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DEVELOPMENT OF (Na^+–K^+)-ATPase IN RAT CEREBRUM: CORRELATION WITH Na^+-DEPENDENT PHOSPHORYLATION AND K^+-*para*NITROPHENYLPHOSPHATASE[1]

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Abstract—The activities of (Na^+–K^+)-ATPase and its proposed partial reactions, K^+-*p*NPPase and Na^+-dependent phosphorylation, all increase tenfold relative to microsomal protein between 5 days prior to birth and 60 days postnatally in NaI-treated rat cerebral microsomes, and all reach half of their adult values between the fifth and tenth postnatal day. These increases are concurrent with the most rapid changes in cerebral wet weight. Increases in the amount of the related phosphorylatable polypeptide during development, as estimated by densitometry of Coomassie-stained polyacrylamide gels after electrophoresis of constant amounts of microsomal protein dissolved in sodium dodecylsulfate, parallel the increments in levels of Na^+-dependent phosphorylation. The fraction of total phosphorylation that is Na^+-dependent increases steadily during development, suggesting a precursor role for some of the Na^+-independent fraction. The results are consistent with a single biosynthetic control for the enzymatic sites critical to the partial reactions of (Na^+–K^+)-ATPase. No changes in turnover number or affinity for substrate or ligands were found during development. Little similarity was noted among the age-related changes of Mg^{2+}-ATPase activity, Mg^{2+}-*para*nitrophenylphosphatase activity, and Na^+-independent phosphorylation levels. The most rapid changes in (Na^+–K^+)-ATPase take place during the period corresponding to glial proliferation and neuronal arborization.

(Na^+–K^+)-ATPASE, EC 3.6.1.3, widely distributed in plasma membranes in a variety of tissues, appears to represent the biochemical basis of the membrane Na^+–K^+ pump (SKOU, 1957, 1965). SDS-PAGE of partly purified (Na^+–K^+)-ATPase from mammalian brain reveals two major polypeptide components with molecular weights of about 53,000 and 94,000 (UESUGI *et al.*, 1971). These correspond to the two polypeptides of purified enzyme from other tissues. The overall (Na^+–K^+)-ATPase reaction can be expressed as a series of partial reactions observed *in vitro*, including Na^+-dependent phosphorylation of the larger polypeptide and its subsequent K^+-dependent dephosphorylation. K^+-*p*NPPase activity is believed to represent the phosphatase moiety of the enzyme (see DAHL & HOKIN, 1974). However, some authors disagree on the role of these partial reactions under physiologic conditions *in situ* (SKOU, 1965; ROBINSON, 1970).

Prior studies of (Na^+–K^+)-ATPase activity in rat brain fail to agree on the precise time for the major developmental changes in this enzyme (ABDEL-LATIF *et al.*, 1967, 1970; SAMSON *et al.*, 1964; SAMSON & QUINN, 1967; COTE, 1964; GARCIA ARGIZ *et al.*, 1967; VALCANA & TIMERAS, 1969; BANIK & DAVISON, 1969; MEDZIHRADSKY *et al.*, 1972; TIRRI *et al.*, 1973; MEISAMI & TIMERAS, 1974; KISSANE & HAWRYLEWICZ, 1975; MEISAMI & MANOOCHEHRI, 1977). Information on the kinetics of foetal rat brain (Na^+–K^+)-ATPase is sparse (WILSON & HUNT, 1974) and there have been no reports on the changes in enzyme protein in rat brain during development.

This study attempts to resolve the schedule for development of rat cerebral microsomal (Na^+–K^+)-ATPase in the context of the brain growth spurt by assaying it at several selected ages from 5 days prior to birth to 60 days after birth. We were also interested in observing whether developmental changes would reveal any differences among four parameters presumed to be related to the enzyme: steady-state levels of Na^+-dependent phosphorylation, K^+-*p*NPPase activity, (Na^+–K^+)-ATPase activity, and estimation of the amount of the phosphorylatable polypeptide. Kinetics of foetal and adult (Na^+–K^+)-ATPase are also reported.

MATERIALS AND METHODS

[γ-^{32}P]ATP was obtained from ICN (Irvine, CA). Imidazole, Tris ATP, Tris base, paranitrophenylphosphate, mercaptoethanol, EDTA, Coomassie brilliant blue, phosphorylase a, bovine serum albumin, glucose-6-phosphate de-

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Abbreviations used: *p*NPPase, *para*nitrophenylphosphatase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

hydrogenase, and catalase were obtained from Sigma (St. Louis, MO); sucrose, trichloroacetic acid, glycerol, and KCl, from Malinckrodt (New York, NY); acrylamide, bisacrylamide and bromphenol blue dye from Eastman (Rochester, NY); NaCl and glycine from MCB (Norwood, OH); sodium dodecylsulfate and ammonium persulfate from Fisher (Fairlawn, NJ); and $MgCl_2$ from Baker Chemical Co. (Phillipsburg, NJ).

Preparation of NaI-*enriched microsomes.* Unanesthetized Sprague–Dawley rats of ages 0,1,2,5,10,20 and 60 days were decapitated and the cerebrum, cerebellum, and brain stems were removed into ice-cold 0.32 M-sucrose within 2 min. Fetuses of ages −5, −2, and −1 days prior to birth (gestational ages 16, 19, and 20 days) were obtained by Cesarian section and placed in ice-cold 0.32 M-sucrose within 2 min after the dam was decapitated and the cerebrum, cerebellum, and brain stem were removed. The cerebra were separated from the brain stem and cerebella by an incision through the mesencephalon. Wet weights of the pooled cerebra were obtained. From 9 to 45 brains were utilized in each microsomal preparation.

The cerebra were homogenized in 10 vol of ice cold 0.32 M-sucrose with three 15 s pulses with 30 s pauses in between pulses, at setting of 5 of a Polytron (Brinkmann Inst., Westbury, NY). The homogenate was centrifuged in a Sorvall Superspeed RC2-B at 2°C for 10 min at 600 *g* (2200 rev./min, Sorvall SS 34 rotor). The supernatant was spun at 20,000 *g* (13,000 rev./min) for 1 h. The resulting pellet (PII) was resuspended with six strokes of a hand homogenizer in ice cold 5 mM-EDTA in 20 mM-Tris–HCl (pH 7.4) using one ml of EDTA–Tris per gram of cerebral wet weight. Protein determinations were performed on this suspension and sufficient EDTA–Tris was added to reach 5 mg/ml protein. Then, equal volumes of NaI reagent (NAKAO *et al.*, 1965) were added slowly over ice with slow stirring for 30 min, and the material was centrifuged at 20,000 *g* (13,000 rev./min, SS 34 rotor) for 1 h. The pellet was resuspended in a Virtis model 23 mixer at medium setting at 4°C with three 15 s duration mixings and 30 s pauses in EDTA–Tris and then centrifuged at 40,000 *g* (18,500 rev./min, SS 34 rotor) for 40 min. The resuspension and 40,000 *g* centrifugation were repeated for a total of three washes. Finally, the pellet (final microsomes) was resuspended with six strokes of a hand homogenizer in cold deionized distilled water, 0.25 ml for each gram of cerebral wet weight, and stored in liquid nitrogen until use. 'Adult' rat cerebral NaI-treated microsomes from rats 8–12 weeks old were also used in some of the kinetics studies. Measurements of contaminating $[Na^+]$ in the final microsomal preparations were performed with a Model I Flame Photometer (Technicon Instruments, Chauncey, NY).

Assays. Protein determinations were carried out according to the method of LOWRY *et al.* (1951) with bovine serum albumin used as the protein standard.

The formation of acid-stable non-exchangeable phosphoprotein was measured as follows. Incubations were done at 0°C for 45 s and were initiated by the addition of microsomes, 80 μg of protein, to media containing (final concentration) 75 mM-imidazole–HCl (pH 7.4), 0.1 mM-Tris-[γ-^{32}P]ATP (2×10^8 c.p.m./mol), 3 mM-$MgCl_2$, and either 100 mM-NaCl or 100 mM-KCl in a final volume of 50 μl. Na^+-dependent phosphorylation was taken as the difference between values obtained in the presence of sodium and in the presence of potassium. Na^+-independent phosphorylation was taken as the value obtained in the presence of potassium minus the value obtained after denaturing the enzyme with trichloroacetic acid prior to addition of reaction mixture. Phosphorylation was terminated by the addition of 50 μl of cold 10% trichloroacetic acid and the remaining steps were carried out as described (SIEGEL & ALBERS, 1967).

ATPase activity was measured at 37°C for 10 min in media containing 2–8 μg microsomal protein in (final concentrations) 75 mM imidazole–HCl (pH 7.4), 3 mM-Tris-[γ-^{32}P]ATP ($8 - 25 \times 10^5$ c.p.m./mol), 3 mM-$MgCl_2$, 10 mM-KCl, in the presence and absence of 80 mM-NaCl, in a final volume of 40 μl. The reaction was terminated by the addition of 10 μl of 5% ammonium molybdate in 4 N-H_2SO_4. After addition of 50 μl of isobutanol, the tubes were vortexed vigorously for 15 s and centrifuged at 2000 *g* for 10 min. Ten μl of the upper phosphomolybdate-containing nonaqueous layer was added to 5 ml of Packard Instafluor and counted in a Packard Model 2420 liquid scintillation counter. (Na^+–K^+)-ATPase activity was taken as the increment due to 80 mM-Na^+. Mg^{2+}-ATPase activity was taken as the value obtained in the absence of Na^+ minus the value obtained in the absence of microsomes.

*p*NPPase activity was assayed by a spectrophotometric method (ALBERS & KOVAL, 1972) after incubation at 37°C for 20 min in media containing 2–8 μg microsomal protein in final concentrations 75 mM-imidazole–HCl (pH 7.4), 5 mM-*p*-nitrophenylphosphate, 5 mM-$MgCl_2$, in the presence and absence of 20 mM-KCl in a final volume of 40 μl. K^+-stimulated *p*NPPase activity was taken as the increment due to 20 mM-KCl. Mg^{2+}-*p*NPPase activity was the difference between the values obtained in the absence of K^+ and the values in the absence of microsomes.

Sodium dodecylsulfate polyacrylamide gel electrophoresis and estimation of amount of catalytic subunit. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) of microsomal samples taken at each age was performed according to published methods (HOKIN *et al.*, 1973). Acid-stable non-exchangeable phosphoprotein was prepared from adult brain as described above and subjected to SDS–PAGE at 4°C. Gels were frozen and sliced horizontally into 1 mm slices with a Bio-Rad (Richmond, CA) gel slicer. Slices were dissolved overnight in 150 μl of 30% H_2O_2 at 50°C in loosely stoppered vials after which Packard Instagel solvent was added to the vials. Radioactivity was measured with a Packard Liquid Scintillation Counter. Another set of companion gels were fixed and stained for protein with Coomassie brilliant blue (FAIRBANKS *et al.*, 1971). A third set of gels were halved lengthwise with a knife, covered with Saran Wrap, and apposed to Kodak XRl film at −20°C for 2–10 days. The films were developed in a Kodak X-Omatic Processor. Molecular weights were estimated from measurements of R_F, relative to Bromphenol Blue as a tracking dye. Phosphorylase a (94,000), bovine serum albumin (58,000), glucose-6-phosphate dehydrogenase (50,000), and catalase (36,000) were used as standards. Densitometry scans of stained gels were made at 550 nm with a Gilford 2412 quartz cuvette and using the 2410-S linear transport and 2220 adapter coupled to a Beckman DU spectrophotometer. The height of the peaks on densitometry scan of the Coomassie-stained protein bands was measured from the peak to the baseline taken as the lowest point near the tracking dye band. As a test for linearity, samples containing 2.5–37.5 μg of adult NaI-treated rat brain microsomal protein were subjected to electrophoresis. The height of peaks measured as de-

scribed was found to be a linear function of the amount of sample protein with an r value of 0.99.

RESULTS

Yield of NaI-*treated microsomes during development*

The amounts of protein recovered in the PII and final microsomal fractions from rat cerebra at various ages are given in Table 1. The amount of protein (mg) contained in the PII (crude microsomal) fraction per gram wet weight of cerebrum declines from 17.4 at −5 days to 13.7 at birth, increases to 18.8 by 10 days, and then falls during the period of myelination to 10.0 by 20 days where it remains at 60 days. If expressed per brain, the protein in PII raises steadily during development, except for a higher than expected value at 10 days. The chaotropic agent, NaI, has moderately different effects at various ages on the percent recovery of PII protein: there is little difference in recovery between −1 and 5 days, but recoveries are lower prior to −1 day and somewhat higher after 10 days with the peak recovery at 20 days. The yield of protein in the final microsomes per gram wet weight of cerebrum increases fairly steadily and reaches a peak at 10 days after which the yield progressively falls. The smooth increments in mean cerebral wet weight (Table 1, Fig. 1) suggest comparability of the different ages since if any age group were sufficiently undernourished or dehydrated a deviation from the curve of cerebral wet weight would be expected.

$(Na^+$–$K^+)$-ATPase and related reactions during development

Figure 1 shows that rat cerebral mean wet weight, microsomal $(Na^+$–$K^+)$-ATPase and K^+-*p*NPPase specific activities, and steady-state levels of Na^+-dependent microsomal phosphorylation all increase similarly during development. The plateau between 20 and 60 days represents approximately ten times the near-term fetal values in each case. A useful reference for purposes of comparison is the time at which 50% of the maximal or adult level is attained (half-adult value). The half-adult value for each of the curves for mean wet weight, $(Na^+$-$K^+)$-ATPase and K^+-*p*NPPase activities, and steady-state levels of Na^+-dependent phosphorylation falls between 5 and 10 days after birth. The half-adult values for $(Na^+$–$K^+)$-ATPase activity, K^+-*p*NPPase activity, and Na^+-dependent phosphorylation calculated per gram wet weight or per whole brain from the data in Table 1 are also found between 5 and 10 days. The parallel increases of $(Na^+$–$K^+)$-ATPase activity and Na^+-dependent steady-state phosphorylation mean that the turnover number (ratio of $(Na^+$–$K^+)$-ATPase activity to the level of phosphorylated intermediate) remains fairly constant over the intervals measured. Thus, the major increments in specific activity cannot be attributed to activational molecular changes in the enzyme during development.

*Mg^{2+}-ATPase, Mg^{2+}-p*NPPase, *and Na^+-independent phosphorylation during development*

There is little similarity in the age-related plots of rat cerebral microsomal Mg^{2+}-ATPase and Mg^{2+}-*p*NPPase activities and steady-state Na^+-independent phosphorylation (Fig. 2). Mg^{2+}-ATPase activity increases 2–3 times while Mg^{2+}*p*NPPase activity changes little between −5 and 60 days. These activities presumably are not directly related to $(Na^+$–$K^+)$-ATPase although it is plausible that they could be

TABLE 1. SUBCELLULAR PROTEIN FRACTIONATION IN DEVELOPING RAT CEREBRUM

Age (days)	−5	−2	−1	0	1	2	5	10	20	60
Number of brains per homogenate	36	45	37	34	41	21	23	16	16	9
Mean cerebral wet weight (g)	0.0352	0.0796	0.101	0.147	0.197	0.222	0.467	0.763	1.10	1.26
Protein (mg) in PII	22.0	53.6	53.0	68.4	126	78.8	199	229	176	106
Protein (mg) in PII/g cerebral wet weight	17.4	14.9	14.2	13.7	15.6	16.9	18.5	18.8	10.0	9.34
Protein (mg) in PII/brain	0.612	1.19	1.43	2.01	3.07	3.75	8.64	14.3	11.0	11.8
Protein (mg) in final microsomes	1.37	5.17	7.68	8.75	21.0	12.5	34.0	49.2	51.8	22.6
Protein (mg) final microsomes/g cerebral wet weight	1.08	1.44	2.06	1.74	2.57	2.69	3.16	4.03	2.95	1.95
Protein (mg) final microsomes/brain	0.038	0.115	0.208	0.257	0.512	0.595	1.48	3.08	3.24	2.51
Protein (mg) final microsomes/protein (mg) in PII	0.0623	0.0965	0.145	0.128	0.167	0.159	0.171	0.215	0.294	0.213

Weighing of cerebra, preparation of microsomes, and protein determinations are described in Methods.

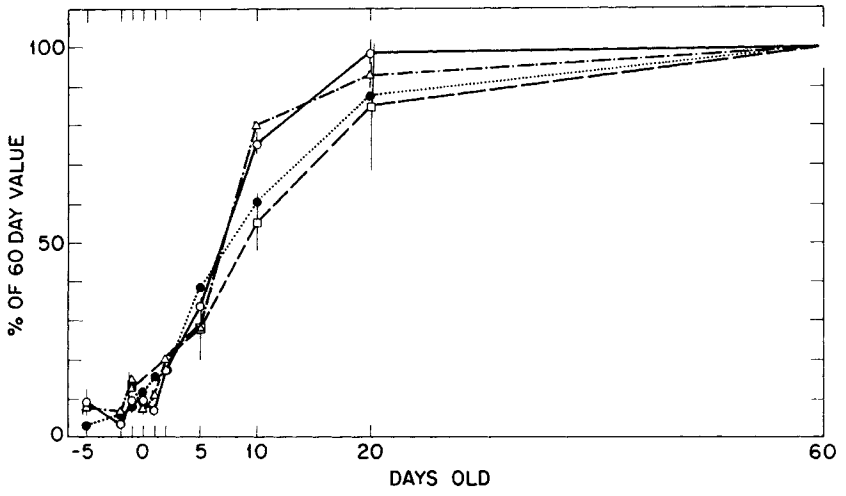

FIG. 1. Age-related development in rat cerebrum of wet weight ●...●, microsomal steady-state Na^+-dependent phosphorylation □---□, K^+-*p*NPPase activity △—·—△, and (Na^+–K^+)-ATPase activity ○——○, expressed as percent of the value at 60 days. At 60 days, the mean wet weight is 1.26 g and the mean ± S.E.M. of at least four separate determinations are steady-state Na^+-dependent phosphorylation 58.9 ± 1.22 nmol·mg^{-1}, K^+-*p*NPPase 100 ± 3.11 nmol·mg^{-1}·min^{-1}, and (Na^+–K^+)-ATPase 416 ± 29.8 nmol·mg^{-1}·min^{-1}.

spuriously elevated slightly due to contaminating Na^+ or K^+ in the microsomal preparations. However, flame photometric determinations of Na^+ in similar rat brain NaI treated microsomal preparations from our laboratory consistently show very low values and after appropriate calculations for final dilutions the contaminating [Na^+] during incubation would be less than 0.01 mM.

The levels of steady-state Na^+-independent phosphorylation decline remarkably during development (Fig. 2). The decline might be due either to loss or inactivation of some specific protein(s) during growth. It is of interest that a graph of the ratio of Na^+-dependent: total phosphorylation (Fig. 3) at each time point very closely approximates the developmental curves of (Na^+–K^+)-ATPase (Fig. 1). This observation would be consistent with a conversion during development of some of the Na^+-independent portion into some of the Na^+-dependent portion of steady-state phosphorylation. The Na^+-dependent fraction

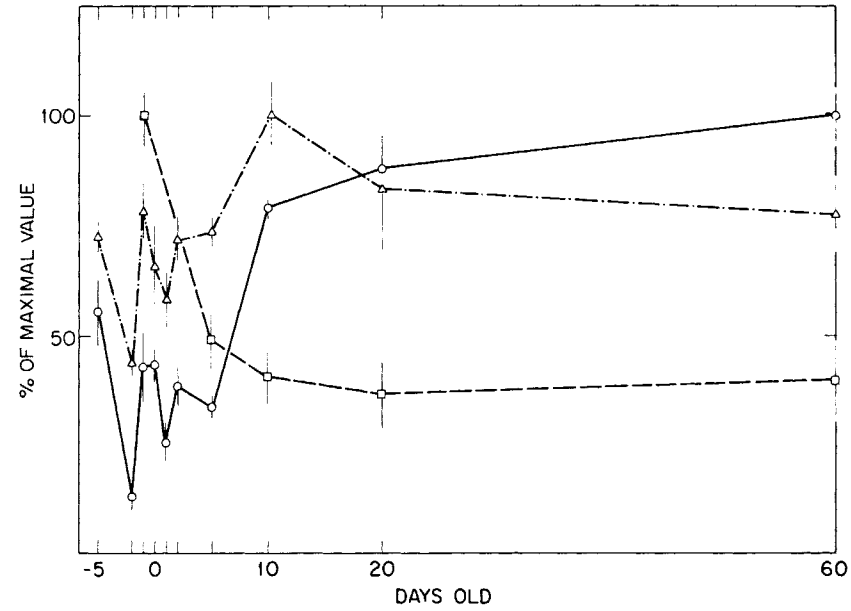

FIG. 2. Age-related development of rat cerebral microsomal steady-state Na^+-independent phosphorylation □---□, Mg^{2+}-*p*NPPase activity △---△, and Mg^{2+}-ATPase activity ○——○, normalized for maximal values. At 60 days, the mean ± S.E.M. of at least four separate determinations are Na^+-independent phosphorylation 31.0 ± 5.55 nmol·mg^{-1}, Mg^{2+}-*p*NPPase 16.1 ± 1.59 nmol·mg^{-1}·min, and Mg^{2+}-ATPase 112 ± 6.05 nmol·mg^{-1}·min^{-1}.

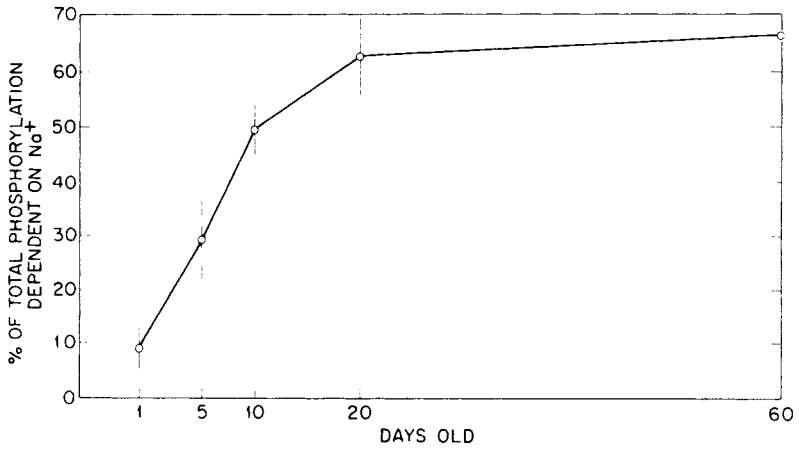

FIG. 3. The fraction of total steady-state phosphorylation that is Na^+-dependent in rat cerebral microsomes at different ages, expressed as means ± S.E.M. of at least four separate determinations.

accounts for no more than 2/3 of the total, a value which is approached asymptotically in the older age ranges (Fig. 3). Of course, these parallel changes may be coincidental and unrelated to each other. The nature of the Na^+-independent phosphorylated products in these foetal and adult brain fractions is not known.

Estimation of the phosphorylatable subunit at various ages

The protein that is phosphorylated by [γ-^{32}P]ATP in the presence of Na^+ migrates as a single band corresponding to a molecular weight of 92,000 ± 3,000 during SDS–PAGE (Fig. 4). After Coomassie staining of gels containing adult rat cerebral microsomal protein, this 92,000 molecular weight chain exhibits the densest band. The R_F of the ^{32}P-labelled phosphoprotein, obtained both by autoradiography of the intact gel and by scintillation counting of 1 mm slices, is identical to the R_F of the densest Coomassie positive band. On densitometry scans, the peak height of the 92,000 molecular weight peptide from adult rat cerebral microsome varies directly with the amount of protein applied to the gel within acceptable error (Fig. 5). The developmental increases in peak height of the 92,000 molecular weight peptide after electrophoresis of 40 μg of microsomal protein for each age (Fig. 6) parallel the time course of steady-state Na^+-dependent phosphorylation (Fig. 1) which is expressed per mg microsomal protein. The baseline (−1 and 0 days) peak height of about 110 mm conceivably is due to incomplete separation of proteins that may or may not have relevance to (Na^+-K^+)-ATPase, or it may reflect levels of inactive precursor(s) of (Na^+-K^+)-ATPase with a molecular weight nearly identical to that of the adult chain. The baseline value for steady-state phosphorylation is arbitrarily defined as the amount of acid-stable ^{32}P-binding in the presence of K^+ and Mg^{2+}. Although the selection of such baselines is open to question, there is no doubt that the increments in densitometry peak height of the 92,000 molecular weight polypeptide follow the same time course as do the increments in Na^+-dependent phosphorylation levels during development (Fig. 1 and 6).

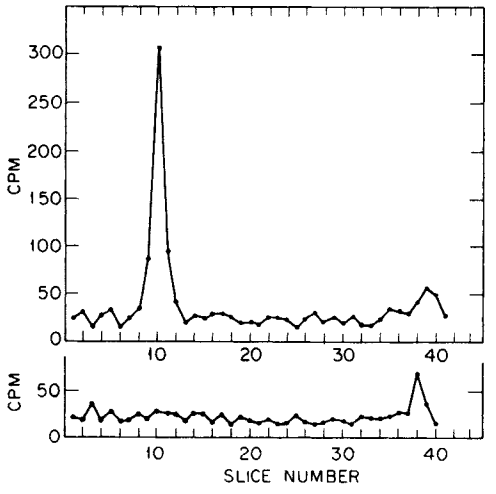

FIG. 4. [^{32}P]c.p.m. as a function of gel slice number after formation of phosphoprotein in the presence of Na^+ (upper tracing) and in the presence of K^+ (lower tracing) and subsequent SDS–PAGE (see Methods). The R_F of the Na^+-dependent peak is 0.24. Autoradiography of gels sliced lengthwise also showed a single Na^+-dependent peak with an R_F of 0.24, and after Coomassie staining the densest band was also found to have an R_F of 0.24, corresponding to a molecular weight of 92,000.

Kinetic activation studies

In order to determine whether the affinity of (Na^+-K^+)-ATPase for physiologic ligands changes during cerebral growth, activation curves were compared for cerebral microsomal preparations obtained from −2 day old and adult rats. Figure 7 shows remarkable similarity in activation curves for Na^+, K^+, Mg^{2+}, and ATP. Although the $[S]_{0.5}$ for ATP for the adult enzyme is 1.1 mM compared to 0.5 mM for the foetal enzyme, it is not clear that this constitutes a difference

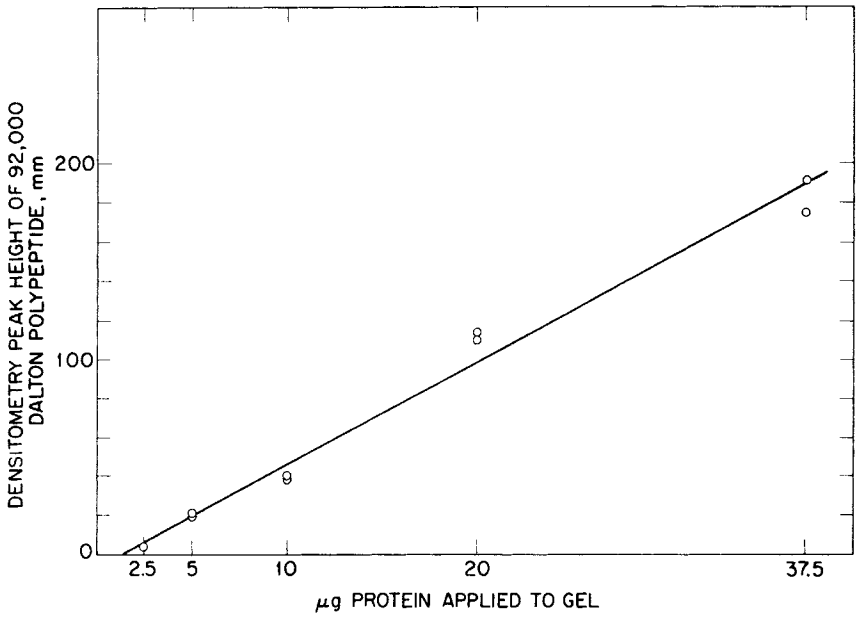

FIG. 5. Linearity of densitometry peak height of Coomassie-stained 92,000 molecular weight polypeptide with varying amounts of adult rat cerebral microsomal protein added per gel. Settings for densitometry measurements were: 550 nm, paper speed 120 inches per h, gel speed 1 cm per min, ratio 0.1, full scale 3, slit width 0.18 mm. Solid line was obtained by linear regression, with $r = 0.99$.

in substrate site affinity. Part of the discrepancy is referable to relatively greater inhibition of the foetal enzyme at 6 mM-ATP.

Finally, Fig. 8 shows that the affinity of rat cerebral microsomal Mg^{2+}-ATPase for ATP does not change during development. With respect to Mg^{2+}, however, there is a small difference of uncertain significance. $[Mg^{2+}]_{0.5}$ values for foetal and adult tissues are 1.9 and 1.0 mM, respectively.

DISCUSSION

Increases in enzymatic specific activity during development such as those reported here for rat cerebral (Na^+-K^+)-ATPase in enriched microsomal fractions may be due to many factors. These include relative decreases in amount of total microsomal protein, age-related shifts in the distribution of the enzyme in subcellular fractionation, changes in turnover number,

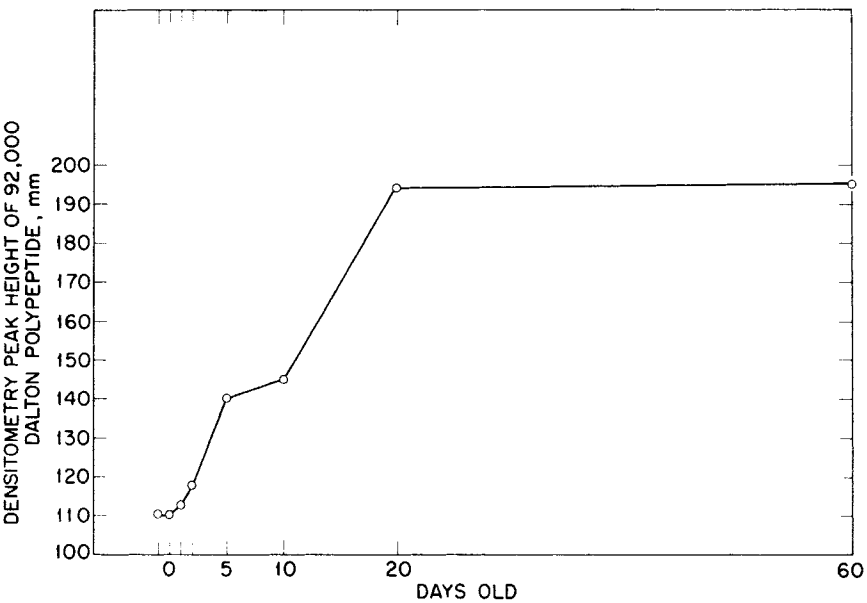

FIG. 6. Estimation of amount of 92,000 molecular weight polypeptide in rat cerebral microsomes in development. The densitometry peak height was determined using the same settings as in Fig. 5 except for a slit width of 0.21 mm. 40 μg of microsomal protein was added to each gel.

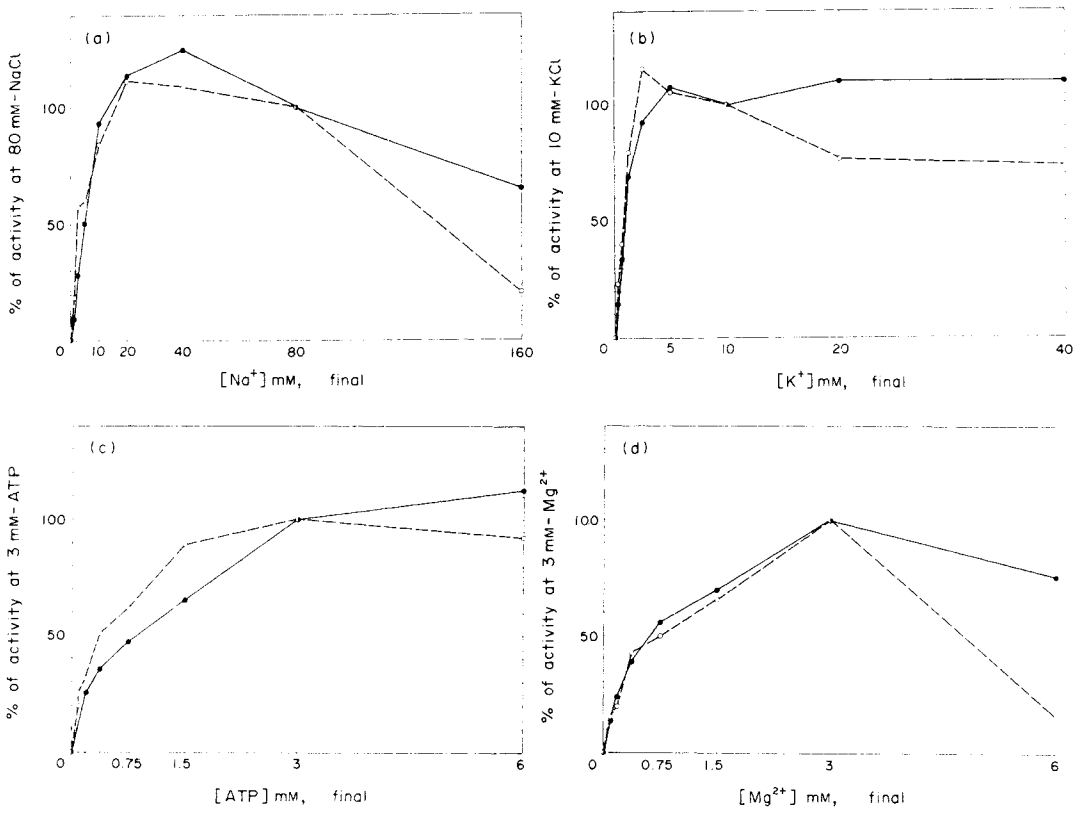

FIG. 7(a). Na^+ activation of (Na^+-K^+)-ATPase in rat cerebral microsomes for −2 days (○) and adult (●). Each point is the mean of two determinations normalized to 80 mM-Na^+ values. (b) K^+ activation of (Na^+-K^+)-ATPase in rat cerebral microsomes for −2 days (○) and adult (●). Each point is the mean of two determinations normalized to 10 mM-K^+ values. (c) ATP activation of (Na^+-K^+)-ATPase in rat cerebral microsomes for −2 days (○) and adult (●). Each point represents the difference in activity ±80 mM-NaCl and is the mean of two determinations normalized to 3 mM-ATP values. (d) Mg^{2+} activation of (Na^+-K^+)-ATPase in rat cerebral microsomes for −2 days (○) and adult (●). Each point is the difference in activity ±80 mM-NaCl and represents the mean of duplicate determinations normalized to 3 mM-Mg^{2+} values.

changes in ligand affinity, changes in amounts of endogenous inhibitory or activating agents, and actual increases in amount of enzyme protein. The data in Table 1 show no decrease in amount of total microsomal protein during the greatest increases in the specific activity of (Na^+-K^+)-ATPase. GARCIA ARGIZ *et al.* (1967) found no difference in the subcellular distribution of (Na^+-K^+)-ATPase activity in the cerebrum of rats 5 and 40 days old. BANIK & DAVISON (1969) reported that microsomes account for 17.4, 15.2, 14.8, and 15.5%, respectively, of total brain homogenate (Na^+-K^+)-ATPase activity obtained from 12, 15, and 21 day old and adult rats. Developmental changes in enzyme turnover number or affinity for ligands and substrate were not found in this study and could not explain the observed increases in enzyme activity. The possibility of activating or inhibitory substances appearing or disappearing during development cannot be rigorously disproved and was not pursued. However, the concurrent increases in estimated amounts of the phosphorylatable polypeptide and Na^+-dependent steady-state phosphorylation levels are consistent with the simplest explanation that the changes in specific activity are due to proportional increases in the amount of enzyme per mg of extracted microsomal protein. It follows that the adult cerebral membranes from which the microsomes are extracted have a higher average density of enzyme molecules per mg membrane protein than do those of foetal brain.

The simultaneous development of Na^+-dependent phosphorylation, K^+-*p*NPPase activity and (Na^+-K^+)-ATPase activity bears on the validity of the proposed partial reaction scheme as a model for (Na^+-K^+)-ATPase (see DAHL & HOKIN, 1974, for references). SKOU (1965) has raised the possibility that Na^+-dependent phosphorylation may be solely an *in vitro* artifact and not an *in situ* enzyme function. In addition, despite the fact that purified (Na^+-K^+)-ATPase preparations from various sources are invariably co-enriched in (Na^+-K^+)-ATPase *and* K^+-*p*NPPase activities indicating a single enzyme, there is considerable dispute over the identity of the active sites and role of K^+-*p*NPPase in the overall reaction scheme. It has been shown (ALBERS & KOVAL, 1972; AHMED & FOSTER, 1974; ASKARI & KOYAL, 1968) that

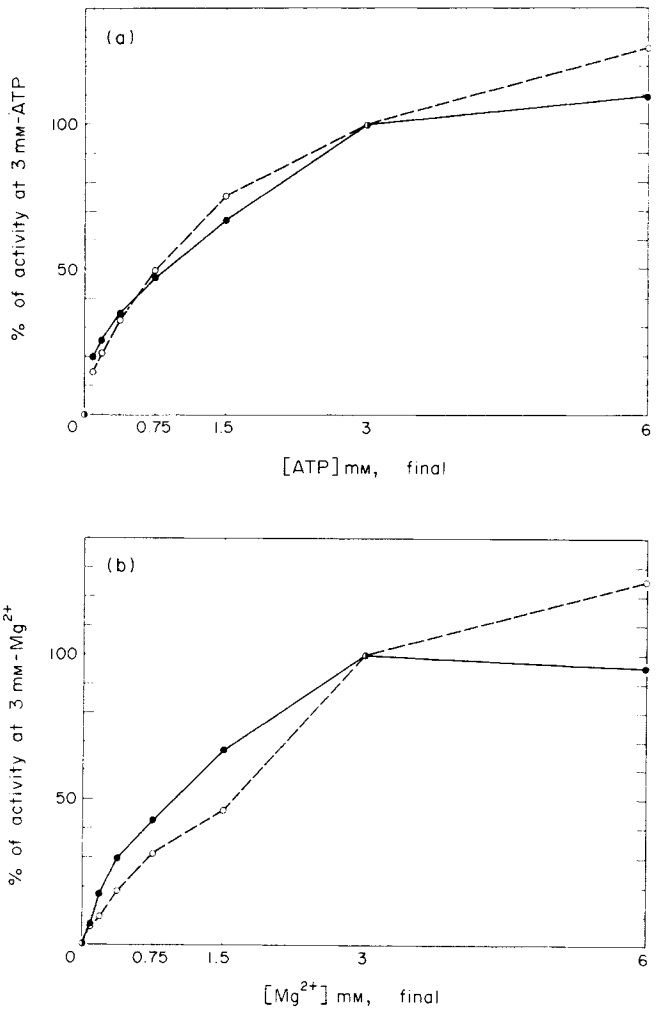

FIG. 8(a). ATP activity of Mg^{2+}-ATPase in rat cerebral microsomes for −2 days (○) and adult (●). Each point is the mean of two determinations normalized to 3 mM-ATP values. (b) Mg^{2+} activation of Mg^{2+}-ATPase in rat cerebral microsomes for −2 days (○) and adult (●). Each point is the mean of two determinations normalized to 3 mM-Mg^{2+} values.

(Na^+-K^+)-ATPase and K^+-*p*NPPase activities can be dissociated by specific agents. ROBINSON (1970) has reported the presence of a separate (Na^+–K^+)-activated phosphatase activity in rat brain microsomes and suggests that its activity and not that of the K^+-activated phosphatase represents the final step in the overall (Na^+–K^+)-ATPase reaction sequence. The results reported here indicate concurrent development of Na^+-dependent steady-state phosphorylation, K^+-*p*NPPase activity and (Na^+–K^+)-ATPase activity and provide additional evidence that these are functions of one enzyme. At the very least, the enzyme properties critical to these activities, whether or not related to identical sites, are apparently under the same biosynthetic control throughout development.

The disappearance of substantial Na^+-independent phosphorylation during maturation remains unexplained although the time course is consistent with a precursor role for some of the Na^+-dependent portion. It is not known, however, what products are phosphorylated in the absence of Na^+. Although about one third of total acid-precipitable phosphorylated product in cerebral microsomal preparations from adult rats is Na^+-independent, there is no labelled band evident after SDS–PAGE of such material (Fig. 4).

Our finding that rat cerebral microsomal (Na^+–K^+)-ATPase activity reaches half-adult values between 5 and 10 days after birth confirms prior studies utilizing whole rat brain homogenates (SAMSON & QUINN, 1967), bulk-isolated glial cell fractions obtained from rat cerebral cortex (MEDZIHRADSKY *et al.*, 1972) and rat cerebral synaptosomes (KISSANE & HAWRYLEWICZ, 1975). Only ABDEL-LATIF *et al.* (1967) reported earlier half-adult values (1 day after birth), in a study of the nerve ending fraction of the crude mitochondrial pellet prepared from whole rat brain. Several other investigators obtained later half-adult points, between 10 and 25 days, in studies employing rat brain homogenates (SAMSON *et al.*, 1964; GARCIA

ARGIZ *et al.*, 1967; COTE, 1964; TIRRI *et al.*, 1973; MEISAMI & TIMERAS, 1974; MEISAMI & MANOOCHEHRI, 1977), microsomes (BANIK & DAVISON, 1969), and deoxycholate treated heavy microsomes (VALCANA & TIMERAS, 1969). These variations are not due to differences in expression of (Na^+-K^+)-ATPase (per mg protein, per g wet weight, or per brain) since half-adult levels in this study are found between 5 and 10 days regardless of the method of expression. It is possible that variations among strains or even colonies of rats make comparisons of studies difficult, and for this reason it is advisable to present any developmental change in the context of another dynamic developmental parameter, such as cerebral wet weight.

Regarding other milestones in brain ontogenesis, both the development of (Na^+-K^+)-ATPase and the coincident brain growth spurt precede the deposition of myelin and succeed neuronal proliferation. Histologically, myelin does not even appear in rat cerebrum until days 10–12 (JACOBSON, 1963), and the appearances of half-adult levels of several myelin markers in rat brain clearly postdate the rapid growth phase: sulfatide incorporation of ^{35}S (KISHIMOTO *et al.*, 1965), galactocerebroside and sulfatide (KISHIMOTO *et al.*, 1965; WELLS & DITTMER, 1967), both light and heavy myelin protein (FUJIMOTO *et al.*, 1976), and recoverable myelin in purified fractions (NORTON & PODUSLO, 1973). In this series, 60% of the adult cerebral wet weight is already reached prior to the start of myelin deposition and about 84% before myelin deposition even reaches its peak. [^{3}H]thymidine incorporation studies (ALTMAN, 1963; ALTMAN & DAS, 1965; HICKS & D'AMATO, 1968; KAPLAN & HINDS, 1977) demonstrate that neuronal proliferation ceases before birth in the rat isocortex although certain microneurons continue to divide in the olfactory bulb and dentate gyrus.

A physiologic change that closely succeeds the rise in (Na^+-K^+)-ATPase activity is the reported increase in intracellular $[K^+]$ and decrease in intracellular $[Na^+]$ in rat cortex (VERNADAKIS & WOODBURY, 1962). It cannot be assumed, however, that these ion shifts are due entirely to cation pump action, since immature membrane may not be as selectively permeable to K^+. At any rate these reported intracellular ionic concentration changes have the expected time course if secondary to development of a cation pump.

Two important anatomic changes that also coincide with major increases in (Na^+-K^+)-ATPase activity are multiplication of glial cells and proliferation of cellular processes. The most rapid rate of change in rat brain DNA occurs near postnatal day 9 (BRASEL *et al.*, 1970). This reflects mainly glial cell division since nearly all neuronal multiplication in rat cerebrum ceases prior to birth. The mean numbers of both axons and dendrites per mm^2 in rat cerebral cortex each reach half-adult values between 6 and 12 days and the greatest rates of change also are observed between 6 and 12 days (EAYRS & GOODHEAD, 1959). The greatest contribution to the growth in rat cerebral cortical thickness during the first two to three postnatal weeks is not in changes in cell body volume but in the intercellular spaces with proliferation of complex intercellular connections (CALEY & MAXWELL, 1971). Lectin-like hemagglutination activity found in soluble rat brain fractions shows a peak on the eighth postnatal day (SIMPSON *et al.*, 1977) and apparently immediately precedes intercellular contact and synapse formation. AGHAJANIAN & BLOOM (1967) found the peak appearance of recognizable rat cerebral synaptic junctions between 12 and 26 days.

The question arises as to whether the major ATPase changes are related to neuronal or glial cell processes. It is of note that MEDZIHRADSKY *et al.*, (1972) found much greater (Na^+-K^+)-ATPase activity in the bulk-prepared glial cell fraction than in the neuronal cell body fraction. However, as they emphasized, the neuronal perikarya were stripped of nearly all arborizations. The use of somewhat milder density gradient bulk separation of beef and rabbit glial and neuronal fractions by HAMBERGER *et al.* (1970) resulted in no detectable difference in (Na^+-K^+)-ATPase activities in the homogenates. It is apparent from the published photomicrographs that there is greater preservation of neuronal processes in Hamberger's method, but also perhaps greater glial contamination. Histochemical studies demonstrate that most of the K^+-*p*NPPase activity in adult rat cerebrum is on fine cellular processes rather than on cell bodies (GUTH & ALBERS, 1974; STAHL & BRODERSON, 1976*a*, 1976*b*), but the methods used were incapable of differentiating dendritic from axonal, or glial from neuronal processes. A recent immunocytochemical study of the brain of the black ghost knifefish *Sternarchus albifrons* (WOOD *et al.*, 1977), in which antisera to *Electrophorus electricus* electric organ (Na^+-K^+)-ATPase was used, demonstrated immunoreactive product on both glial and neuronal plasma membranes.

It is proposed that the biosynthesis of specialized neuronal and/or glial plasma membranes with an increased density of (Na^+-K^+)-ATPase molecules (per mg of extractable membrane protein) during the growth spurt in rat cerebrum is the anatomic counterpart of the rapid changes in (Na^+-K^+)-ATPase reported here. Considerably more information is needed regarding the distribution of (Na^+-K^+)-ATPase within membranes of the mammalian nervous system and the factors such as neuronal–glial interactions and lectins possibly related to its changes during development.

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