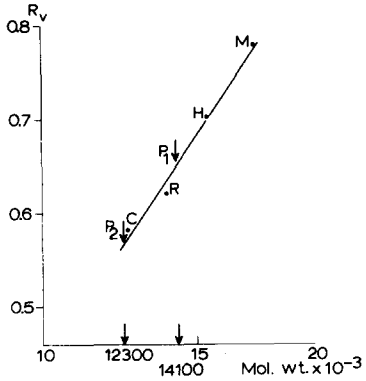
Protein	*Mol. wt.*	R_v
Cytochrome *c*	12 400	0.582
Ribonuclease	13 700	0.615
Hemoglobin chains	15 500	0.709
Apomyoglobin	17 200	0.760
P_1 basic protein	14 100	0.662
P_2 basic protein	12 300	0.579

TABLE II

RELATIONSHIP BETWEEN *f* VALUE AND MOLECULAR WEIGHT

Protein	*Mol. wt.*	*Frictional ratio (f)*
Cytochrome *c*	12 400	1.248
Lysozyme	14 300	1.303
Trypsin	23 300	1.521
Hemoglobin chains	15 500	1.352
Ribonuclease	13 700	1.286
P_1 basic protein	14 300	1.300
P_2 basic protein	12 400	1.248

plotted against molecular weights and the molecular weights of the two basic proteins were estimated (Fig. 8). A molecular weight of 14300 ± 600 was found for P_1 and 12400 ± 600 was found for P_2.



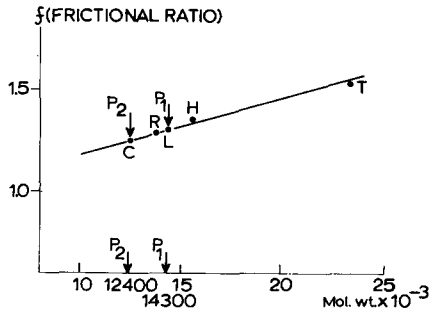


Fig. 7. Relationship between R_V values of the proteins listed in Table I and their molecular weights. C, reduced cytochrome *c*; R, reduced ribonuclease; H hemoglobin chains; M, apomyoglobin. Arrows show R_V values of peripheral nerve basic proteins.

Fig. 8. Relationship between frictional ratios (*f*) of different proteins (estimated by polyacrylamide gel electrophoresis) *versus* their molecular weights using five reduced standard proteins. C, cytochrome *c*; R, ribonuclease; H, hemoglobin chains; T, trypsin; L, lysozyme. The arrows show frictional ratio of peripheral nerve basic proteins. The frictional ratios of these proteins were determined for changes in acrylamide concentrations of 4%.

TABLE III

AMINO ACID COMPOSITION OF PURIFIED BASIC PROTEINS PREPARED FROM BOVINE INTRADURAL ROOT (PERIPHERAL NERVE)

Values are the mean of six determinations.

Amino acid	*moles/100 moles*		
	P_1	P_2	*Central nerve basic protein (ref. 14)*
Lysine	14.4	14.0	7.6
Histidine	0.8	1.8	5.9
Arginine	6.4	5.8	10.8
Aspartic acid	11.0	9.7	7.1
Threonine	10.9	8.7	4.5
Serine	7.3	7.3	9.5
Glutamic acid	8.0	10.1	6.4
Proline	1.8	3.3	7.7
Glycine	9.2	10.5	13.6
Alanine	4.5	4.9	7.5
Half-cystine	0	0	0
Valine	8.6	6.8	1.9
Methionine	2.0	1.6	1.3
Isoleucine	4.8	3.9	1.8
Leucine	7.7	8.7	6.1
Tyrosine	1.2	1.8	2.6
Phenylalanine	4.0	3.9	5.0
Tryptophan	1.4	1.1	0.7

Amino acid analysis. Table III shows the mean values of the amino acid composition of the two basic proteins. The P_1 basic protein contained 21.6 mole % of basic amino and 19.0 mole% of acidic amino acid. The P_2 basic protein contained 21.6 mole % of basic amino acid and 18.8 mole% of acidic amino acid. The amino acid composition differs from that of the amino acid composition of the central nerve basic proteins. The main differences are in the higher content of lysine, isoleucine, aspartic acid, threonine, and valine as well as in the lower content of arginine, glycine, histidine, and proline in the peripheral basic proteins as compared to central nerve myelin basic proteins. No N-terminals were found in the two methods used. There was no difference between the basic proteins purified at acidic pH and those purified at basic pH. The amino acid analysis and molecular weights were identical.

DISCUSSION

WOLFGRAM AND ROSE[1] and FOLCH *et al.*[4] reported as early as 1961 differences in the protein composition of the central nerve (the white matter) and the peripheral nerve. They found mainly differences in extractability of the two tissues with chloroform–methanol mixtures and the difference in amount of the chloroform–methanol-soluble protein, the so-called "Folch–Lees proteolipid". ENG *et al.*[6] also showed differences in the protein composition of sciatic nerve and central nerve myelin. They have reported that the basic protein forms 30 % of the total proteins of central nerve myelin and 20% of the sciatic nerve myelin proteins.

Other studies[5,7] consisted of disc electrophoretic comparison of peripheral nerve myelin and central nerve myelin in different solubilizing and depolymerizing systems like phenol–acetic acid–water–urea and phenol–formic acid–urea. WOLFGRAM AND KOTORII[5] were able to extract basic protein from peripheral nerve myelin without purifying these proteins. The total basic fraction was different in amino acid composition from the one extracted from the central nerve system. We have confirmed the existence of two separate basic proteins extracted and purified from the ox peripheral nerve and which were shown to be constituents of the myelin membrane. The homogeneity and purity of the two proteins were confirmed by disc electrophoresis in different systems and pH values.

The peripheral nerve basic proteins were found to be different in molecular weights, P_1 with a molecular weight of 14200 and P_2 wit a molecular weight of 12300. We cannot exclude the possibility of proteolytic activity *in situ*, although the difference in amino acid composition of the P_1 and P_2 basic proteins makes it difficult to see that P_2 could be derived from P_1.

The delipidation procedure can be varied. No differences were found between the proteins extracted after chloroform–methanol treatment and after the use of chloroform–butanol mixtures for delipidation of the tissue. The recoveries, elution volumes, and amino acid composition of the basic proteins were indentical. The basic proteins recovered after the butanol extraction of myelin were very similar to the basic proteins recovered after delipidation and acidic extraction.

Although these proteins are definitely constituents of myelin, the quantities of these proteins in myelin requires further studies on purity of peripheral nerve myelin preparations. Enzymatic studies to assess purity of peripheral nerve myelin are now in progress. Studies on localization[17], structure, and physical properties[18–22] of the

basic proteins were done mainly in view of the importance of these proteins as a model for autoimmune diseases and the study of their role in the pathogenesis of multiple sclerosis. It was shown that basic proteins are constituents of the myelin membrane and are bound to phospholipids[16,36,37]. The studies mentioned above in which the open conformation of the basic proteins was shown and the regular spacing of non-polar residues allows for more detailed studies of lipid–protein interactions in model systems.

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REFERENCES

1 F. WOLFGRAM AND A. S. ROSE, *J.Neurochem.*, 8 (1961) 161.
2 J. S. O'BRIEN, E. L. SAMPSON, M. B. STERN, *J. Neurochem.*, 14 (1967) 357.
3 G. SCHMIDT, E. L. HOGAN, N. I. FELDMAN, J. JOSEPH, T. TANAKA, K. OKABE, A. KJETA-FYDE, R. A. COLLINS AND R. W. KENNAN, *Proc. 152nd Meeting Am. Chem. Soc., 1966.*
4 J. FOLCH, M. LEES AND S. CARR, *Exp. Cell Res. Suppl.*, 5 (1958) 58.
5 F. WOLFGRAM AND K. KOTORIL, *J. Neurochem.*, 15 (1968) 15.
6 L. F. ENG, F. C. CHAO, B. GERSTL, D. PRATT AND M. G. TARASTSJERNA, *Biochem.*, 7 (1968) 4455.
7 E. MEHL AND F. WOLFMAN, *J. Neurochem.*, 16 (1969) 1091.
8 B. BENCINA, P. R. CARNEGIE, T. A. MCPHERSON AND G. ROBSON, *FEBS Lett.*, 4 (1969) 9.
9 R. H. LAATSCH, M. W. KIES, S. GORDON AND E. C. ALVORD, JR., *J. Exp. Med.*, 115 (1962) 777.
10 H. HIRCHFLED, D. TEITELBAUM, R. ARNON AND M. SELA, *FEBS Lett.*, 7 (1970) 317.
11 R. E. MARTENSON, G. E. DEIBLER AND M. W. KIES, *Biochim. Biophys. Acta*, 206 (1970) 1353.
12 A. NAKAO, W. J. DAVIS AND E. R. EINSTEIN, *Biochim. Biophys. Acta*, 130 (1966) 163.
13 E. H. EYLAR, J. SALK, G. C. BEVERIDGE AND L. V. BROWN, *Arch. Biochem. Biophys.*, 132 (1969) 34.
14 F. B. PALMER AND R. M. C. DAWSON, *Biochem. J.*, 111 (1969) 629.
15 E. H. EYLAR, *Proc. Natl. Acad. Sci. U.S.A.*, 63 (1970) 1425.
16 F. B. PALMER AND R. M. C. DAWSON, *Biochem. J.*, 111 (1969) 637.
17 J. P. DICKSON, K. M. JONES, S. R. APURICIO AND C. E. LUMSDEN, *Nature*, 227 (1970) 113.
18 E. H. EYLAR AND M. THOMPSON, *Arch. Biochem. Biophys.*, 129 (1969) 468.
19 L. P. CHAO AND E. ROBOZ EINSTEIN, *J. Neurochem.*, 17 (1970) 1132.
20 G. A. HASHIM AND E. H. EYLAR, *Arch. Biochem. Biophys.*, 129 (1969) 635.
21 R. F. KIBLER, R. SHAPIRA, S. MCKNEALLY, J. JENKINS, P. SELDEN AND F. CHOU, *Science*, 164 (1969) 577.
22 E. H. EYLAR, *Proc. Natl. Acad. Sci U.S.A.*, 67 (1970) 1425.
23 L. A. AUTILIO, W. T. NORTON AND R. D. TERRY, *J. Neurochem.*, 11 (1964) 17.
24 R. E. MARTENSON AND F. N. LE BARON, *J. Neurochem.*, 13 (1966) 1469.
25 R. VERGER, G. H. DE HAAS, L. SARDA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 188 (1969) 272.
26 A. H. MADDY, *Biochim. Biophys. Acta*, 117 (1966) 193.
27 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
28 C. J. F. BÖTTCHER, C. M. VAN GENT AND C. PRIES, *Anal. Chim. Acta*, 24 (1961) 203.
29 K. TAKAYMA, *Arch. Biochem. Biophys.*, 114 (1966) 223.
30 G. E. DEIBLER, R. E. MARTENSON, M. W. KIES, *Biochim. Biophys. Acta*, 200 (1970) 342.
31 C. R. PARISH, J. J. MARCHALONIS, *Anal. Biochem.*, 34 (1970) 436.
32 O. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
33 M. K. GAITONDE AND T. DOVEY, *Biochem. J.*, 117 (1970) 907.
34 C. GROS AND B. LUBOUESSE, *Eur. J. Biochem.*, 7 (1969) 463.
35 R. R. PORTER AND F. SANGER, *Biochem. J.*, 42 (1948) 1287.
36 F. GONZALES-SASTRE, *J. Neurochem.*, 17 (1970) 1049.
37 D. H. ADAMS AND E. A. CASPARY, *J. Neurol. Sci.*, 11 (1970) 187.