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STUDIES ON BRAIN-SPECIFIC MEMBRANE PROTEINS

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SUMMARY

The M_1-1.0 synaptic membrane fraction was isolated according to the method of De Robertis and compared electrophoretically and immunologically with other subcellular fractions from the brain and other organs. Two non-myelin, non-mitochondrial proteins were detected in the synaptic membranes, synaptic vesicles and brain microsomes. Both proteins appeared to be specific to the brain. Parallel studies with complement fixation test revealed organ specificity of the brain proteins and cross-reactivity between the synaptic membranes and the brain microsomes.

INTRODUCTION

Technical development during the past decade has made possible the isolation of the various subcellular fractions from the brain, including the nerve ending particles and their components. Earlier, MAHLER AND COTMAN[1] and MEHL[2] separately conducted a general survey of the electrophoretic protein patterns of the synaptic membranes and the related fractions from rat brain. Although they were able to show differences in band pattern among the various subcellular fractions, definitive evidence for the brain specificity of any of the protein bands was not obtained. The current investigation differed from the earlier works in two respects: emphasis was placed on the detection of brain-specific proteins; immunological studies were conducted in parallel with the electrophoretic comparisons.

MATERIALS AND METHODS

Subcellular fractionation

Unless otherwise specified, subcellular fractions were isolated from the cat cerebral cortex. Adult cats of either sex, weighing about 6 to 8 lb, were killed by exsanguination and 10 to 12 g of cerebral cortex, free of the meninges and the bulk of white matter, was usually obtained from one cat. Synaptic membranes were isolated from the cerebral cortex according to Method III of RODRÍGUEZ DE LORES ARNAIZ *et al.*[3]. In this method the M_1 fraction (a pellet obtained from the "crude mitochondrial fraction" after hypoosmotic shock), which contained a mixture of myelin, mitochondria and the synaptic membranes, was separated in a discontinuous gradient consisting

of the following sucrose solutions (top to bottom): 0.8 M; 0.9 M; 1.0 M; 1.2 M. The following fractions were obtained: (1) M_1-0.8, myelin; (2) M_1-0.9, synaptic membranes; (3) M_1-1.0, synaptic membranes; (4) M_1-1.2, synaptic membranes; (5) M_1-P, mitochondria. Here the designation of RODRÍGUEZ DE LORES ARNAIZ *et al.*[3] where a fraction was named according to the sucrose concentration above which it banded, was adopted. M_1-P was the pellet collected below 1.2 M sucrose.

Pure myelin was isolated by a modification of the procedure of SOTO[4]. A 10 % homogenate in 0.32 M sucrose was prepared from 1.5 g of white matter dissected from one cat brain. The fraction collected between 900 × *g* (10 min) and 23000 × *g* (10 min) was resuspended in 20 ml of 0.8 M sucrose. This was layered with 12 ml of 0.32 M sucrose and centrifuged at 25000 rev./min for 2 h in a Spinco SW 25 rotor. The material banded at the interface was aspirated, suspended again in 0.8 M sucrose and the gradient centrifugation step was repeated. After this the material collected between 0.32 and 0.8 M sucrose was subjected to hypoosmotic shock by diluting the sucrose concentration to 0.05 M. A final centrifugation yielded the purified myelin used for the current study.

Pure mitochondria were obtained by the discontinuous sucrose gradient centrifugation in the absence of osmotic shock[5]. Microsomes were collected between 30000 × *g* (20 min) and 100000 × *g* (1 h). The post-microsomal supernatant fraction was taken as the soluble proteins. Synaptic vesicles were purified by the method of LAPETINA *et al.*[6].

All the subcellular fractions were dialyzed and lyophilized, and materials obtained from five cats were pooled for electrophoretic and immunological studies. For the fractionation of the liver, the cats were starved for 24 h in order to reduce the glycogen content.

Polyacrylamide gel slab electrophoresis

This was a modification of the procedure of MEHL[2]. Gel slabs, 3 mm in thickness and consisting of 14 % monomer, were prepared in an apparatus previously described[7]. The gel mixture was made up of 1 part Solution A, 4 parts Solution C and 3 parts of 0.14 % (w/v) ammonium persulfate (see DAVIS[8]). Spacer and sample gels were omitted. After soaking overnight in 1.5 l of distilled water to eliminate the soluble materials, the gel was equilibrated for 2 weeks with 400 ml of the phenol mixture consisting of phenol–formic acid–water (14:3:3, w/v/v), taking into account the amount of water already present in the gel. Electrophoresis was carried out horizontally in an apparatus made of polypropylene. The gel was trimmed to fit the platform of the apparatus so that no channel existed between the two electrode compartments. Two pieces of glass plate placed on top of the gel helped to secure it in position. Sample solutions, at a concentration of 10 mg lyophilized material per ml of the phenol mixture, were introduced to one edge of the gel through pieces of Whatman No. 3 MM paper. The paper was cut to 3 mm × 8 mm; two pieces were stacked together to carry 15 μl (150 μg) of sample to the gel. A constant current of 3.5 mA/cm^2 was applied for the first 30 min and 7.0 mA/cm^2 for the next 2.5 h. The gels were stained for 24 h with 250 ml of 1 % Amido black in 7 % acetic acid. Destaining consisted of several changes of 7 % acetic acid. In order to facilitate the recognition of artifacts and variations from gel to gel, duplicate samples and duplicate gels were used.

Immunological procedures

Male albino rabbits, weighing 5 to 6 lb, were each inoculated with 5 mg of the lyophilized M_1-1.0 synaptic membrane fraction. The antigen was suspended in 1 ml of isotonic saline and mixed thoroughly with 1 ml of the complete Freund's adjuvant (Difco). About 0.25 ml was injected into each of the four foot pads and the remainder was distributed subcutaneously in two sites around the scapular regions. The rabbits were bled 4 weeks following immunization. There were no signs of allergic encephalomyelitis. Sera from 3 to 5 rabbits showing similar antigenic titers were pooled. The antisera were decomplemented by incubation at 56° for 30 min. When assayed against the M_1-1.0 fraction, the antibody titer in the pooled rabbit antiserum was 85 times higher than that in the control serum obtained prior to immunization.

Antigenic content of the subcellular fractions was assayed by a quantitative complement fixation test. This was a modification of the method of MOORE AND PEREZ[9] which shows the highest degree of sensitivity of all the methods tested. Our method differed from the original mainly in scaling up the volume and in the use of membrane protein rather than soluble protein as antigen. Mercaptoethanol was omitted. Biological reagents were products of Baltimore Biological Laboratory, and the NaCl veronal buffer of KABAT AND MAYER[10] was used for dilution. The procedure consisted of mixing 0.1 ml of a suspension of antigen with a 0.1-ml mixture of guinea pig complement *plus* decomplemented antiserum. Fixation was allowed to proceed for 18 h at 4°. After that 0.1 ml of sensitized sheep erythrocytes was added and the mixture incubated at 37° for 1 h. Following incubation 1.7 ml of the cold NaCl–veronal buffer was added and the entire mixture centrifuged at 2000 rev./min for 10 min to sediment the unlyzed erythrocytes. The concentration of hemoglobin released into the supernatant was determined with a Beckman spectrophotometer at 413 nm. The amount of erythrocytes used was adjusted to give an absorbance of 1.4 upon complete hemolysis. The amount of complement used was adjusted to cause a 50 % hemolysis in the absence of fixation by the antigen–antibody reaction. Control tubes included (1) erythrocytes, (2) erythrocytes + complement, (3) serum + erythrocytes + complement, (4) antigen + erythrocytes + complement. The amount of complement fixed was expressed as the decrease in absorbance (ΔA) compared to the blank (50 % hemolysis).

Protein determination

The membrane fractions were suspended in 1 M NaOH and kept at room temperature for 1 h. The samples were then diluted to 0.1 M with respect to NaOH and determined for protein by the colorimetric method of LOWRY *et al.*[11], using crystalline bovine serum albumin as standard.

Electron microscopy

Pellets collected from the gradient were fixed overnight at 4° by layering with a solution of 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The fixed pellets were cut into small pieces with a razor blade and postfixed for 45 min with 1.33 % osmium tetroxide in 0.067 M collidine buffer (pH 7.4)[12]. Following dehydration in graded concentrations of ethanol and propylene oxide, the pieces were embedded in Epon 812[13]. Thin sections were stained with uranyl acetate and lead citrate[14]. All specimens were examined and photographed with an RCA 3-G electron microscope.

Enzyme determinations

Succinate dehydrogenase was assayed by the *p*-iodonitrotetrazolium method described by ADAMS *et al.*[15]. The reaction mixture contained 1.0 ml of 63.5 mM phosphate buffer (pH 7.4), 0.05 ml of 0.1 M EDTA, 0.1 ml of 15 mM KCN, 0.15 ml of a 10 mM menadione suspension, 0.2 ml of 0.5 M succinate, and 0.1 ml of brain sample. The menadione suspension was freshly prepared by mixing 3 ml of ethanol containing 25 mg of vitamin K_3 with 12 ml of 0.1 M sodium phosphate (pH 7.4) containing 0.2 % bovine serum albumin. The reaction was started by the addition of 0.1 ml (2 μmoles) of a fresh solution of *p*-iodonitrotetrazolium in 20 % acetone (10 mg/ml), and the mixture was incubated at 37° for 10 min. After terminating the reaction with 1 ml of 10 % trichloroacetic acid, the formazan was extracted with 4.0 ml of ethyl acetate and read at 490 nm. Under the assay condition 1 μmole of reduced *p*-iodonitrotetrazolium gave an absorbance of 5.45 (1 cm light path).

Acetylcholinesterase was estimated by means of the dithiobisnitrobenzoate reagent, using a procedure modified from that of ELLMAN *et al.*[16]. Incubation was started by adding 0.1 ml of a brain sample to 1.0 ml of a working solution freshly prepared by mixing 15 ml of 0.1 M sodium phosphate (pH 8.0), 0.1 ml of 0.075 M (12.67 mg/ml) acetylthiocholine iodide, and 0.5 ml of a stock dithiobisnitrobenzoate reagent. The dithiobisnitrobenzoate stock solution was prepared by dissolving 39.6 mg of dithiobisnitrobenzoate in 10 ml of 0.1 M sodium phosphate buffer at pH 7.0, followed by the addition of 15 mg of $NaHCO_3$. After incubation at 37° for 10 min, the reaction was terminated by the addition of 0.1 ml of a 2 % (w/v) eserine sulfate and read at 412 nm. When calibrated with a sample of erythrocyte acetylcholinesterase (Sigma Chemical Co.) under the current assay condition, an absorbancy of 10.5 was observed per μmole of the acetylthiocholine hydrolyzed.

The incubation mixture for (Na^+–K^+)-ATPase consisted of: 0.5 ml of 0.32 M Tris–HCl (pH 7.4) containing 0.2 mM EDTA, 0.1 ml of 0.03 M $MgCl_2$, 0.1 ml of 1.5 M NaCl, 0.1 ml of 0.2 M KCl, 0.1 ml of 0.02 M ATP (Tris salt), and 0.1 ml of the enzyme solution. The ATP solution was added after a 2-min preincubation period. After 20 min at 37°, the entire mixture was deproteinized with 0.1 ml of a 50 % trichloroacetic acid solution and the amount of orthophosphate in the supernatant fraction was measured by the method of KING[17]. The values were corrected for enzyme activities in the absence of Na^+ and K^+.

All the special reagents for enzyme assay were purchased from the Sigma Chemical Co.

All the results of enzyme assay were averages of at least two determinations.

RESULTS

Electron microscopic and enzymic identification

Fig. 1 shows the ultrastructure of the major subfractions of M_1 obtained by the sucrose gradient centrifugation. Fractions M_1-0.8 and M_1-P appeared to be myelin and mitochondria, respectively. Fraction M_1-1.0 was rich in synaptic membranes and synaptic complexes. The distribution of the marker enzymes are shown in Table I. The relative specific activities of acetylcholinesterase and (Na^+–K^+)-ATPase, considered to be positive markers for the synaptic membranes, were high in the M_1-0.9, M_1-1.0 and M_1-1.2 fractions. The activity of succinate dehydrogenase,

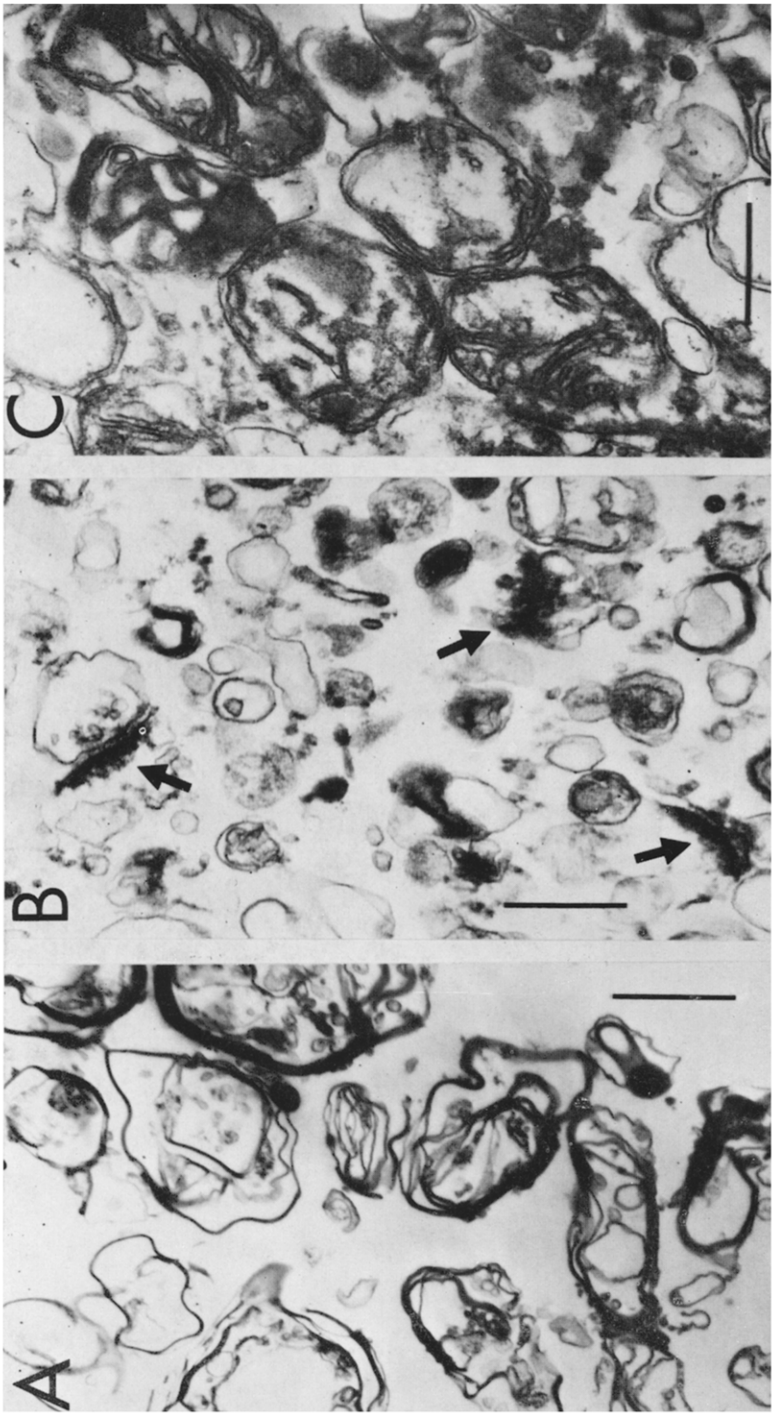

Fig. 1. Electron micrographs of the subcellular fractions. (A) Taken from M_1-0.8, consists of structures typical of myelin. (B) Taken from M_1-1.0, shows numerous membranous materials consistent with synaptic membranes, some of which possess the pre- and postsynaptic components (arrows). (C) Taken from the M_1-P, reveals aggregates of partially damaged mitochondria, which are typical of this method of subcellular fractionation[3]. The scales represent 1 μm for A and 0.5 μm for B and C.

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TABLE 1

ENZYME ACTIVITIES OF THE SUBFRACTIONS OF M_1

Absolute values for protein and enzyme activities correspond to the amounts present in the cerebral cortex of one cat brain. Enzyme activities are expressed as μmoles substrate converted per min at 37°. Rel. spec. act. = % activity/% protein weight.

Fraction	*Protein*		*Succinate dehydrogenase*			*Acetylcholinesterase*			*$(Na^+–K^+)$-ATPase*		
	mg	*% wt.*	*Total activity*	*% Activity*	*Rel. spec. act.*	*Total activity*	*% Activity*	*Rel. spec. act.*	*Total activity*	*% Activity*	*Rel. spec. act.*
M_1-0.8	56.1	41.1	0.18	2.5	0.06	0.75	28.5	0.69	2.56	35.3	0.86
M_1-0.9	3.8	2.0	0.03	0.5	0.25	0.42	15.9	7.79	0.82	11.7	5.75
M_1-1.0	6.1	4.4	0.08	1.1	0.25	0.54	20.5	4.62	1.56	21.6	4.88
M_1-1.2	20.4	14.9	0.88	12.3	0.82	0.63	23.9	1.60	2.28	31.4	2.11
M_1-P	50.2	37.5	5.99	83.6	2.23	0.29	11.2	0.29	<0.01	<0.1	0.01
Sum	136.6	100.0	7.16	100.0	—	2.63	100.0	—	7.22	100.0	—

a mitochondrial enzyme, was highest in the M_1-P fraction. Thus, the results of electron microscopic and enzymic studies of the subfractions of M_1 were in agreement with those of RODRÍGUEZ DE LORES ARNAIZ *et al.*[3] corroborating their original observation that M_1-0.8 and M_1-P were myelin and mitochondrial fractions, respectively, whereas M_1-0.9, M_1-1.0 and M_1-1.2 were the three synaptic membrane-rich fractions.

Electrophoretic studies

In order to detect subtle differences in electrophoretic protein patterns, samples obtained from the sucrose gradient were applied to the acrylamide gel slab in the same order as they appeared in the gradient (Fig. 2). Thus in the resulting protein map individual bands that were in common among various subcellular fractions could easily be discerned. The myelin fraction contains two major bands, "a" and "b", corresponding to the proteolipid protein and the encephalitogenic protein, respectively (see MEHL AND WOLFGRAM[18]). CARNEGIE *et al.*[19] proposed the use of cytochrome *c* as a reference for expression of electrophoretic mobility (M_c value). The M_c values for the "a" and "b" bands of the cat myelin were determined in the current investigation to be 0.38 and 0.71, respectively, obtained by dividing the distance traveled by the band in question by the distance traveled by cytochrome *c*. However, for other bands in the gel, we found it more accurate to express the mobility relative to the myelin "a" band (M_a value) since cytochrome *c* moves too far away from most of the bands observed in the gel.

A few of the myelin bands have extended into the M_1-0.9 synaptic membrane fraction. Likewise some of the mitochondrial bands are present in the M_1-1.2 fraction. Thus from electrophoretic evidence it seems likely that both the myelin and the mito-

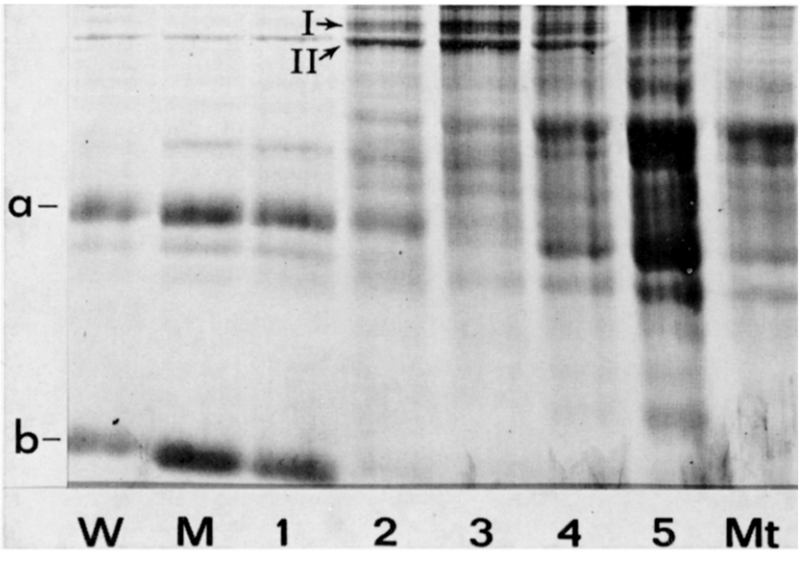

Fig. 2. Electrophoretic protein map for the subfractions of M_1. Samples 1 to 5 are obtained from the consecutive bands of the sucrose gradient. 1, M_1-0.8; 2, M_1-0.9; 3, M_1-1.0; 4, M_1-1.2; 5, M_1-P; W, total particulate material from the white matter (corpus callosum and centrum semiovale); M, myelin purified by a modification of the method of SOTO[4]; Mt, brain mitochondria purified by the method of DE ROBERTIS *et al.*[5]. The origin of electrophoretic migration is on top.

chondria contaminate their adjacent fractions, namely, the M_1-0.9 and M_1-1.2, respectively. This is not too difficult to understand because of their physical proximity in the sucrose gradient. By the same token, fraction M_1-1.0, which is farthest from both the myelin and the mitochondria in the sucrose gradient, should be expected to be least contaminated and this is indeed what appears in the protein map. That M_1-1.0 is the purest among the three synaptic membrane fractions was also observed by RODRÍGUEZ DE LORES ARNAIZ *et al.*[3] on electron microscopic evidence.

Despite the possibility of contamination from other subcellular particles as discussed above, the synaptic membrane fractions do contain protein bands not found in myelin and mitochondria. The bands most distinctive of the synaptic membranes are those designated I and II in Fig. 2. The relative mobility of protein I is 0.05 with respect to cytochrome *c* (M_c) and 0.13 with respect to myelin "a" band (M_a); that of protein II is 0.09 and 0.22, respectively. Protein I has a tendency to be doublets and triplets and might consist of a group of interrelated proteins or polypeptide subunits. Although a trace of protein II is seen in the myelin fraction, it is very unlikely that this protein originates from the myelin for the following reasons: protein II is much more intense in the synaptic membrane fractions than in myelin; the two major bands of myelin are absent in some synaptic membrane fractions where protein II is prominent. In addition to proteins I and II, other bands characteristic of the synaptic membrane fractions are seen near the middle part of the gel, but they are less conspicuous.

Other subcellular fractions from the brain were also electrophoresed. As shown in Fig. 3, proteins I and II are also present in the brain microsomal and the synaptic vesicle fractions. The purified synaptic vesicle fraction contains protein I and a trace of protein II, whereas the brain microsomes have slightly more of protein II than of protein I. All other particulate and soluble fractions of the brain do not exhibit proteins I and II. Unlike the brain microsomes, liver microsomes are devoid of these

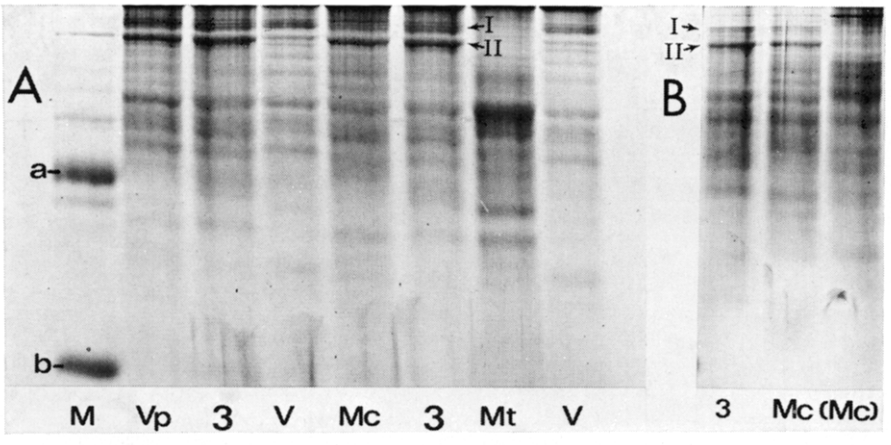

Fig. 3. Electrophoretic protein patterns of various subcellular fractions. Origin on top. A and B are two separate pieces of gel slabs. Mc, microsomes; V, purified synaptic vesicles, corresponding to M_2A of LAPETINA *et al.*[6]; V_p, pellet below 0.5 M sucrose during the purification of the synaptic vesicles, reported to be contaminated with synaptic membranes, corresponding to M_2B of LAPETINA *et al.*[6]. Parentheses denote fraction obtained from cat liver; all other fractions are from the brain. See legend to Fig. 2 for meanings of M, Mt and 3.

proteins. In fact, the two microsomal fractions would have been quite similar in electrophoretic pattern were it not for the presence of these two proteins in the brain. In another experiment where the procedure for the isolation of the synaptic membranes was carried out on the liver, the simulated "synaptic membrane" fraction from the liver still did not reveal any of the two proteins.

Proteins I and II are so predominant in the brain that they were detectable even in the total particulate fraction. On the other hand, when the particulate fractions from other organs of the cat were electrophoresed, the two protein bands were not clearly demonstrable in the gel (Fig. 4). Among the organs compared, only the cardiac and skeletal muscles had a prominent band exhibiting mobility close to the two proteins in question. However, their identify with either of the two proteins was excluded on the ground that there was no overlapping of bands and that the muscle band existed mainly in the material sedimented below the microsomal fraction. Electrophoretic evidence also suggested that the two proteins are different from the following: acetylcholinesterase, butyrylcholinesterase, the neurotubule protein and cerebral actomyosin. Proteins I and II can be detected in the brains of all the animals examined, as shown in Fig. 5, despite some variations in other bands.

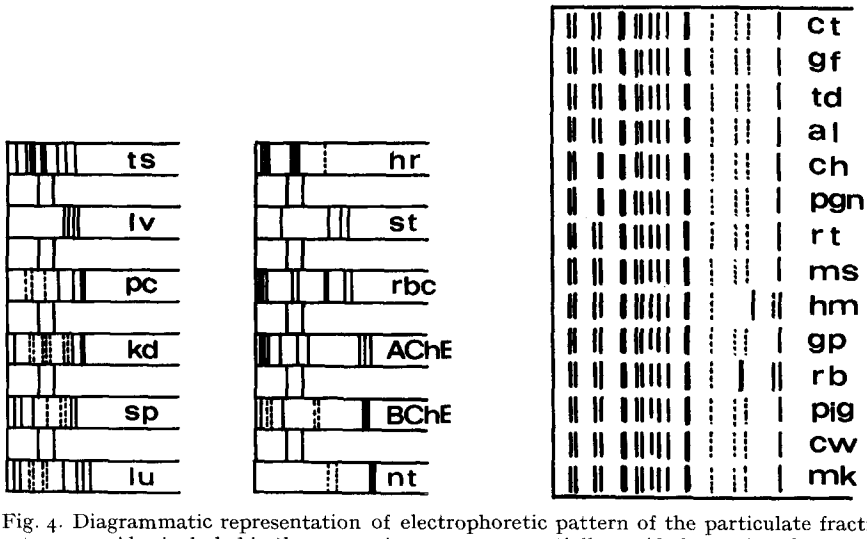

Fig. 4. Diagrammatic representation of electrophoretic pattern of the particulate fractions from cat organs. Also included in the comparison are some partially purified proteins. Origin on the left. The locations of proteins I and II are indicated by two lines in alternate spaces. ts, testis; lv, liver; pc, pancreas; kd, kidney; sp, spleen; lu, lung; hr, heart; st, stomach; rbc, human red cell ghosts; AChE, bovine erythrocyte acetylcholinesterase (Sigma Chemical Co.); BChE, horse serum butyrylcholinesterase (Sigma); nt, neurotubule protein[25,26]. Also compared but not represented are cat diaphragm, cat cerebral actomyosin[27], and electric eel acetylcholinesterase (Sigma). None of these samples have bands overlapping those of proteins I and II.

Fig. 5. Electrophoretic pattern of the brain membrane proteins from various vertebrates. The particulate material sedimented between 9000 × g·min and 600000 × g·min was used. Origin on the left. Proteins I and II are the first two lines close to the origin. ct, cat; gf, goldfish; td, toad; al, alligator; ch, chicken; pgn, pigeon; rt, rat; ms, mouse; hm, hamster; gp, guinea pig; rb, rabbit; pg, pig; cw, cow; mk, monkey.

Immunological studies

Antigenic specificity of the various subcellular fractions was compared, employing the quantitative complement fixation test. Pooled rabbit sera immunized against

cat synaptic membrane (M_1-1.0) was used as a source of antibody. Fig. 6 shows the linear relationship between the amount of complement fixed and the amount of the membrane antigen used. As to be expected, the highest antigenic titer was demonstrated in the homologous synaptic membrane fraction (Table II). Among the membrane materials isolated from the same gradient, *i.e.* the various subfractions of M_1, the

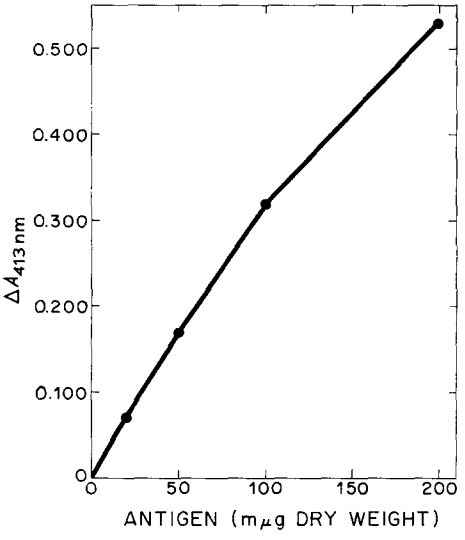

Fig. 6. Complement fixation curve of M_1-1.0 synaptic membranes titrated against the homologous antiserum. The amount of complement fixed is represented by the difference in absorbance.

TABLE II

IMMUNOLOGICAL SPECIFICITY OF THE SUBCELLULAR FRACTIONS

Subcellular fractions were titrated with the antiserum against the M_1-1.0 fraction from the cat cerebral cortex. Antiserum dilution, 1:1600. The fixation values are relative to the M_1-1.0 and are taken from the linear portion of the complement fixation curves. Parentheses indicate fractions isolated from the liver; all others are from the brain.

Fraction	*Relative amount of complement fixed*	
	Dry weight basis	*Protein basis*
M_1-0.8	64.8	97.5
M_1-0.9	67.4	68.5
M_1-1.0	100.0	100.0
M_1-1.2	90.5	60.0
M_1-P	42.0	23.0
Synaptic vesicles	47.3	46.8
Nuclei	15.0	24.0
Microsomes	91.9	100.0
Myelin	32.4	47.5
Soluble proteins	10.7	6.5
(M_1-1.0)	22.5	24.5
(Microsomes)	10.4	8.8
(Mitochondria)	10.5	7.8
(Nuclei)	14.1	13.5
(Soluble proteins)	13.0	6.5

antigenic titer decreased in both directions away from the M_1-1.0. The only exception to this was the M_1-0.8, which was predominantly myelin. Nevertheless, the titer exhibited by the purified myelin was low. Various degrees of cross-reactivity were found in other particulate fractions of the brain, among which the brain microsomes showed the highest titer, followed by the synaptic vesicle fraction. In contrast to the brain fractions, liver particulate components did not cross-react significantly with the synaptic membranes. Other cat organs behaved similarly. Thus immunological evidence roughly corroborates electrophoretic results with respect to the specificity of brain membrane proteins.

DISCUSSION

Although several organ-specific proteins were detected before in the soluble fraction of the brain homogenate, some of which have been intensively characterized[20], studies on the membrane-bound proteins specific to the nervous tissue, except for the myelin components, have been handicapped by the difficulty of subjecting the membranes to analytical procedures. In the present investigation the problem was approached by a combination of three different techniques: subcellular fractionation, polyacrylamide electrophoresis and immunochemistry. Several methods for the isolation of nerve ending particles and synaptic membranes have been reported, each carrying its own advantages and shortcomings. Inasmuch as the method developed in the laboratory of RODRÍGUEZ DE LORES ARNAIZ *et al.*[3] has been intensively studied, both morphologically, enzymatically and pharmacologically, we decided to use the fractions isolated by this procedure, so that the results gathered by us could be integrated with or interpreted in the light of the knowledge already obtained in their laboratory.

Since in any centrifugation procedure it is very difficult to completely eliminate the possibility of cross-contamination, in the current work we assumed that the presence of a protein band in more than one subcellular fraction is a result of contamination from the fraction where the band is found in the largest amount, so that we would be erring on the safe side in our attempt to identify proteins specific to some morphological structures. In order to carry out a rigorous comparison of the protein components, electrophoresis was conducted with polyacrylamide gel slabs using the phenol–formic acid–water solvent. This system has the following advantages: the membrane proteins are completely solubilized; the samples can be compared under identical electrophoretic conditions; samples can be applied in close proximity to one another making it easy to distinguish protein bands with slightly different mobilities.

The use of electrophoresis for the study of the synaptic membranes has been reported separately by MAHLER AND COTMAN[1] and by MEHL[2]. The two studies differed from ours in that they were intended as general survey of the protein patterns of the various fractions without placing emphasis on organ specificity of the individual protein bands. The possibility of contamination in contributing to the protein pattern of the fractions was not seriously considered. Comparison of our band pattern with those of MAHLER AND COTMAN was handicapped by the relatively poor resolution of their protein bands and by the lack of a reference protein (for calculation of mobility) in their system. Comparison with the results of MEHL was easier and indeed proteins with mobilities similar to those of I and II could be detected in some of his gel patterns.

However, in MEHL's work the two proteins were not intensively studied in terms of their brain specificity. Other differences also exist, such as the difference in experimental animal and in the handling of the gel. In MEHL's work the gels were destained by electrophoresis which in our experience did result in the loss of some protein bands when used in conjunction with the phenol gel. Concurrent immunological studies were not performed in the aforementioned two laboratories.

The existence of proteins I and II in the synaptic membranes, brain microsomes and synaptic vesicles can be interpreted as follows. On the one hand it is possible that some degree of cross-contamination exists between the three fractions; the brain microsomes, for example, have been considered a rather heterogeneous entity[21,22], consisting of the endoplasmic reticulum, small nerve endings, small axons and fragmented plasma membranes. On the other hand, it is conceivable that the brain microsomes, the synaptic vesicles and the synaptic membranes might have a common origin, such as has been postulated for the liver microsomes, membranes of the Golgi apparatus and the liver plasma membranes (MORRÉ, personal communication). Whether the multiple distribution pattern of the two proteins reported in this paper results from contamination, common origin or both remains to be determined by further work, including the purification of the individual proteins and study of their ultrastructural localization by immunoelectron microscopy.

Limitations do exist in the use of acrylamide gel electrophoresis. In general, the more sensitive a method is, the more protein bands will be detected, and the greater will be the chance of band overlapping. This could result in uncertainty in comparing protein samples in that relatively similar gel patterns might be observed despite large differences in antigenicity.

Numerous immunological studies of the brain proteins, particularly with the use of immunoelectrophoresis, have been reported in the literature. But since only about 30–40 % of the cerebral proteins are water soluble, such studies did not reflect a complete picture of the antigenicity of brain proteins. Complement fixation test, by virtue of its independence on the solubility of the antigen, is useful in estimating the total antigenic content of a protein fraction, especially the membrane proteins. Further advantage is provided by its high degree of sensitivity and the feasibility of a quantitative assay. Although complement fixation test has the drawback of revealing nothing about the individual protein components other than the total antigenic content of a sample, employing the method in parallel with acrylamide electrophoresis provides a more complete picture of the proteins in the subcellular fractions than either method employed alone.

The quantitative complement fixation test of MAYER *et al.*[23] was modified by WASSERMAN AND LEVINE[24], and more recently by MOORE AND PEREZ[9]. The last method was adopted for the present study because of its sensitivity. Although the method was originally intended for a soluble protein, we have demonstrated its applicability to the membrane proteins, provided that proper volumes are used and that care is taken to effect a homogeneous dispersion of the membrane fragments during pipeting.

The complement fixation test has potential use in the isolation of brain-specific insoluble proteins. In principle it would be best to obtain a purified protein before doing any immunological study. In practice, however, membrane proteins are difficult to fractionate; whereas a complement fixation test, even with a relatively crude antiserum, can rapidly provide a general idea as to whether antigenic differences exist

between two membrane samples. Such preliminary information could be very useful before attempting a laborious chemical fractionation. Furthermore, the antiserum induced by a relatively crude protein sample can be purified by absorption with the undesirable proteins; the absorbed serum will then be a valuable aid for identifying the brain-specific antigens in fractions separated by a conventional preparative biochemical procedure (such as column chromatography). Following this the partially purified brain protein can be used to obtain a batch of antiserum of higher specificity, which, after exhaustive absorption, can again monitor the further purification of the antigen. Such recycling steps should result in the isolation of proteins specific to the nervous tissue or to a particular neuronal structure such as the synaptic membranes.

Earlier, DE ROBERTIS[22] investigated the immunological differences among the various subcellular fractions of the cat brain using complement fixation. The method differed significantly from ours in that it was at most semiquantitative and that antibodies rather than antigens were assayed. Nevertheless, some results were comparable between his study and ours. In both instances cross-reactivity between the synaptic membrane and the microsomal fraction was demonstrated. Comparison of the electrophoretic protein patterns, however, was not conducted by this group of investigators.

Either acrylamide gel electrophoresis or complement fixation test alone has been previously employed for the study of the cerebral membrane proteins. The present investigation was unique in that the two powerful techniques were used concertedly for the same subcellular fractions. The two techniques complemented each other in indicating the presence of brain-specific proteins in the synaptic membrane and related fractions. The persistence of the two proteins (I and II) in the vertebrate brain throughout the phylogenetic scale implies their participation in some unknown physiological role indispensable for the activities of the nervous system.

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