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# Endosomal association of a protein phosphatase with high dephosphorylating activity against a coronavirus nucleocapsid protein

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On the assumption that dephosphorylation of the neurotropic coronavirus JHM (JHMV) nucleocapsid protein (N) may be connected with initiation of the infectious cycle we searched for a relevant host enzyme activity. Analysis of subcellular fractions from L-2 murine fibroblasts, separated by dual Percoll density gradients, revealed the presence of a phosphoprotein phosphatase (PPPase), co-sedimenting with the endosomal/prelysosomal material, which possesses high activity against N. With purified [$^{32}$P]N as substrate it was demonstrated that this PPPase, distinguishable from acid and alkaline phosphatases, acts optimally at neutral pH in the presence of $Mn^{2+}$ following treatment with a detergent. Complete inhibition with okadaic acid at 0.9–4.5 μM but not at 1–10 nM relegates this PPPase to a type 1 protein phosphatase. Similar PPPase activity for N was present in the endosome fraction of a rat Roc-1 astrocytoma-oligodendrocyte cell line and in homogenates of brain and cultured oligodendrocytes. Our data suggest that the phosphorylated N of the inoculum may be modified by the endosomal PPPase in host cells, including those from the CNS so as to facilitate the JHMV infectious process.

Phosphoprotein phosphatase; Endosome; Coronavirus; Nucleocapsid protein

## 1. INTRODUCTION

Protein phosphorylation is known to be fundamental in the regulation of numerous cellular processes [1]. In the case of viruses differential states of phosphorylation can profoundly modulate cell–virus interactions and generally influence the infection [2, 3]. Protein phosphatases (PPases) of several well-categorized classes [4], acting in concert with protein kinases, also affect many cellular processes but have not been shown, heretofore, to be involved directly in viral functions.

Coronavirus JHM (JHMV), a neurotropic strain of mouse hepatitis virus (MHV), causes demyelinating and encephalitic diseases in rats and mice. Demyelinating lesions in the white matter of the rat CNS presumably occur due to the tropism of JHMV for glial cells of the oligodendrocytic lineage [5]. Evidence from several studies indicates that the infectious process which commences with penetration may be initiated at or near the cell surface, perhaps following sequestration of the inoculum in an endosomal compartment with a neutral pH milieu [6–8].

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*Abbreviations:* EDTA, Ethylenediaminetetraacetate, disodium salt; EGTA, Ethyleneglycol-bis(β-aminoethyl ether)-*N, N, N', N'*-tetraacetic acid; PMSF, phenyl methyl sulfonyl fluoride; *p*NPP, *p*-nitrophenyl phosphate.

In this paper, we present data regarding partial characterization of a phosphoprotein phosphatase, associated with the endosomal fraction of host cells (PPPase), which is highly active against the nucleocapsid protein (N) of JHMV. Among the 3 major structural proteins of JHMV, N is phosphorylated [9] and has the capacity to bind nucleic acid [10]. Implications of dephosphorylation of N on early host–virus interactions are discussed.

## 2. EXPERIMENTAL

### 2.1. *Materials*

Okadaic acid was purchased from Moana Bioproducts, Hawaii. Acid phosphatase of potato was obtained from Nutritional Biochemical Corporation, Ohio, alkaline phosphatase of *E. coli* from Sigma, St. Louis and Percoll from Pharmacia.

### 2.2. *Virus and cell cultures*

Propagation of L-2 murine fibroblasts [11] as monolayers and coronavirus JHMV, including plaque assays (pfu), followed procedures described previously [5]. Suspension cultures of L-2 cells for biochemical studies were grown in MEM supplemented with 8% Nu-Serum plus 2% FBS. The immortalized Roc-1 cell–cell hybrid line, of rat $C_6$ glioma × primary oligodendrocyte (a kind gift from Dr. F.A. McMorris, The Wistar Institute, Philadelphia, PA), was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1.2 g/l $NaHCO_3$ and 1 × HAT supplement (Gibco). Primary cultures enriched for rat oligodendrocytes (95% pure) were isolated and cultured for 10 days as described previously [12].

### 2.3. *Homogenates and subcellular fractionation and characterization*

Homogenates obtained by a Dounce homogeniser of oligodendrocyte and whole brain from 2-day-old rats were suspended in

TPEL buffer (10 mM Tris·HCl (pH 7.4), 1 mM EGTA, 50 μg PMSF per ml and 2 μg leupeptin per ml).

Subcellular fractionation was achieved by means of centrifugation through dual Percoll gradients as in Merion and Perez [13]. The fractions were collected starting from the bottom of the tube.

### 2.4. *Marker enzyme assays*

Acid phosphatase was assayed according to Pallen and Wang [14]. Lactate dehydrogenase (EC 1.1.1.27) using the Sigma Diagnostic reagent kit (LD-L) (Sigma Diagnostics, St. Louis, MO), alkaline phosphatase as described by Lee et al. [15], (except that the reaction mixture, made up to a volume of 1.0 ml with distilled water, contained 1 μmol *p*NPP, 20 μmol $MgCl_2$ and 50 μmol Tris·HCl, pH 9.0), the ouabain-sensitive ($Na^+$,$K^+$)-dependent ATPase activity as described by Cates and Holland [16] and β-galactosidase as in Hall et al. [17]. Protein was measured according to Lowry et al. [18].

### 2.5. *Electron microscopy of subcellular fractions*

The biochemically characterized lysosomal and endosomal fractions were sedimented at $10^5 \times g$ for 60 min into pellets. These were fixed with 1% buffered glutaraldehyde, post-fixed in $O_5O_4$ and processed for ultramicrotomy and examination in a Philips EM 300 as described previously [19].

### 2.6. *Purification of $^{32}$P-labelled viral nucleocapsids*

Confluent L-2 cell monolayers in 600 $cm^2$ trays were inoculated with JHMV at an m.o.i. of 0.01 pfu/cell. When 10–15% of the monolayer became fused into syncytia, usually 12 h after infection at 32°C, the cultures were incubated for 2 h in phosphate-free medium at 37°C. Then 2 mCi [$^{32}$P]orthophosphate (DuPont, Canada, specific activity: 8500 Ci/mmol) was added and syncytiogenesis allowed to proceed to completion. The cell material was washed thrice with cold PBS, scraped and suspended in 1.5 ml $H_2O$ per tray, then disrupted by squeezing through a syringe tipped with a 30-gauge hypodermic needle. The resulting lysate, buffered with TMEN-6 (Tris-maleate 50 mM, EDTA 1 mM and NaCl 0.1 M, at pH 6.0) was centrifuged at $7000 \times g$ for 15 min to obtain a supernatant fraction containing the nucleocapsid component. Membranes were solubilized with NP-40 at a final concentration of 0.5% by shaking at 0°C for 30 min. Following centrifugation at $1.3 \cdot 10^5 \times g$ for 20 h through a 15 to 50% (w/w) sucrose gradient in TMEN-6 containing 0.1% NP-40, layered over a 65% sucrose cushion, 1 ml fractions were collected from the bottom and analyzed for N by SDS polyacrylamide gel electrophoresis (SDS-PAGE) [20] and where appropriate, also by Western blotting [21]. The bottom 4 gradient fractions containing concentrated N were pooled for preparative SDS-PAGE. Visualization in the gel by 4 M sodium acetate [22] permitted precise excision and extraction of the N band from the gel, as described by Pruslin and Rodman [23]. The extract was clarified by centrifugation and filtration and dialyzed against distilled water. Homogeneity and purity of the product was assessed by autoradiography and Western blotting, using a monoclonal (Mab) anti-N antibody (kindly provided by M. Buchmeier of the Scripps Clinic and Research Foundation, La Jolla).

### 2.7. *Phosphoprotein phosphatase assays*

The reaction mixture consisted of 25 mM Tris-maleate buffer (pH 7.0), 1 mM $MnCl_2$, 0.1% Triton X-100, $^{32}$P-labelled N and the enzyme fraction (20–100 μg protein) in a total volume of 100 μl. After incubation at 30°C for 90 min the reaction was terminated by addition of 400 μl ice-cold 25% TCA plus 50 μl 1% BSA and left overnight at 0°C. $^{32}$P released into the supernatant by enzyme action was assayed by the method of Maeno and Greengard [24]. Qualitative assessment of dephosphorylation was obtained by comparing the autoradiogram of SDS-PAGE separations of control and experimental reaction mixtures on 10% acrylamide gels.

## 3. RESULTS

A protein phosphatase activity against purified N protein, which could be activated with Triton X-100 was detected in L-cell homogenates. Subcellular fractionation intended to separate membraneous organelles was carried out by means of dual Percoll gradients [13]. The data regarding distribution of marker enzymes obtained from such fractionation are summarized in Fig. 1 and Table I. The markers for lysosomes and endosomes were acid phosphatase and β-galactosidase. The activities were distributed in 3 peaks in gradient I (Fig. 1, Percoll I). Peak 1, at the bottom of the gradient presumably contained the denser lysosomes. Peak 2, contained both lysosomal/endosomal marker enzymes and the plasma membrane ouabain sensitive $Na^+$,$K^+$-ATPase. Peak 3 was associated with cytosolic material, marked by the presence of lactate dehydrogenase and alkaline phosphatase. When subcellular materials isolated in peak 2 of the first Percoll gradient were centrifuged through the less dense second Percoll gradient, endosomal marker enzymes became distributed near the bottom, clearly separated from the plasma membrane

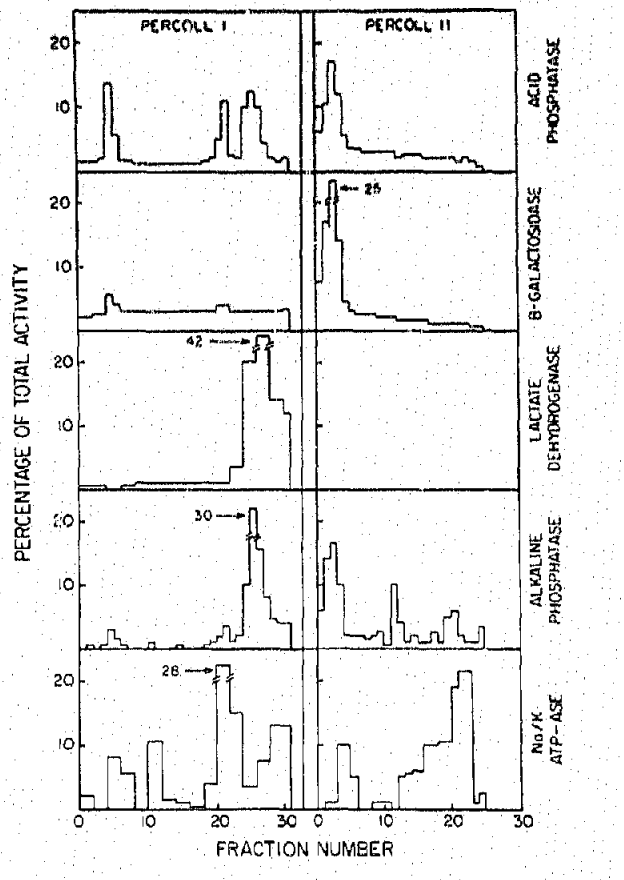


Fig. 1. Profiles of enzyme activities present in L-2 subcellular fractions separated by centrifugation through gradients. Profiles of marker enzyme distribution describe activities measured in fractions isolated from Percoll I (panels on left) and Percoll II (panels on right) gradients. Isolated fractions 21 and 22 from Percoll I were combined and separated by Percoll II gradient. The representative data shown were derived from one of several similar experiments.

Table I

Subcellular distribution of a phosphoprotein phosphatase from L-2 cells, active on JHMV nucleocapsids

| Cell fraction | % Standard activity |
|---|---|
| Total homogenate | 100 |
| Total homogenate without detergent | 29 |
| Nuclear pellet | 249 |
| Post-nuclear supernatant | 147 |
| Lysosomal | 512 |
| Cytosolic | 443 |
| Endosomal | 2550 |

Preparation of cell fractions and enzyme assays were described in section 2. Standard activity is the measure of dephosphorylation obtained with 50 μg cell homogenate in the presence of 0.1% Triton X-100 at 30°C for 90 min. Approximately 0.5 μg of purified $^{32}$P-labelled N protein, with a content of about 1000 cpm, was present as the substrate. The protein-bound, remaining radioactivity was quantitatively recoverable in TCA precipitates. Triton X-100 (0.1%) was present in all reactions except where indicated. Data calculated as the percentages of standard activity represent dephosphorylating activities in different subcellular fractions containing equal amounts of protein (50 μg). The data shown here are representative of several experiments.

enzyme (Fig. 1, Percoll II). The peak 1 fractions from the 1st Percoll gradient and the bottom fractions in Percoll gradient II were enriched, respectively, in lysosomal and endosomal components. Electron microscopic observations, illustrated in Fig. 2, revealed the presence of larger membraneous structures, characteristic of lysosomes, in fraction 5 under peak 1 of Percoll gradient I and much smaller vesicles of the size and morphology associated with endosomes in fraction 3 near the bottom of Percoll gradient II, supporting our biochemical evidence.

The data comparing PPPase activities against [$^{32}$P]N as substrate, relative to the activity in the total cell homogenates, were obtained on subcellular fractions and are shown in Table I. It is evident from these data that the endosomal fraction was enriched 25-fold in this PPPase. Material from Percoll gradient II in the fractions enriched in plasma membranes contained only minor dephosphorylating activity (data not shown). The effect of the endosomal PPPase on the [$^{32}$P]N substrate, was also demonstrated autoradiographically, as illustrated in Fig. 3, supporting the quantitative results.

The endosomal PPPase activity was stable during storage at −20°C for over two months, but profoundly decreased upon freezing and thawing more than once. The pH optimum, basd on measurements in buffers containing sodium acetate, Tris-maleate and Tris-HCl, providing the appropriate pH range, occurred at about neutrality (data not shown). $Mn^{2+}$ at 1 mM was the required divalent cation for optimum PPPase activity,

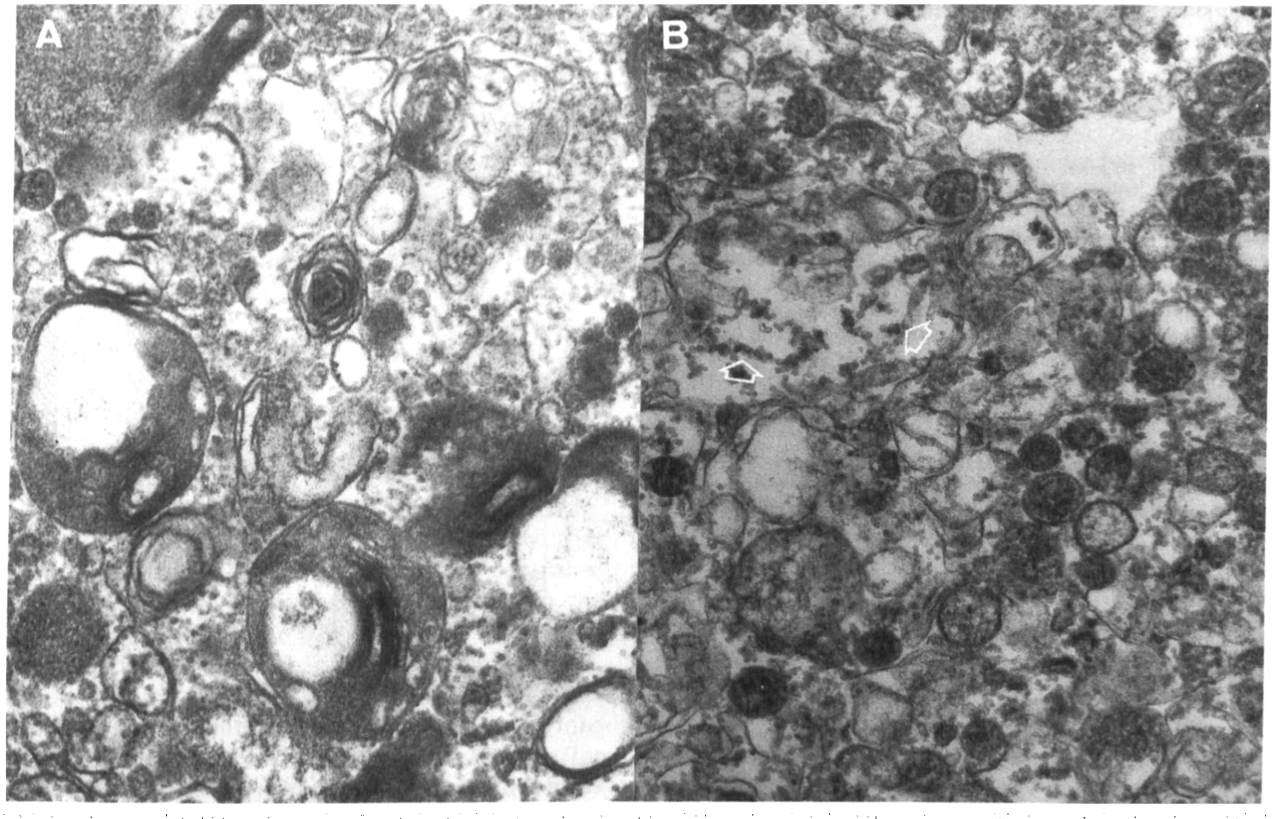


Fig. 2. Selected examples of thin sections prepared from pellets of cell fractions characterized biochemically as lysosomes (A) and endosome (B). The membrane enclosed organelles in (A) contain large quantities of multilayered membraneous material. In (B) the vesicles are smaller and some contain dense material. The 'granular' background is due to aggregates of spherules of uniform size (arrows), most probably the remnants of Percoll used in gradients for separation of cell fractions. A and B magnified × 53 000.

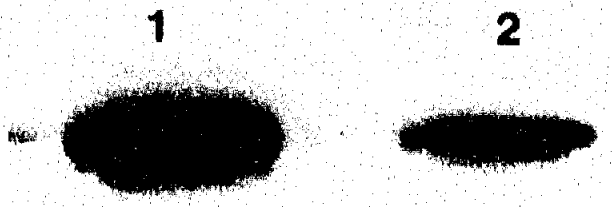


Fig. 3. Dephosphorylation of N by endosomal PPPase. The assay was performed as described in section 2. The figure illustrates an autoradiogram of $^{32}$P-labelled N separated by SDS-PAGE after incubation in the absence (lane 1) and presence (lane 2) of the endosomal fraction of L-2 cells. In a parallel quantitative experiment the enzyme preparation released over 40% of the phosphorous from N.

Table II

Effect of divalent cations, sodium fluoride and okadaic acid on PPPase and acid phosphatase from L-2 cell endosomes

| Addition | Activity of endosomal fraction on | |
|---|---|---|
| | [$^{32}$P]N at pH 7.0 (% dephosphorylation) | pNPP at pH 5.5 (nm/mg protein) |
| None | 9.1 | - |
| $Fe^{2+}$, 1 mM | 0.0 | - |
| $Zn^{2+}$, 1 mM | 3.0 | - |
| $Ca^{2+}$, 1 mM | 14.0 | - |
| $Mg^{2+}$, 1 mM | 14.0 | - |
| $Mn^{2+}$, 1 mM | 28.8 | 424.5 |
| $Mn^{2+}$, 1 mM + NaF, 30 mM | 19.5 | 17.3 |
| $Mn^{2+}$, 1 mM + O.A., 0.9 μM | 6.05 | - |
| $Mn^{2+}$, 1 mM + O.A., 4.5 μM | 1.15 | - |

Activity of PPPase on [$^{32}$P]N was monitored by determining the release of $^{32}$P from the substrate as described in section 2 and expressed as % dephosphorylation. Acid phosphatase was assayed with *p*-nitrophenyl phosphate as substrate. The reactions were run for 90 min. '-' denotes not done; O.A., okadaic acid.

Table III

Activity of acid and alkaline phosphatases on the N protein substrate

| Enzyme used | pH of reaction | PPP'ase activity (% dephosphorylation) |
|---|---|---|
| L-cell endosomes | 7.0 | 51[a] |
| Acid phosphatase from potato | 5.5 | 0 |
| Acid phosphatase from potato | 7.0 | 0 |
| Alkaline phosphatase from *E. coli* | 9.0 | 0 |
| Alkaline phosphatase from *E. coli* | 7.0 | 0.2 |

Commercially obtained acid phosphatase from potato and alkaline phosphatase from *E. coli* were tested initially at concentrations which possessed the same activities with *p*-nitrophenyl phosphate as substrate as the endosomal PPPase from L-2 cells. The reactions were run for 90 min at 30°C. Similar results were obtained using 10-fold concentrations of the commercial enzymes.

[a]In this experiment the endosomal preparation had exceptionally high dephosphorylating activity, the usual values being 30–40%.

while $Ca^{2+}$ and $Mg^{2+}$ were less effective and $Zn^{2+}$ and $Fe^{2+}$ were inhibitory (Table II). The PPPase in the endosomal fractions could be differentiated from any contaminating acid phosphatase by the degree of inhibition in the presence of 30 mM NaF: the acid phosphatase was reduced by 96%, while the neutral PPPase by only 33%, as evident from Table II.

Okadaic acid, a known inhibitor of protein phosphatases, was used to demonstrate that the endosomal PPPase belongs to the type 1 or 2A enzyme category of Cohen et al. [25]. Pretesting the inhibition by okadaic acid in a series of concentrations (data not shown) revealed that in the range 0.9–4.5 μM inhibition was 80–96% (Table II) whereas inhibition was absent at 1–10 nM. On this basis we relegated the endosomal PPPase to a type 1 serine-threonine protein phosphatase rather than to a tyrosine-specific phosphatase [26]. This was confirmed by demonstrating in standard assays dephosphorylation of $^{32}$P-labelled casein and histone 2B (data not shown). Specificity of the viral N protein as a substrate for the endosomal PPPase was shown by a lack of dephosphorylating activity with acid (from potato) and alkaline (from *E. coli*) phosphatases (data in Table III). For an objective comparison of these data it should be noted that assays were conducted employing [$^{32}$P]N at the pH and concentration appropriate for each of the 3 enzymes, following preliminary testing using *p*NPP as a non-specific substrate.

In the context of our more general interest in infections by JHMV within the CNS of rodents [30], we examined neural cells and tissues for the presence of

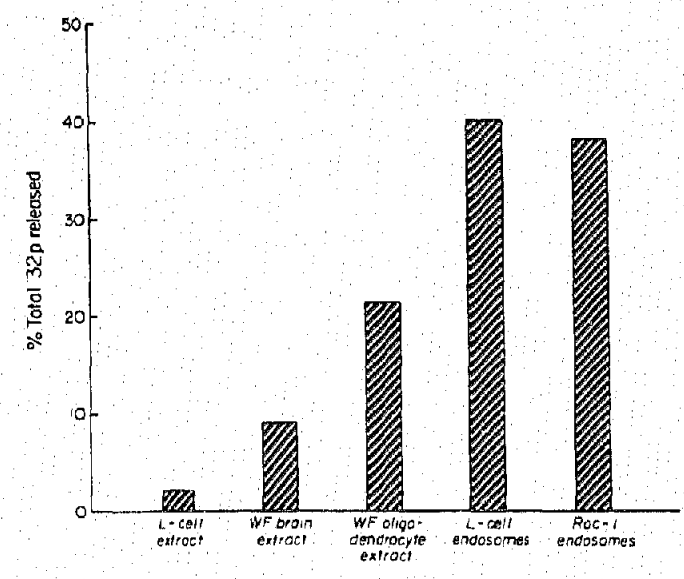


Fig. 4. Comparison of PPPase activity in brain tissue and neural cells with that of L-2 cells. The data from one of several representative experiments are expressed as percent dephosphorylation of [$^{32}$P]N by enzyme in samples containing 50 μg protein during 90 min at 30°C. Comparable assays on neonatal rat liver and kidney homogenates gave PPPase values of 1.5% and 2% respectively. WF = Wistar Furth rats.

PPPase(s) acting on [$^{32}$P]N substrate. We compared PPPase assays on rat material, including extracts of neonatal brain, primary oligodendrocytes and a defined endosomal fraction from oligodendrocyte × $C_6$ astrocytoma Roc-1 cells, with endosomes from L-2 cells. Since it is impossible to determine from which particular cell type endosomes originate when starting with the heterogenous cell population in brain tissue and was not possible to obtain a sufficient number of purified primary oligodendrocytes for isolating endosomes, we were unable to obtain any data directly on endosomal PPPase in these two materials. It is, however, evident from Fig. 4, that homogenates from brain and primary oligodendrocytes contained respectively about 5 and 12 times more PPPase, per 50 μg protein, than homogenates of L-2 cells. Assays made on liver and kidney tissue homogenates demonstrated that the comparable PPPase activities, 1.5% and 2% respectively, were lower than those in brain and approximated those found in whole homogenates of L-2 cells. The endosomal preparations from L-2 and Roc-1 cells contained comparable PPPase activity. Therefore, the PPPase which may play a role in the infection of the CNS by JHMV is abundantly active in neural cells.

## 4. DISCUSSION

Presence of a serine-threonine type 1 endosomal PPPase activity, evidently specific in the dephosphorylation of a viral nucleocapsid protein N, can be detected in murine L cell fibroblasts, rat glial cell line, explanted oligodendrocytes and brain tissue, drawing attention to the possible role of this enzyme in the infectious process of a neurotropic coronavirus JHMV. Activation of this PPPase in the presence of detergent suggests that the enzyme is latent, perhaps residing on the endosomal membrane rather than in the fluid milieu of the endosome. It has not been established whether the PPPase occurs at the external or cytoplasmic face of this organelle. The presumed role for a neutral PPPase localized in endosomes during early virus-cell interactions is consistent with clear evidence that coronaviruses, unlike many other enveloped agents, do not pass through an acidic compartment to initiate their infectious cycles [8]. It is, of course, well established that contents of endosomal vesicles progress from neutral to an acidic milieu as these organelles migrate from the surface towards the interior where, due to membrane fusions and proton pumps they become prelysosomes, then lysosomes with an acidic content [28, 29]. The RNA genomes of coronaviruses may gain access into the cytosol near the cell surface, conceivably soon after inoculum virions have been sequestered inside early endosomes. Thus a neutral PPPase concentrated in early endosomes would be in a strategic position to dephosphorylate the coronavirus N component of nucleocapsids after internalization of the inoculum virions.

As to the biological significance of specific dephosphorylation of the viral component by the endosomal PPPase, our previous studies [30, 31] indicated that molecules of N out of which the protective coat around the RNA genome is made, become rapidly hydrolized after infection. Dephosphorylation may be the initial step required for the processing of N. This idea led us to hypothesize that the PPPase activity, described here, initiates dissociation of N from the nucleocapsid so as to promote uncoating of the RNA. The role of the PPPase may also be of relevance in infections within the CNS, where JHMV is specifically tropic for cells of the oligodendrocytic lineage both in young rats and CNS explants [5]. Susceptibility to JHMV infection is lost when the progenitors acquire the phenotype of mature oligodendrocytes. The block due to maturation, which is apparent subsequent to attachment and sequestration, could involve the uncoating step. The non-permissive state of mature oligodendrocytes for JHMV could be related to the cAMP dependent protein kinase metabolism, specifically involving a notable upregulation of the regulatory subunit RI of protein kinase type I [30]. Since RI can suppress the endosomal PPPase when added to an in vitro assay system [31], there appeared to exist in oligodendrocytes an interrelationship between induction of R and inhibition of the endosomal PPPase. The consequence of reduced PPPase activity in oligodendrocytes might be an adverse effect on the uncoating of JHMV affecting viral expression in the CNS.

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