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Incorporation of ^{3}H-Leucine into Insoluble Proteins of Neuronal and Glial Cell Fractions *in Vitro*

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Rat brain cerebral cortex was dispersed by collagenase-hyaluronidase digestion and neurons and glia were separated by gradient centrifugation. The cell fractions obtained were studied by electron microscopy and incubated with ^{3}H-leucine following uptake and incorporation into soluble and insoluble proteins. Insoluble proteins were separated by polyacrylamide gel electrophoresis and specific radioactivities of individual protein bands were determined. (a) Uptake of ^{3}H-leucine proceeds more rapidly in glia reaching maximal radioactivity at 20 min. (b) Incorporation of ^{3}H-leucine into protein is 7 fold higher in neurons compared to glia at 20 min but the ratio increases up to 2 h. In both cell fractions the insoluble protein reaches around 5 times higher specific radioactivity than the soluble protein. In neurons the radioactivity increases up to 2 h while in glia a plateau is reached at 20 min. (c) Electrophoretic patterns of the proteins of the cell fractions differ only slightly. The highest specific radioactivities are found in the slowly moving neuronal bands.

Advances in preparative and analytical techniques have opened new aspects of brain protein metabolism in recent years. Extraction of brain tissue with aqueous media leaves a bulky residue containing 98 to 50 % of the total brain protein depending on the media used.[1,2] The insoluble residue, including largely membrane proteins, has been shown to label rapidly with radioactive precursors motivating a detailed study of the insoluble protein. Polyacrylamide gel electrophoresis introduced by Ornstein and Davis [3] can be adapted to separation of insoluble proteins by performing electrophoresis in the presence of detergents in acidic,[4,5] basic,[6] or neutral milieu.[7] Such techniques have been applied for analysing patterns of insoluble proteins from myelin,[6–8] synaptosomes,[9,10] and mitochondria.[11]

In this paper insoluble proteins of neuronal and glial cell fractions are characterized. The fractions obtained by collagenase-hyaluronidase digestion and gradient centrifugation [12,13] are incubated for various times with radioactive leucine *in vitro* and incorporation into protein is determined. Insoluble proteins are separated by polyacrylamide gel electrophoresis and specific radioactivities of stained protein discs are measured.

MATERIALS AND METHODS

Preparation of neuronal and glial fractions. Cerebral cortices from 8 rats, six weeks old, were used for each experiment. Tissue was digested in a collagenase-hyaluronidase incubation medium for 60 min and cells separated by gentle sieving on a ball vibrator as previously described.[12] Neuronal and glial fractions were separated in a Ficoll-sucrose gradient by centrifuging in the Spinco SW-25.1 rotor at 63 000 *g* for 90 min.[13] The neuronal fraction containing about 4.5 mg of protein was collected at the 30 % Ficoll-2 M sucrose interphase and the glial fraction containing 5.5 mg of protein at the 10 – 20 % Ficoll interphase. The morphology of the cells and purity of the fractions was characterized by light microscopy as described recently.[13] For electron microscopy the cell fractions were fixed in 2.5 % glutaraldehyde in 0.32 M sucrose and 0.2 M phosphate buffer, pH 7.2 for 90 min and sedimented at 800 *g*. The pellet was prepared according to Sabatini *et al.*[14]

Incorporation of ^{3}H-leucine

The cell fractions obtained from the gradient were repeatedly washed with buffer sedimenting at 300 *g*. Finally 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mM glucose was added. Aliquots containing 0.5 ml of the cell suspensions were shaken at a rate of 60/min on a water bath at 37° for 10 min. After the preincubation 10 μCi 4,5-^{3}H-leucine (specific activity 17 Ci/mmol, Amersham) in a volume of 10 μl was added into each tube and incubation was continued for various times (1, 5, 20, and 120 min) under air.

Stopping incubation. At the end of the incubation the cell suspensions were rapidly poured into 100 ml cold Krebs-Ringer phosphate buffer and sedimented for 3 min.[13] The supernatants were decanted and the walls of the tubes were carefully wiped with paper, holding the tubes upsidedown. 2 ml of cold 10 mM Tris buffer, pH 7.0, was added and the cellular pellets were collected with a Pasteur pipette for homogenization in a Potter-Elvehjem homogenizer about 5 min after stopping the incubation.

The homogenate was frozen and thawed three times and centrifuged at 100 000 *g* for 30 min. By definition the proteins recovered in the 100 000 *g* pellet were called insoluble and those recovered in the supernatant were called soluble.

TCA-soluble fraction and soluble proteins. The 100 000 *g* supernatant was mixed with an equal volume of cold 12 % TCA and the solution centrifuged. An aliquot of the supernatant was added to Bray's scintillation fluid to determine the TCA-soluble radioactivity representing intracellular ^{3}H-leucine. Results for TCA soluble radioactivity were expressed per unit protein summing the soluble and insoluble protein. The TCA-precipitate was washed three times with 6 % TCA, twice with ethanol and once with ethanol-chloroform, ethanol-ether, and ether. The residue was dissolved in 0.5 N NaOH. Protein content of this alkaline solution was measured according to Lowry *et al.*[15] The remaining NaOH solution was added into Bray CAB-O-SIL gel for determination of radioactivity of the soluble protein.

Insoluble proteins. The 100 000 *g* pellet containing the insoluble proteins was extracted twice with Tris buffer to wash the soluble radioactivity away. The resulting pellet was dissolved by repeated freeze-thawing in 250 μl of the sample solvent containing 50 mM K_2SO_3, 8 M urea, 10 % mercaptoethanol and 5 % Triton X-100, pH 9 – 10.[5] A 100 μl aliquot of this solution was precipitated by 6 % TCA and protein and radioactivity were determined as above. The remaining 150 μl were saved for electrophoresis.

Polyacrylamide gel electrophoresis of insoluble proteins

Polyacrylamide gel electrophoresis was performed according to Lim and Tadayyon [5] by replacing *N,N'*-methylene bisacrylamide by ethylene diacrylate (Borden Chemical Co.) in the small pore solution to make the gel alkali labile.[16] Tubes 15 cm long were used with a current of 2.5 mA/tube until the methyl green dye had reached the

cathode end of the gel. Protein (400 – 600 μg) was applied in a volume of 150 μl onto each gel for electrophoresis. Staining was performed in a solution containing 1 % amido black, 10 % acetic acid, and 10 % methanol for 15 min. For destaining the gels were floated in acetic acid-methanol solution for several days. Relative mobilities of the bands were calculated in relation to the methyl green front.

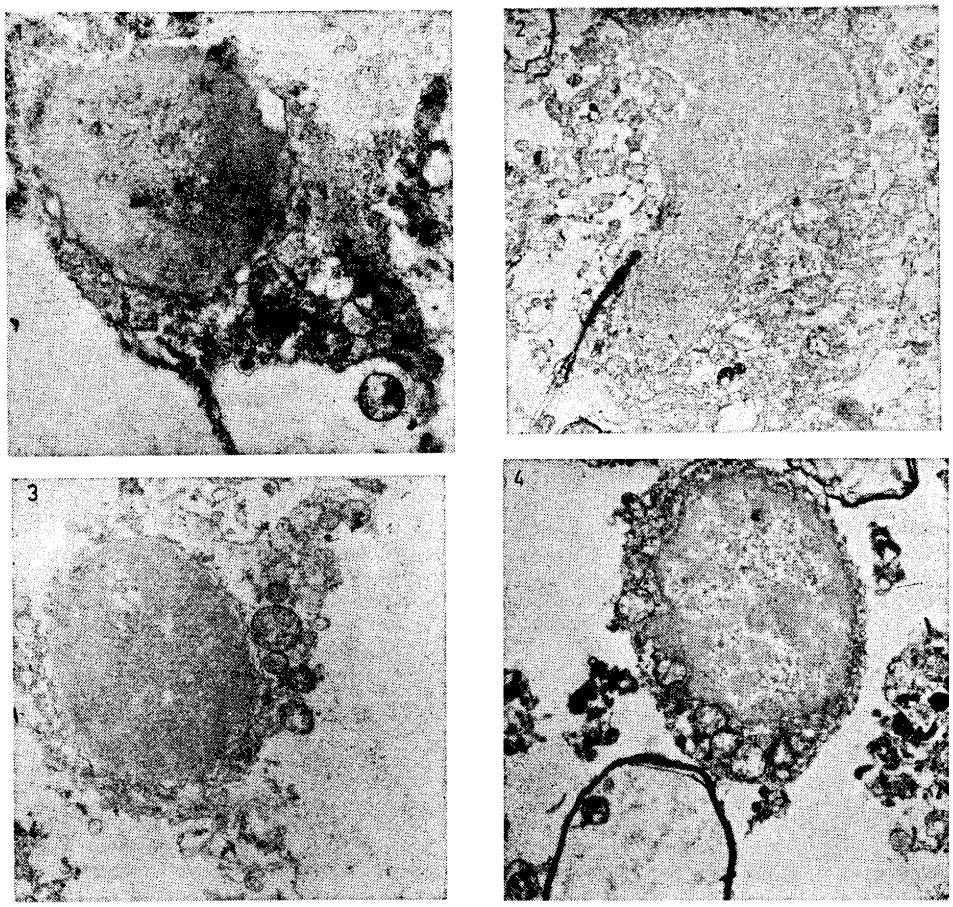
Determination of specific radioactivities of proteins in gels. The protein discs developed in the gels were cut out on an apparatus set for slices of 2 mm by thickness. 8 gels were cut at a time carefully checking that each disc was entirely contained in a slice. The slices were depolymerized in 0.5 ml of 0.5 N NaOH. Optical density of amido black was measured at 625 nm for protein [17] and 10 ml Bray CAB-O-SIL was added for liquid scintillation counting. External standardization was used to correct for quenching. Results are expressed as dpm/mg of protein. The technique for determination of specific radioactivities of protein discs has been shown to be reproducible [18] within standard errors of less than 10 %. Soluble and insoluble brain protein separated by electrophoresis and stained were cut out of the gel and amido black measurement was followed by protein measurement using Lowry's method. A linear relationship between protein content and binding of amido black was demonstrated, parallelling the findings of Davies [17] for serum albumin.

RESULTS

Integrity of cell structure as interpreted by electron microscopy. The neuronal fraction (Figs. 1 and 2) contained cells with relatively abundant cytoplasm and rich Nissl substance surrounded by a plasma membrane, frequently discontinuous. Conforming well to criteria established *in situ*,[19] these cells were identified as neurons. A small number of glial cells and endothelial cells were also present. Cells in the glial fraction (Figs. 3 and 4) could be classified at least in two groups: One containing cells with long, thin processes, a uniformly dense nucleus and mitochondria as the most prominent cytoplasmic organelle (Fig. 3); the other consisted of round cells with a thin rim of cytoplasm and a nucleus with dense islands of chromatin (Fig. 4). These cell types closely resemble those described by Raine *et al.*[20] and accordingly the former is suggested to be astroglia and the latter oligodendroglia. Plasma membrane interruptions were also encountered in these cells.

Accumulation of ^{3}H-leucine into the TCA-soluble pool and cellular protein. Uptake of radioactive leucine by the neuronal and glial fractions (Fig. 5a) proceeded rapidly to a level characteristic of the cell type. The neurons accumulated radioactive leucine maximally at 270 000 dpm/mg protein compared to 430 000 dpm/mg protein of the glia. Maximal labelling of the intracellular precursor pool occurred at 20 min in glia and later, though not precisely determined, in neurons. Incorporation of radioactive leucine into neuronal proteins (Fig. 5b) increased up to 2 h. The specific radioactivity of the insoluble protein was on average 5 times higher than that of the soluble protein. As the dilute buffer used to extract soluble proteins dissolved 20 % of the neuronal protein, the total radioactivity present in the soluble protein fraction is only 4 %.

The glial incorporation (Fig. 5c) differed in two respects from the neuronal. First, incorporation into the insoluble protein reached a maximum at 20 min; second, the rate of incorporation is initially only 35 % of the neuronal incorporation dropping to 15 % at 20 min. The proportion of radioactivity incorporated into the glial soluble protein is, like in neurons, around 20 % of the total. The amount of extractable protein is higher in the glia, *i.e.* 35 %.



Figs. 1 to 4. Electron micrographs of neuronal and glial pellets fixed in 2.5 % glutaraldehyde. *Fig. 1.* A single cell from a neuronal pellet, 4000 ×, showing some mitochondria, plasma membrane and a synapse. *Fig. 2.* A neuron 2300 ×, with typically prominent rough endoplasmic reticulum. *Fig. 3.* A cell from a glial pellet identified as astroglia, 4000 ×, with processes, prominent mitochondria, very little rough reticulum and a nucleus with dense chromatin. *Fig. 4.* A cell from a glial pellet identified as oligodendroglia, 4000 × with narrow but dense cytoplasm and a nucleus with chromatin islands.

Patterns of insoluble proteins in polyacrylamide gels. Gel electrophoretic patterns of the neuronal and glial insoluble proteins (Fig. 6) appear to show both quantitative and qualitative differences. Bands N2 to N6 seem to be unique for the neuronal fraction while glial band G13 cannot be detected in the neuronal gel. The intensities of bands N2 to N5 in the neuronal fraction varied from one gel to another and by electrophoretic mobility they were concluded to originate from erythrocytes contaminating the neuronal fraction by 6 %.[13] The patterns of the slowly moving proteins in the two gels, G16 to G22 and N8 to N12, are on the other hand roughly similar.

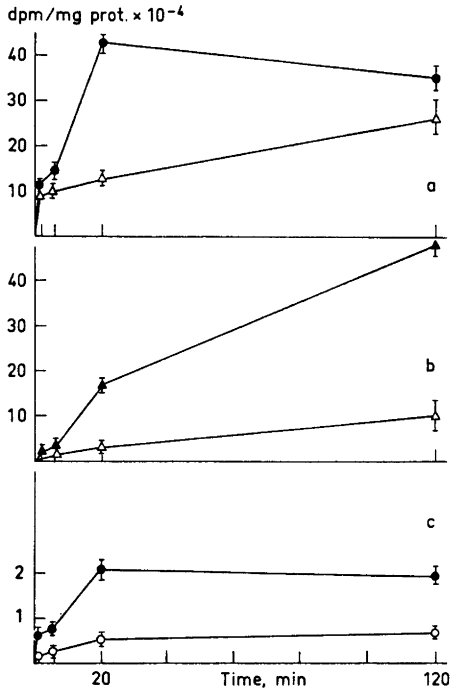


Fig. 5. Uptake and incorporation of[^{3}H]-leucine by the neuronal and glial cell fractions. Cells were incubated in 0.5 ml with 10 μCi [4,5-^{3}H]leucine for various times. (a) TCA-soluble radioactivity of neurons △ and glia ●. (b) TCA-precipitable radioactivity of neurons; insoluble proteins ▲, soluble proteins △. (c) TCA-precipitable radioactivity of glia; insoluble proteins ●, soluble proteins ○. Means of two experiments, ranges indicated.

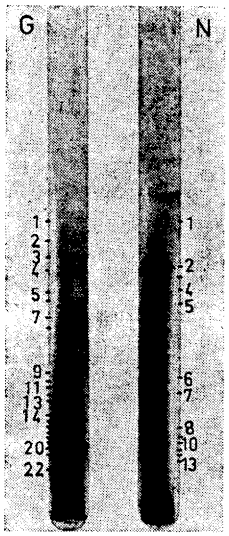


Fig. 6. Polyacrylamide gels of neuronal (N) and glial (G) insoluble proteins prepared according to Lim and Tadayyon.[5] Origin is at the bottom. Numbering refers to Tables 1 and 2 listing specific radioactivities of the individual bands.

Specific radioactivities of the protein discs. Specific radioactivities of the most prominent bands seen and numbered in Fig. 6 are shown in Tables 1 and 2. Specific radioactivities of the neuronal and glial proteins follow the labelling of the total insoluble fractions quoted in Fig. 5. The highest specific activities in the neuronal fraction are detected in the bands moving slowest. The difference between the smallest and the largest specific activity is only 7 fold indicating that all insoluble proteins analysed incorporate ^{3}H-leucine at a rather uniform rate. Radioactivities determined for glial discs also follow kinetics determined by the radioactivity of the precursor pool, most of the bands labelling maximally at 20 min.

Table 1. Relative mobilities and specific radioactivities of individual insoluble proteins of the neuronal fraction after polyacrylamide gel electrophoresis. Cell fractions were incubated with 10μCi of [4,5-^{3}H]leucine. Results are expressed as means of two experiments.

Disc No.	Relative mobility	Specific radioactivity (dpm/mg of protein) × 10^3 at various times (min)		
		5	20	120
N1	0.55	23.6	37.6	173
N2	0.47	5.3	27.8	98.5
N3	0.44	4.4	41.8	100.3
N4	0.42	5.1	30.7	97.4
N5	0.39	11.7	45.0	97.1
N6	0.26	11.3	50.3	144
N7	0.23	11.9	44.0	188
N8	0.16	9.4	55.3	268
N9	0.15	14.2	119	418
N10	0.14	23.6	109	690
N11	0.12	20.6	137	465
N12	0.11	18.2	198	422
N13	0.10	27.0	196	640

Table 2. Relative mobilities and specific radioactivities of individual insoluble proteins of the glial fraction. Results are expressed as means of two experiments.

Disc No.	Relative mobility	Specific radioactivity (dpm/mg of protein) × 10^3 at various times (min)		
		5	20	120
G1	0.53	15.2	15.6	13.4
G2	0.51	9.6	13.6	14.0
G3	0.48	8.3	15.6	12.6
G4	0.45	28.3	26.1	10.5
G5	0.41	16.4	35.1	15.3
G6	0.40	10.2	30.0	20.9
G7	0.36	18.2	25.5	31.1
G8	0.34	14.9	22.7	19.4
G9	0.26	21.8	48.8	30.8
G10	0.25	23.3	23.1	17.1
G11	0.23	5.2	29.0	18.6
G12	0.22	34.0	30.7	26.8
G13	0.21	10.2	23.6	22.3
G14	0.19	7.1	24.5	19.5
G15	0.17	18.1	22.4	24.3
G16	0.16	15.1	35.1	25.4
G17	0.15	10.6	27.9	16.3
G18	0.14	21.4	16.5	24.1
G19	0.14	11.9	69.0	22.9
G20	0.12	9.9	38.8	21.1
G21	0.11	6.2	24.3	25.3
G22	0.08	7.9	28.1	28.9

DISCUSSION

Electron microscopy of brain cell fractions has been presented by several authors [21–23] but satisfactory morphology and cell classification has been published only by Raine *et al.*[20] after careful examination of the preparative procedures. In this paper we show cells essentially similar to those of Raine *et al.*[20] using standard electron microscopic preparation. Comparing the fractions shown here to preparations *in situ* [19,24] it is evident that the separation procedure has affected the cells; membranes are frequently discontinuous and organelles show swelling and rarefaction. This must be kept in mind when considering the data presented. They primarily describe the function of organelles associated with glia and neurons, respectively, and they can be extrapolated to the level of intact cells only with extreme caution. For comparison it is necessary to obtain similar data on the function of isolated organelles and such work is indeed being carried out in our laboratory.

The glial cell fraction accumulates ^{3}H-leucine from the incubation medium more rapidly than the neuronal, reaching three times higher radioactivity. At 20 min the precursor has freely mixed with the endogenous amino acid pool in glia, while a longer time is needed for neurons. These results are in agreement with our earlier observations [13] quoting two times higher TCA-soluble radioactivity for glia after one hour incubation. Similarly Hamberger [25] has recently reported a higher glial capacity for accumulation of a number of amino acids. Rose [26] has found that the glial amino acid pool is more rapidly metabolized than the neuronal, although smaller. Kinetically the uptake of labelled amino acids by brain slices is largely analogous to the cell suspension equilibrating with the endogenous pool at 30 min.[27–29]

Incorporation of amino acids into protein is close to linear up to 20 min in the cell suspension. Neuronal proteins and glial soluble proteins continue incorporation at a decreasing rate up to 2 h bearing analogy to brain slices. By contrast, incorporation into the glial insoluble protein reaches a maximum at a level of 25 000 dpm/mg protein showing rapid degradation or decreased protein synthesis. Interestingly much of the brain proteolytic activity has been found in membraneous fractions [11] and particularly in grey matter and myelin [30] closely related to glia. Yet no exceptional *in vivo* turnover of the glial protein has been observed by Blomstrand and Hamberger.[31]

The early leveling of glial incorporation curve suggests a relatively more dominant role for mitochondrial protein synthesis in glia compared to neurons. Incorporation of isolated mitochondria usually levels off at around 20 min while brain polysomes incorporate in a linear fashion for some hours.[32,33] Such an observation is further supported by electron microscopy showing an abundance of mitochondria compared to rough endoplasmic reticulum in isolated glia (Figs. 3 and 4). Additionally, higher specific radioactivities are measured in glial than in neuronal mitochondria after *in vitro* incubation.[34]

The radioactivities of the individual neuronal protein bands (Table 1) constantly increase with time indicating net deposition of radioactive protein. Judged by electrophoretic controls of isolated subcellular organelles (Hemminki, in preparation) the slowly moving bands (Fig. 6) most likely originate from microsomal and mitochondrial membrane structures. The rapid labelling

of these proteins parallels our finding *in vivo* (Hemminki, in preparation) accentuating the active metabolism of membrane proteins in brain. Most of the glial proteins on the other hand label maximally at 20 min showing an equilibrium between protein synthesis and degradation. Whether the apparently different maxima of some proteins (Table 2) imply existence of protein pools with unique incorporation kinetics cannot be estimated with the present accuracy of the technique. Although the rate of incorporation into neuronal and glial proteins is strikingly different, the protein patterns are largely similar in gel electrophoresis (Fig. 6). The few bands, probably unique for the cell fraction, show no exceptional incorporation features. Packmann *et al.*[35] reported analogous results for neuronal and glial soluble proteins.

Quantitative differences of the neuronal and glial protein incorporation *in vitro* have been a subject of several studies [26,36,37, 13] generally quoting 2 to 5 times higher incorporation into the neuronal fraction. In this study the difference is found to depend on the incubation time. If the 20 min values are taken for comparison the difference is 7 fold. As the specific activity of the precursor is probably much lower in the neuronal fraction, the efficiency of incorporation may be at least 15 times higher in neurons. The actual rate of protein synthesis in the two cell types cannot be derived from these data because the specific radioactivity of the precursor is not known and rapid degradation of protein may very much obscure the estimation.

For comparison some approximations can be given. The rate of incorporation of labelled leucine is around 8 pmol (mg protein h)$^{-1}$ for the neuronal insoluble protein and 0.5 pmol (mg protein h)$^{-1}$ for the glial insoluble protein. To derive the absolute rate of incorporation the specific activity of the precursor pool should be known. The concentration of labelled leucine was 1.2 μM and around 1 mg protein was in each incubation vessel. Arbitrarily taking the data of Jones and McIlwain [29] on tissue amino acid pools, we can very approximately calculate that for absolute incorporation the above rates should be multiplied by around four, and yet no provision is made for the rapid release of amino acids during the incubation as described by Jones and McIlwain.[29] Tiplady and Rose [37] presented similar data quoting an incorporation of 4 to 11 pmol (mg protein h)$^{-1}$ for labelled lysine.

At least two principal techniques are currently available for preparing cell suspensions from brain tissue. One introduced by Rose [21] based on mechanical sieving, has been characterized by light and electron microscopy and by enzymatic and metabolic studies [21,38,22] and is now used in several modifications.[31,23] The other is based on enzymatic digestion with trypsin [39] or with collagenase-hyaluronidase.[12] The cells prepared by collagenase-hyaluronidase incubation have been characterized by light and electron microscopy and their ability to accumulate amino acids and potassium, to consume oxygen and to incorporate radioactive amino acids in protein.[13] In this study electron microscopic findings are presented to show the relatively well preserved structural organisation of the preparation. Uptake and incorporation of ^{3}H-leucine by the neuronal and glial cell fractions largely follows kinetics quoted for brain slices. High specific radioactivities incorporated into protein and relatively large yields of material obtained recommend collagenase – hyaluronidase preparation even for detailed studies of individual proteins.

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