Pediat. Res. 9:143–146 (1975)

Cerebrospinal fluid
Herpesvirus hominis encephalitis
thymidine kinase

Thymidine Kinase Activity in Cerebrospinal Fluid of Rabbits with *Herpesvirus hominis* Encephalitis

MILO D. HILTY,[22] D. C. THOMAS, R. E. HAYNES, AND H. G. CRAMBLETT

Department of Pediatrics and Medical Microbiology, The Ohio State University College of Medicine and the Children's Hospital Research Foundation, Columbus, Ohio, USA

Extract

The rabbit model of *Herpesvirus hominis* (HVH) encephalitis was utilized to determine whether thymidine (TdR) kinase (EC. 2.7.1.2.1), an early enzyme in the HVH replicative cycle, is present in the cerebrospinal fluid (CSF) of rabbits with HVH encephalitis. The mean TdR kinase activity in CSF from infected rabbits was 2.30 ± 1.69 pmol product/120 min/100 μl CSF, while the mean TdR kinase activity of uninfected CSF was 0.34 ± 0.25. The enzyme appeared to be cell associated since the TdR kinase activity in the sedimented cell suspension was 5.08 ± 2.93 (seven values) while the supernatant activity was 0.14 ± 0.31 (seven values). To allow a comparison of TdR kinase from infected rabbit CSF with HVH and rabbit mononuclear cell TdR kinase, the mean ratio of TdR kinase activity at pH 6.0/pH 8.0 was calculated. The mean ratio of thymidine kinase activity, pH 6.0/pH 8.0, for infected rabbit CSF is intermediate between the ratio for HVH TdR kinase ($P < 0.001$) and rabbit mononuclear cell TdR kinase ($P < 0.05$).

Speculation

The presence of TdR kinase in CSF from HVH-infected rabbits with biochemical properties intermediate between HVH and mononuclear cell TdR kinase suggests that abortive HVH infection occurs in CSF mononuclear cells during the course of HVH encephalitis.

Herpesvirus hominis is rarely recovered from the CSF of patients with HVH encephalitis, and the isolation of HVH from brain tissue specimens is usually necessary to make a definite diagnosis (1, 3, 8, 11, 17). Since intact infectious HVH is so infrequently recovered from the CSF, we have elected to search for a nonvirion product of HVH replication in the CSF. HVH thymidine kinase was chosen because it is a nonvirion protein, an early enzyme in the HVH replicative cycle, and an enzyme which has had some of its biochemical properties established (4–6, 10). The rabbit model of HVH encephalitis was utilized to evaluate some of the quantitative and qualitative aspects of this enzyme in CSF of infected rabbits.

MATERIALS AND METHODS

VIRUS

Herpesvirus hominis, designated strain 0-1116, was isolated from a brain biopsy specimen of a 15-year-old boy with HVH encephalitis. Original isolation was performed in human diploid fibroblast (W1-38 cells) and the virus was identified as HVH type 1 (HVH-1) by Dr. André J. Nahmias of Emory University.

CELL CULTURE

W1-38 human diploid fibroblasts were maintained in Eagles minimum essential medium (Eagle's MEM) which contained 2% fetal calf serum, $NaHCO_3$ 30 mEq/liter, penicillin 250 units/ml, streptomycin 200 μg/ml, polymyxin 12.5 units/ml, and chlortetracycline 25 μg/ml.

Monkey kidney (BGM) cells, an established line of epithelial cells derived from green monkey kidneys, were grown in 32-ounce Brockway bottles and maintained with Eagle's MEM as described above, and used for *in vitro* thymidine kinase production.

VIRUS ISOLATION

Brain tissue was removed within 12 hr after rabbits died and placed in collecting media which consisted of Eagle's MEM, 3% fetal calf serum, 1.0 M Tris buffer, pH 7.0, amphotericin B 5 μg/ml, penicillin 500 units/ml, polymyxin 25 units/ml, chlortetracycline 50 μg/ml, and streptomycin 400 μg/ml. The brain tissue was then frozen at −70° until attempts to isolate virus were carried out. To isolate the virus from brain tissue, the brain specimens were thawed and immediately macerated with a Tenbröck tissue grinder and the suspension was inoculated into W1-38 cells (0.3 ml/tube). Adsorption was carried out for 60 min, and the medium was exchanged. Cultures were then observed for cytopathogenic effect (CPE) daily for 7–10 days. HVH was identified by the appearance of its characteristic CPE 1–3 days after inoculation. The CSF specimens were frozen at −70° until they were thawed and inoculated (0.2 ml/tube) onto W1-38 cells and observed for CPE as described previously.

ENZYME

An extract containing thymidine (TdR) kinase activity was prepared using HVH-1 strain 0-1116 which had been "passaged" in W1-38 cells. Each 32 ounce Brockway bottle containing a confluent sheet of BGM cells was infected with 2×10^6 $TCID_{50}$ HVH 0-1116 and incubated for 20–40 hr at 37°. The culture medium was then removed, the infected cells were washed with Eagle's MEM and the enzyme was released from cells by freezing and thawing. The resulting cell suspension was centrifuged at 2000 × *g* for 30 min and the supernatant was decanted, pooled, and frozen at −70° until TdR kinase assays were performed.

THYMIDINE KINASE ASSAY

The method of Ives *et al.* (9) was used with some modifications. The reaction mixture contained ATP 25 mM, $MgCl_2$ 12.5 mM, Tris-maleate buffer 250 mM, 2-[^{14}C] thymidine 80 μM (specific activity 57 mCi/mmol) at pH 6.0, 7.0, or 8.0. Reaction mixture (25 μl) plus 100 μl sample (infected rabbit CSF, negative control rabbit CSF, positive control HVH-1 enzyme, or rabbit mononuclear cells) were mixed together in the cold before incubation at 37° for 120 min (final concentration of reagents: ATP 5 mM, $MgCl_2$ 2.5 mM, Tris buffer 50 mM, 2-[^{14}C] thymidine 19 μM). The zero time assays contained 100 μl sample which had boiled for 2 min and cooled before the addition of 25 μl reaction mixture. Zero time reaction mixtures were then incubated in parallel with the 120-min reaction time samples at 37°. After the incubation period, all tubes were boiled for 2 min. The supernatant (10 μl) was then applied to 2 cm SB-2 resin-loaded ion exchange paper (18) in duplicate. The discs were then washed in 0.0001 M ammonium formate on a model 3623 Thomas rotating apparatus for 20 min in 1,000-ml beakers (8–10 discs/150 ml). The ammonium formate was changed once during agitation. The discs were then removed, dried, and placed in plastic liquid scintillation vials which contained 15 ml scintillation mixture (13) *p*-bis-(*O*-methylstyryl)-benzene 3.42 g, 2-(4-*t*-butylphenyl)-5-(4-biphenylyl-1,3,4-oxadiazole) 34.2 g, and 2,6-di-*t*-butyl-1,4-methylphenol in 3.8 liters toluene) and counted for at least 8,000 counts/vial in a Packard Tri-Carb liquid scintillation spectrometer. The counting efficiency in this system is 60%. The thymidine kinase activity is expressed either as picomoles of thymidine phosphorylated per minute per liter × 10^6 white blood cells (WBC) or picomoles of thymidine phosphorylated per 120 min/100 μl sample. In the present assay system, 1 pmole thymidine phosphorylated/unit time represents a difference of 125 dpm between the zero time incubation tube and the experimental tube; only results with a difference of 375 dpm (3 pmol thymidine phosphorylated/unit time) between zero time tube and experimental were accepted.

MONONUCLEAR CELLS

Mononuclear cells were separated on a Ficoll Hypaque gradient as described by Boyum (3). With this procedure, the preparation contained 90–95% mononuclear cells. The TdR kinase was released from the cells by freezing and thawing the cell suspension and was sedimented at 2,000 × *g* in a refrigerated centrifuge. The supernatant was removed, aliquoted, and frozen at −70° until TdR kinase assays were performed.

RABBITS

Encephalitis was produced in 8–10-week-old New Zealand White rabbits by abrading the cornea and inoculating each eye with 10^3–10^4 $TCID_{50}$ of HVH-1 0-1116. The neurovirulence of the virus was enhanced by repeated intracerebral inoculation of rabbits and recovery in W1-38 cells. The rabbits utilized in this study were infected by corneal inoculation and developed encephalitis 5–7 days after inoculation and had either died or were killed by the 21st day.

The CSF specimens from uninfected and HVH-1-infected rabbits were collected by cisternal puncture. Immediately after collection, the CSF specimens were placed on ice, an aliquot was removed for a mononuclear cell count, and the remainder was frozen at −70° until the specimen was assayed for TdR kinase and HVH.

RESULTS

The rabbit model of HVH-1 encephalitis has features which are similar to our experience with HVH-1 encephalitis in children. The mononuclear cell counts ranged from 100–500 cells/mm^3, seizures were common, temperature elevation was present in all animals, and HVH was not recovered from any of the CSF specimens from infected rabbits.

The CSF specimens from 10 uninfected rabbits and 7 rabbits with HVH-1 encephalitis, documented by recovery of HVH-1 from brain tissue, were assayed for TdR kinase activity at pH 6.0. The mean TdR kinase activity in CSF from infected rabbits was 2.30 ± 1.69 pmol thymidine phosphorylated/120 min/100 μl CSF, whereas the mean TdR kinase activity of uninfected rabbit CSF was 0.34 ± 0.25. The difference is statistically significant at $P < 0.01$. The TdR kinase activity in infected rabbit CSF was most reliably detected when the mononuclear cell count/mm^3 was 500 or greater (Fig. 1). When these values utilizing >375 dpm difference are used, there is a direct relation between TdR kinase activity and WBC per cubic millimeter.

To determine this relation, the TdR kinase activity in CSF from infected rabbits was confined to the cellular portion, CSF from seven infected rabbits was centrifuged at 2,000 × *g* for 20 min, and TdR kinase assays were performed on the sedimented cell suspension and the supernatant. Enzyme activity in the sedimented cell suspension was 5.08 ± 2.93 (seven values) while the supernatant activity was only 0.14 ± 0.31 (for seven values), a difference which was significant at $P < 0.001$. Two possible explanations for the localization of TdR kinase to the cellular fraction of the CSF were considered and evaluated. (*1*) The TdR kinase activity represents the TdR kinase normally present in mononuclear cells which responds to an inflammatory process (14). (*2*) The TdR kinase activity represents TdR kinase induced by HVH-1 in mononuclear cells. To evaluate the source of TdR kinase in CSF, the pH optimum was utilized to differentiate HVH-1 from mononuclear cell TdR kinase. The pH optima of the two TdR kinase preparations were evaluated at pH 6.0, pH 7.0, and pH 8.0 (Fig. 2). The HVH-1 TdR kinase had maximal activity at pH 6.0, whereas the mononuclear cell TdR kinase had maximal activity at pH 8.0.

To evaluate the effectiveness of measuring TdR kinase at pH 6.0 and pH 8.0 to differentiate HVH-1 TdR kinase from mononuclear cell TdR kinase, pooled concentrated CSF specimens from three groups of HVH-1-infected rabbits were utilized. There were 18 rabbits in the three groups and HVH was recovered from the brain tissue of 17. Seven sets of pooled CSF specimens were obtained from the three groups 9–17 days after inoculation of the cornea with HVH-1. The WBC and TdR kinase activity of these specimens is presented in Table 1. The WBC in the pooled concentrated CSF specimens ranged from 498/mm^3 to 2,776/mm^3 and the mean activity of TdR kinase at pH 6.0 was less than the mean specific activity at pH 8.0. When these results are compared with the data from

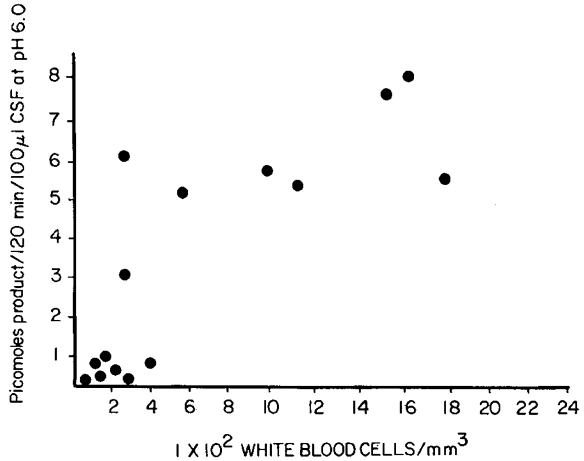

Fig. 1. Comparison of thymidine kinase activity at pH 6.0 with cerebrospinal fluid white blood cell count.

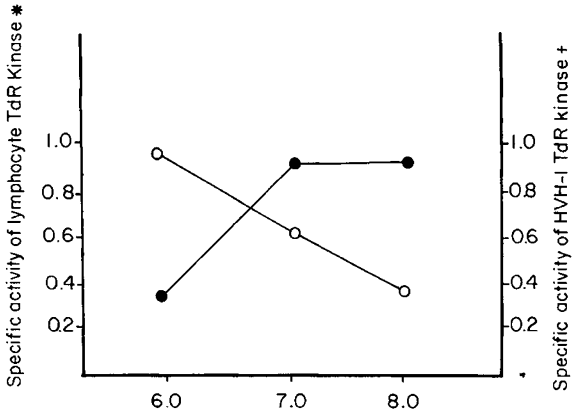

Fig. 2. Thymidine kinase activity of lymphocyte (white blood cells) and herpesvirus hominis type 1 (*HVH-1*) at pH 6.0, 7.0, and 8.0. *: picomoles of thymidine phosphorylated per liter × 10^6 white blood cells/min; †: picomoles of thymidine phosphorylated per microgram of protein per minute. ○: HVH-1 thymidine kinase (*TdK*) activity; ●: rabbit lymphocyte thymidine kinase activity.

Table 1. *Comparison of thymidine kinase specific activity of herpesvirus hominis-infected rabbit cerebrospinal fluid (CSF) mononuclear cells with rabbit peripheral blood mononuclear cells*

	Thymidine kinase specific activity[1]	
White blood cells/mm³	pH 6.0	pH 8.0
Infected rabbit CSF		
498	0.62	0.88
528	0.97	1.52
2,776	0.62	0.64
838	0.45	0.33
2,198	0.45	0.73
768	0.89	0.98
868	0.64	1.48
	0.64 (+0.22)[2]	0.94 (+0.44)[2]
Rabbit peripheral blood mononuclear cells		
3,865	0.38	0.84
512	0.62	1.65
1,576	0.27	0.73
1,296	0.21	0.47
	0.38 (±0.19)[2]	0.92 (±0.51)[2]

[1] Thymidine kinase specific activity expressed as picomoles of thymidine phosphorylated/min/1 × 10^6 white blood cells.

[2] Mean thymidine kinase specific activity (± SD).

peripheral rabbit mononuclear cells, two differences are noted. The mean TdR kinase specific activity of 0.64 from infected rabbit CSF at pH 6.0 is greater than the TdR kinase specific activity of 0.38 from peripheral rabbit mononuclear cells at pH 6.0 ($P < 0.05$). At pH 8.0 the mean specific activity of the two TdR kinase preparations is the same. These results indicate that the CSF from infected HVH rabbits contains TdR kinase with biochemical properties which are different than that of mononuclear cell TdR kinase.

To allow a comparison of TdR kinase from infected rabbit CSF with HVH-1 and peripheral rabbit mononuclear cell TdR kinase, the mean ratio of TdR kinase activity at pH 6.0/pH 8.0 was calculated (Table 2). With this method of expressing the pH optima, the mean ratio of thymidine kinase activity pH 6.0/pH 8.0 for infected rabbit CSF is intermediate between the ratio for HVH-1 TdR kinase ($P < 0.001$) and rabbit mononuclear cell TdR kinase ($P < 0.05$). The presence of TdR kinase in the CSF with activity at pH 6.0 relatively greater than that of uninfected rabbit mononuclear cells is consistent

Table 2. *Comparison of ratio of thymidine kinase activity at pH 6.0/pH 8.0 from infected rabbit cerebrospinal fluid (CSF) with thymidine kinase activity from peripheral blood mononuclear cells and herpesvirus hominis type 1 (HVH-1)*[1]

Source (no. values)	$\frac{\text{(TdR kinase at pH 6.0)}}{\text{(TdR kinase at pH 8.0)}}$
HVH-1 (11)	2.31 ± 0.95[2]
Infected rabbit CSF (7)	0.80 ± 0.30[2]
Peripheral blood mononuclear cells (4)	0.41 ± 0.05[2]

[1] Difference between HVH-1 and infected rabbit CSF was statistically significant at $P < 0.001$ while difference between infected rabbit CSF and peripheral blood mononuclear cells are significant at $P < 0.05$.

[2] Mean thymidase kinase specific activity ± SD.

with the induction of HVH-1 TdR kinase in CSF of mononuclear cells. Other possible explanations for these results are (*1*) mononuclear cells undergoing blastogenic transformation and (*2*) contamination of CSF with peripheral blood mononuclear cells. To evaluate these possibilities, TdR kinase activity from mononuclear cells of infected rabbits and uninfected rabbit mononuclear cells stimulated with phytohemagglutinin (PHA) was determined at pH 6.0 and pH 8.0. The ratio of TdR kinase activity pH 6.0/pH 8.0 for peripheral blood mononuclear cells from infected rabbits was 0.41 and the ratio for PHA-stimulated mononuclear cells was 0.30. These results indicate that the TdR kinase from rabbits with HVH-1 encephalitis has biochemical properties which are different than the TdR kinase of HVH-1 and peripheral rabbit blood mononuclear cells. In addition, the difference does not appear to be the result of blastogenic transformation of mononuclear cells or contamination of CSF specimens with blood mononuclear cells.

DISCUSSION

The presence of TdR kinase in CSF from HVH-1-infected rabbits with properties intermediate between HVH-1 and mononuclear cell TdR kinase suggests that HVH-1 TdR kinase is produced during the course of HVH-1 encephalitis. The site of HVH-1 TdR kinase production in this system appears to be the mononuclear cell and is consistent with the observation that HVH can replicate in leukocytes (12). However, in the HVH leukocyte system, HVH replication occurred only when the leukocytes were stimulated with PHA. Since HVH is rarely recovered from CSF, it is possible that central nervous system leukocytes can not support productive HVH infection and only abortive HVH infection occurs. The presence of TdR kinase in CSF with properties intermediate between HVH-1 TdR kinase and mononuclear cell TdR kinase is consistent with a composite of HVH-1 TdR kinase, produced during an abortive infection in mononuclear cells and mononuclear cell TdR kinase from cells responding to the inflammatory process.

If nonvirion products of HVH replication are present in the CSF with greater frequency than virion products of HVH replication during the course of HVH encephalitis it may explain the paucity of positive results that have been obtained with virus isolation from CSF (1, 3, 8, 11, 17) and demonstration of HVH antigen in CSF with indirect immunofluorescent procedures (7, 16). It appears that a more appropriate diagnostic approach would be the detection of early nonvirion products of viral replication in CSF utilizing either sensitive enzyme assays or immunologic procedures.

SUMMARY

The diagnosis of HVH encephalitis in man is difficult to establish unless the virus is either recovered from brain tissue or CSF. Because HVH is so infrequently recovered from CSF

we have chosen to determine whether TdR kinase, an early enzyme in the HVH replicative cycle, is present in the CSF of rabbits with HVH encephalitis. The rabbit model of HVH encephalitis was utilized to evaluate this hypothesis and to partially characterize the CSF TdR kinase.

Infected rabbit CSF contained significantly more TdR kinase than uninfected rabbit CSF and the enzyme activity was optimal at a pH intermediate between the optimal pH for either HVH or rabbit mononuclear cell TdR kinase. These data suggest that abortive HVH infection occurs in CSF mononuclear cells during the course of HVH encephalitis and that detection of this early enzyme in CSF could be utilized to provide indirect evidence of HVH central nervous system infection.

REFERENCES

1. Armstrong, C.: *Herpes simplex* virus recovered from spinal fluid of suspected case of lymphocytic choriomeningitis. Pub. Health Rep., *58:* 16 (1943).
2. Boyum, A.: Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. Suppl., *97:* 77 (1968).
3. Brunell, P. A., and Dodd, K.: Isolation of herpes virus hominis from cerebrospinal fluid of child with bacterial meningitis and gingivostomatitis. J. Pediat. *65:* 53 (1964).
4. Buchan, A., Tuff, S., and Wallia, C.: Failure to demonstrate the interaction of subunits of thymidine kinase in cells simultaneously infected with herpes virus and a kinaseless mutant. J. Gen. Virol., *9:* 229 (1970).
5. Buchan, A., and Watson, D. H.: The immunological specificity to thymidine kinases in cells infected by viruses of the herpes group. J. Gen. Virol., *4:* 461 (1969).
6. Buchan, A., Watson, D. H., Dubbs, D. R., and Kit, S.: Serologic study of a mutant of herpesvirus unable to stimulate thymidine kinase. J. Virol., *5:* 817 (1970).
7. Dayan, A. D., and Stodes, M. I.: Rapid diagnosis of encephalitis by immunofluorescent examination of cerebrospinal fluid cells. Lancet, *i:* 177 (1973).
8. Hunt, B. P., and Comer, E. O. B. Herpetic meningo-encephalitis accompanying cutaneous herpes simplex. Amer. J. Med., 19: 814 (1955).
9. Ives, D. H., Furham, J. P., and Tucker, V. S.: Rapid determination of nucleotide kinase and nucleotidase activities with tritium-labeled substances. Anal. Biochem., *28:* 192 (1969).
10. Klemper, H. G., Haynes, R. E., Shedden, W. I. H., and Watson, D. H.: A virus-specific thymidine kinase in BHK21 cells infected with herpes simplex virus. Virology, 31: 120 (1967).
11. Nahmias, A. J., Alford, C. A., and Korones, S. B. Infection of the newborn with herpesvirus hominis. Advan. Pediat., *17:* 185 (1970).
12. Nahmias, A. J., Kibrick, S., and Rosan, R. C.: Viral leukoycyte interrelationships. I. Multiplication of a DNA virus—Herpes simplex—in human leukocyte cultures. J. Immunol., *93:* 69 (1964).
13. Pollack, J. D., Somerson, N. L., and Senterfit, L. B.: Characterization and immunogenicity of mycoplasma pneumoniae membranes. Infect. Immun., *2:* 326 (1970).
14. Rabinowitz, Y., and Wilhite, B. A.: Thymidine salvage pathway in normal and leukemic leukoyctes with effects of ATP on enzyme control. Blood, *33:* 759 (1969).
15. Rabinowitz, Y., Wong, P., and Wilhite, B. A.: The effect of phytohemagglutinin on enzymes of thymidine salvage of cultured chronic lymphatic leukemia lymphocytes. Blood, *35:* 236 (1970).
16. Sommerville, R. G.: Rapid identification of neurotropic viruses by an immunofluorscent technique applied to crebrospinal fluid cellular deposits. Arch. Virusforschung, *29:* 63 (1966).
17. Stalder, H., Oxman, M. N., Dawson, D. M., and Levin, M. J.: Herpes simplex meningitis: Isolation of herpes simplex virus type 2 from cerebrospinal fluid. New Engl. J. Med., *289:* 1296 (1973).

18. Reeve Angel, Clifton, N. J.
19. The authors would like to thank William Zaayer for his competent technical assistance.
20. This study was presented in part at the Society for Pediatric Research annual meeting, May 1972, in Washington, D.C.
21. This investigation was supported in part by United States Public Health Service General Research Support Grant no. RR05505 from the Children's Hospital Research Foundation.
22. Requests for reprints should be addressed to: M. D. Hilty, M. D., Children's Hospital, 561 S. 17th. St, Columbus, Ohio 43205 (USA).
23. Accepted for publication November 20, 1974.

Pediat. Res. *9:* 146–150 (1975)

Brain
cerebellum
cerebrum
malnutrition
Na^+-K^+-ATP phosphohydrolase
neonate
protein
synaptosomes

Development of Na^+-K^+-ATPase in Neonatal Rat Brain Synaptosomes after Perinatal Protein Malnutrition

JAMES Q. KISSANE AND ERVIN J. HAWRYLEWICZ[23]

Department of Research, Mercy Hospital and Medical Center, Chicago, Illinois, USA

Extract

Four protein dietary groups of rats were used in this study, namely: 27% casein diet fed throughout gestation and lactation *(group C-C);* 27% gestation, 10% lactation *(group C-D);* 10% gestation, 27% lactation *(group D-C);* 10% throughout gestation and lactation *(group D-D).* Mean body weight of *group D-D* at weaning was 19 g compared with 59 g for *group C-C.*

The specific activity of Na^+-K^+-ATPase in isolated synaptosomes from cerebral and cerebellar tissues increases throughout the lactation period. At 1 and 7 days of age no differences in Na^+-K^+-ATPase activity are noted in the brain regions of *Groups C-C* and *D-D.* At 14 and 21 days, however, the specific Na^+-K^+-ATPase activity was significantly reduced in isolated synaptosomes from both brain regions of the protein-deprived group. ATPase activity in cerebellar synaptosomes from dietary exchange groups *(C-D* and *D-C)*