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DEVELOPMENT OF (Na^+-K^+)-ATPase IN RAT HINDBRAIN: INCREMENTS IN PARALLEL WITH Na^+-DEPENDENT PHOSPHORYLATION AND K^+-*p*NITROPHENYLPHOSPHATASE[1]

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Abstract—Rat hindbrain NaI-enriched microsomal (Na^+-K^+)-ATPase activity, K^+-*p*NPPase activity, and Na^+-dependent steady-state phosphorylation levels all increase approx 10-fold relative to microsomal protein between 5 days prenatally and 60 days postnatally. These activities, as well as the mean wet weight of the hindbrain, are at half of their 60 day values shortly after the 10th postnatal day. For all ages, these hindbrain activities average over twice those found in the forebrain in a companion paper (BERTONI & SIEGEL, 1978). Increases during development in the amount of the related phosphorylatable polypeptide, estimated by densitometry of stained polyacrylamide gels containing fixed amounts of microsomal protein dissolved in SDS, are in agreement with increases in steady-state levels of Na^+-dependent phosphorylation. The fraction of total phosphorylation that is Na^+-dependent rises steadily during development consistent with, but not obligatorily due to, a conversion of some of the previously Na^+-independent portion. Mg^{2+}-ATPase and Mg^{2+}-*p*NPPase activities and steady-state Na^+-independent phosphorylation levels do not increase in parallel during development. These observations add further support to the proposed partial reaction scheme for (Na^+-K^+)-ATPase. The major increments in (Na^+-K^+)-ATPase occur simultaneously with the deposition of specialized plasma membranes, particularly in the molecular layer of the cerebellum, as described in previous studies of rat hindbrain.

(Na^+-K^+)-ATPASE, EC 3.6.1.3, has been extensively studied since its activity was first demonstrated in crab nerve (SKOU, 1957; DAHL & HOKIN, 1974). Prior investigations in this laboratory have established the timetable for development in rat cerebral microsomes of $(Na^{2+}-K^+)$-ATPase, and Na^+-dependent phosphorylation and K^+-*p*NPPase activities and the quantity of phosphorylatable polypeptide (BERTONI & SIEGEL, 1978). These all increase in parallel 10 times relative to microsomal membrane protein during the cerebral rapid growth phase; half of their adult values are attained between 5 and 10 days postnatally. The studies available concerning (Na^+-K^+)-ATPase activity in rat hindbrain fail to agree on the course of development of this enzyme system and extend only 21–50 days into postnatal development (GARCIA ARGIZ *et al.*, 1967; VALCANA & TIMERAS, 1969; MEISAMI & TIMERAS, 1974; KISSANE & HAWRYLEWICZ, 1975; MEISAMI & MONOOCHEHRI, 1977). The maturation of hindbrain follows a different schedule than that of forebrain. The present report shows that this difference is manifested also in the development of (Na^+-K^+)-ATPase, its associated activities, and the density of microsomal protein that exhibits Na^+-dependent phosphorylation.

MATERIALS AND METHODS

[γ-^{32}P]ATP was obtained from ICN (Irvine, CA). Other reagents were obtained as described (BERTONI & SIEGEL, 1978).

Preparation of NaI-*enriched microsomes.* Unanesthetized Sprague–Dawley rats of ages 0, 1, 2, 5, 10, 20, and 60 days were decapitated. Fetuses of ages −5, −2, and −1 days prior to birth (gestational ages 16, 19, and 20 days) were obtained by Cesarian section after the dam was decapitated. After dissection on ice, the brains were divided by an incision through the mesencephalon, and wet weights were obtained for the parts of the brain. NaI-enriched microsomes were prepared as described (BERTONI & SIEGEL, 1978), using the NaI reagent of NAKAO *et al.* (1965).

Assays. Protein determinations were done according to the method of LOWRY *et al.* (1951). The formation of acid-stable non-exchangeable phosphoprotein was measured after a 45 s incubation at 0°C with 80 μg of microsomal protein added to media containing (final concentrations) 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 vol of 50 μl. ATPase activity was assayed after 10 min at 37°C in media

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Abbreviations used: *p*NPPase, *para*nitrophenylphosphatase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PII, crude microsomes; PF, final microsomes.

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 × 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 vol of 40 μl. *p*NPPase activity was measured spectrophotometrically after 20 min at 37°C in media containing (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. Details of assay procedures are previously described (BERTONI & SIEGEL, 1978). [Na^+] in the final microsomes was determined with a KLiNa Flame System (Beckman Instruments, Fullerton, CA) to check for possible Na^+ contamination.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–Page). Microsomal protein was dissolved in SDS and subjected to electrophoresis as described (HOKIN *et al.*, 1973) and stained with Coomassie brilliant blue according to the method of FAIRBANKS *et al.* (1971). Estimation of amount of protein in individual bands by densitometry of peak heights was done as previously described (BERTONI & SIEGEL, 1978).

RESULTS

Yield of microsomes during development

The amounts of protein recovered in the PII (crude microsomal) and the PF (final microsomal) fractions from rat hindbrain at various ages are given in Table 1. The amount of protein in the PII fraction per g hindbrain wet weight varies between 9.86 and 18.7 mg without a clear trend between −5 and 2 days, after which it falls continuously to 6.34 by 20 days and to 3.74 by 60 days. When expressed per brain, however, the protein (mg) in PII rises rather steadily from 0.265 at 5 days to 3.28 at 10 days after which time it starts to decline. The dispersion of crude microsomal protein by the NaI treatment has differing effects on the per cent yield in the final microsomes at various stages in development. The yield fluctuates between 14 and 25% up to day 2 and increases slightly but steadily thereafter. The data for mean wet weight, except for the 1st postnatal day, fit a smooth curve which suggests comparability of the different ages. A state of dehydration or malnutrition sufficient to produce metabolic disturbances would be expected to cause deviations from the curve of hindbrain wet weights.

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

Hindbrain mean wet weight, microsomal (Na^+–K^+)-ATPase and K^+-*p*NPPase specific activities, and steady-state levels of Na^+-dependent phosphorylation all are at 50% of their respective 60-day values shortly after day 10 (Fig. 1). The enzymatic activities generally follow the curve for wet weights, except that the wet weights are lower at 5 days and higher at 20 days compared to the enzyme measurements. The wet weight therefore has a steeper rate of change. At 5 days the mean value for Na^+-dependent steady-state phosphorylation is relatively high but, due to a large variance, it does not differ significantly from either (Na^+–K^+)-ATPase or K^+-*p*NPPase per cent of adult activity at the $P = 0.02$ level (two-tailed test).

The mode of expression for enzyme activity is critical to interpretations. Based on calculations from the data in Table 1, half of the 60-day values for (Na^+–K^+)-ATPase is reached between 2 and 5 days when enzyme activity is expressed per g wet weight of hindbrain, between 5 and 10 days when expressed per brain, and between 10 and 20 days when expressed per mg of microsomal protein (Fig. 2). Although the normalized curves for (Na^+–K^+)-ATPase units (mol product/min) per brain and per mg microsomal protein (Fig. 2) have nearly the same half-adult points, the curve for enzyme units per g wet weight is shifted to the left. A similar but less pronounced finding has been noted in studying the rat forebrain (unpublished observations). This might be explained by the relatively later contribution to wet weight by non-enzyme matter such as myelin or other lipid-enriched substances since, during lipid deposition, microsomal protein becomes a smaller proportion of wet weight.

There is no consistent change in turnover number [ratio of (Na^+–K^+)-ATPase activity to the level of phosphorylated intermediate] during development (Fig. 1) to explain increases in activity through activational molecular changes in the enzyme.

TABLE 1. SUBCELLULAR FRACTIONATION IN DEVELOPING RAT HINDBRAIN

Age (days)	−5	−2	−1	0	1	2	5	10	20	60
Number of brains	36	45	36	34	41	21	23	16	16	9
Mean hindbrain wet weight (g)	0.0269	0.0414	0.0613	0.0782	0.0704	0.0872	0.118	0.228	0.412	0.502
Protein (mg) in PII	9.55	23.1	31.2	27.1	44.1	34.3	44.1	52.5	41.8	16.9
Protein (mg) in PII/gm wet weight	9.86	12.4	14.1	10.2	15.3	18.7	16.2	14.4	6.34	3.74
Protein (mg) in PII/brain	0.265	0.513	0.867	0.797	1.08	1.63	1.92	3.28	2.61	1.88
Protein (mg) in PF	1.70	3.31	5.36	3.87	8.20	4.69	8.79	12.6	10.8	5.45
mg protein in PF/g wet weight	1.76	1.78	2.43	1.46	2.84	2.56	3.24	3.45	1.64	1.21
mg final protein in PF/brain	0.0472	0.0735	0.149	0.114	0.200	0.223	0.382	0.788	0.675	0.606
mg protein in PF/mg protein in PII	0.178	0.143	0.246	0.143	0.186	0.137	0.199	0.240	0.258	0.322

Subcellular fractionation in developing rat hindbrain. Weighing of hindbrains, preparation of microsomes, and protein determinations are described in Methods.

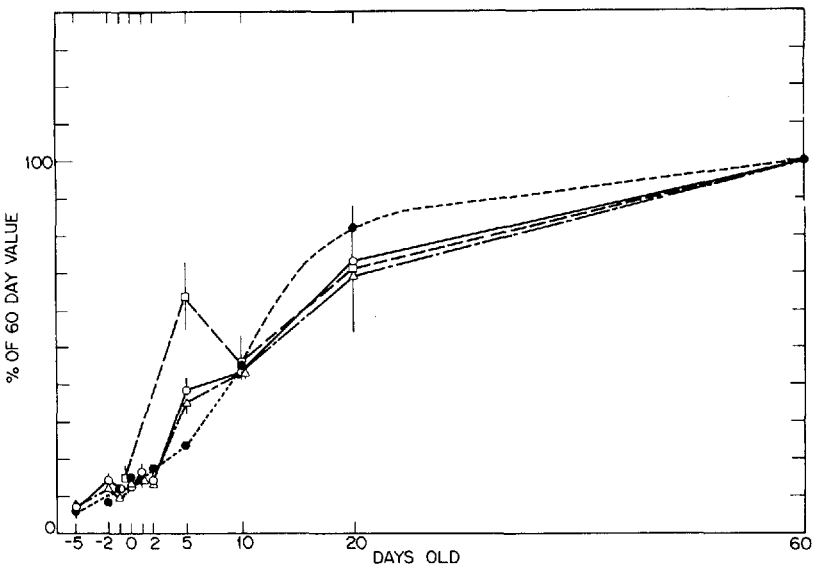

FIG. 1. Development of rat hindbrain. Mean hindbrain weight ●···●, microsomal steady-state Na^+-dependent phosphorylation □---□, K^+-*p*NPPase activity △-·-·-△, and $(Na^+–K^+)$-ATPase activity ○——○ are expressed as per cent of the value at 60 days. At 60 days, the mean wet weight is 0.502 g and the mean ± S.E.M. of at least four separate determinations are: steady-state Na^+-dependent phosphorylation, 113 ± 25.4 nmol·mg^{-1}; K^+-*p*NPPase, 210 ± 10.8 nmol·mg^{-1}·min^{-1}; and $(Na^+–K^+)$-ATPase, 674 ± 43.0 nmol·mg^{-1}·min^{-1}.

Mg^{2+}-ATPase, Mg^{2+}-pNPPase, and Na^+-independent phosphorylation during development

The changes in rat hindbrain Mg^{2+}-ATPase and Mg^{2+}-*p*NPPase activities, and Na^+-independent phosphorylation steady-state levels are not parallel (Fig. 3). Na^+-independent phosphorylation levels fall between 5 and 10 days but slowly rise thereafter. Mg^{2+}-*p*NPPase activity fluctuates up to 10 days and then rises slightly. Mg^{2+}-ATPase activity rises about twofold between −5 and 60 days. The sharp peak seen in this series at the single time post of 2 days is not due to Na^+ contamination, as the measured Na^+ in that preparation of microsomes could account for an incubation concentration of Na^+ of not more than 0.05 mM.

There is a plateau reached near 55% for the ratio of Na^+-dependent phosphorylation/total phosphorylation (Fig. 4) but the ratios of $(Na^+–K^+)$-ATPase/

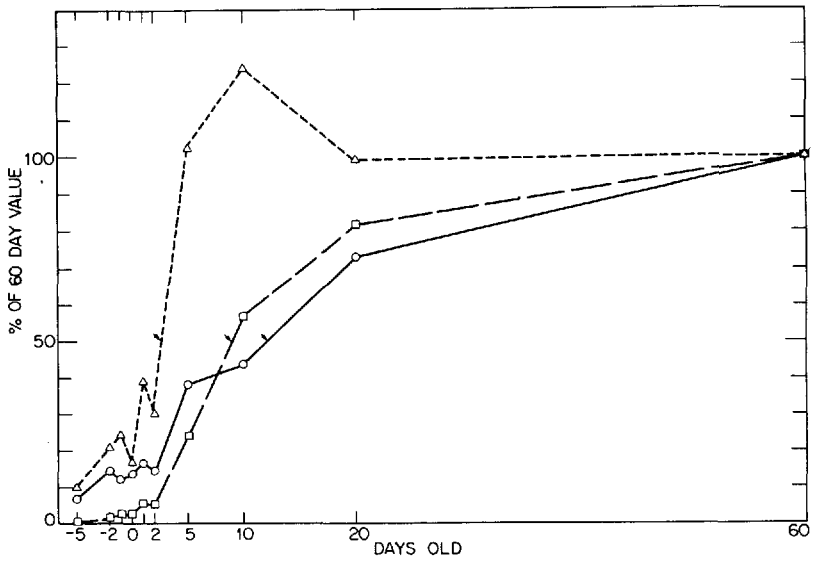

FIG. 2. Differences in the times corresponding to half-maximal values of rat hindbrain $(Na^+–K^+)$-ATPase depending on mode of expression of enzyme activity: per mg microsomal protein ○——○, per brain □--□, and per g hindbrain wet weight △----△. Points are determined from data in Table 1 normalized for 60 day values. Arrows indicate 50% of 60 day value.

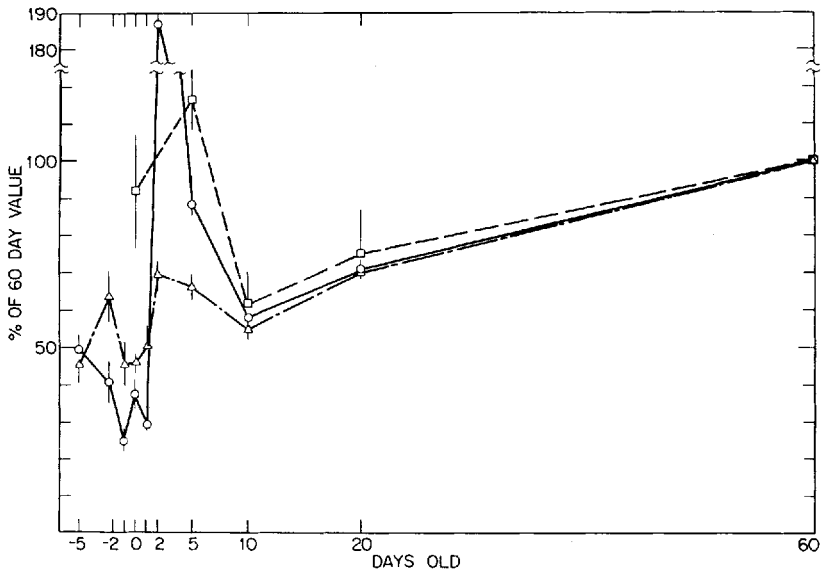

FIG. 3. Development of Na^+-and K^+-insensitive activities in rat hindbrain. Age-related development of rat hindbrain microsomal steady-state Na^+-independent phosphorylation □---□, Mg^{2+}-*p*NPPase activity △·-·-·△, and Mg^{2+}-ATPase activity ○——○, are expressed as percent of the value at 60 days. At 60 days, the mean ± S.E.M. of at least four separate determinations are: Na^+ independent phosphorylation, 107.1 ± 17.1 nmol·mg^{-1}; Mg^{2+}-*p*NPPase, 31.1 ± 2.46 nmol·mg^{-1}·min^{-1}; and Mg^{2+}-ATPase, 123 ± 15.3 nmol·mg^{-1}·min^{-1}.

total ATPase and K^+-*p*NPPase/total *p*NPPase both reach 87% at 60 days (data not shown). It cannot be assumed that all the apparent Mg^{2+}-baseline activities are related to each other. The important observation is that the Na^+- and K^+-dependent enzyme activities with respect to ATP and *p*NPP hydrolysis and phosphorylation are all uniquely related to each other by the extent and rate of their developmental changes in contrast to the other ATPase activities assayed.

It is of additional interest that the fraction of total phosphorylation that is dependent on Na^+ increases steadily during development (Fig. 4). A similar increase in this proportion has also been found for rat cerebrum (BERTONI & SIEGEL, 1978). One explanation for this finding might be that some of the originally Na^+-independent fraction could be converted during development into the Na^+-dependent fraction. There is no further evidence for this, however, and the question bears further study.

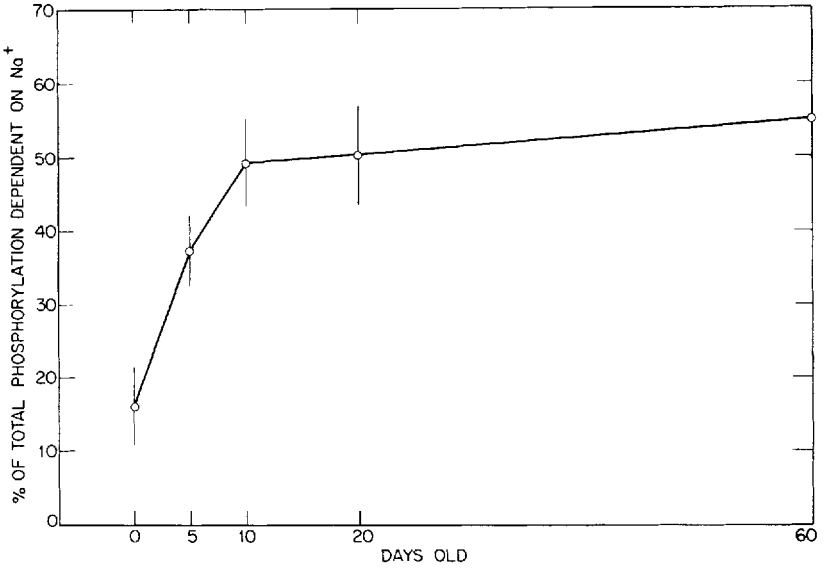

FIG. 4. The fraction of total steady-state phosphorylation that is Na^+-dependent in rat cerebral microsomes at different ages, expressed as means ± S.E.M. of at least 4 separate determinations.

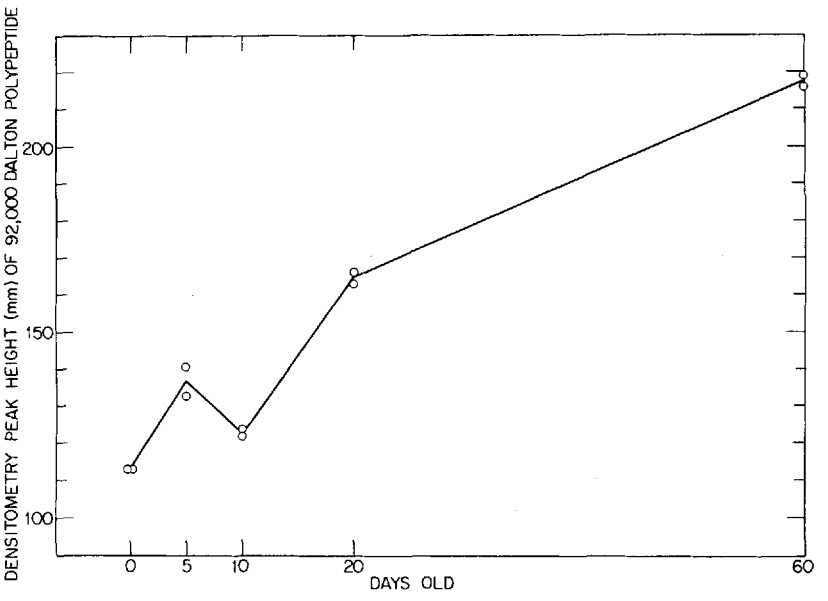

FIG. 5. Estimation of amount of 92,000 molecular weight polypeptide in rat hindbrain microsomes during development. Settings for densitometry were: 550 nm, paper speed 120 in. per h, gel speed 1 cm per min, ratio 0.1, full scale 3, slit width 0.21 mm. Forty μg of microsomal protein were applied to each gel. Linearity of peak height with amount of microsomal protein was established previously (BERTONI & SIEGEL, 1978).

Estimation of the phosphorylatable protein at various ages

The polypeptide labelled by [γ-^{32}P]ATP during Na^+-dependent phosphorylation of rat forebrain microsomes migrates as a single band corresponding to about 92,000 daltons on sodium dodecylsulfate polyacrylamide gels (BERTONI & SIEGEL, 1978). This is the densest Coomassie-stained band on these gels. Developmental increases, measured densitometrically, in this 92,000 molecular weight band in samples from rat hindbrain follow a course (Fig. 5) similar to that for Na^+-dependent steady-state phosphorylation (Fig. 1). The same relationship was also found in rat forebrain. Because of incomplete separation of bands and an error in the method of approx 10%, the data from peak densitometry are best regarded as estimations. It should be pointed out that the baselines for Na^+-dependent phosphorylation and for height of the 92,000 dalton peak by densitometry are chosen arbitrarily but, nonetheless, the changes with time are similar for both parameters.

DISCUSSION

The developmental increases in specific activity of $(Na^+$–$K^+)$-ATPase reported here might be explained by many factors including relative decreases in the total amount of microsomal protein, shifts in enzyme distribution among subcellular fractions, changes in turnover number, and actual increases in average amount of enzyme protein per mg microsomal protein. Table 1 shows that there was a relatively higher than expected yield of both crude and final microsomal protein per brain at 10 days (cf. at 5 days), which might suggest differences in sampling of sedimentable fragments. However, when expressed per gm wet weight, this difference is not apparent. This underscores the importance of the mode of expression of development data since the relative contribution by non-protein constituents to increments in wet weight changes dramatically during myelination. The fact that the Mg^{2+}-dependent activities do not exhibit the same proportional changes as those related to $(Na^+$–$K^+)$-ATPase mitigates against relative decreases in microsomal proteins or shifts in distribution among subcellular fractions as major factors. In addition, BANIK & DAVISON (1969) reported that microsomes account for 17.4, 15.2, 14.8, and 15.5%, respectively, of total homogenate $(Na^+$–$K^+)$-ATPase activity obtained from 12, 15, 21 day old and adult rat whole brains. GARCIA ARGIZ *et al.*, (1967) found no difference in the subcellular distribution of $(Na^+$–$K^+)$-ATPase activity in the cerebellum of 5 and 40 day old rats. Consistent developmental changes in turnover number were not found in this study and the questionable trends could not explain increases in activity. The enzyme obtained from fetal and adult rat cerebra (BERTONI & SIEGEL, 1978) shows no difference in affinity for substrate or ligands and it is not likely that the hindbrain enzyme would differ in this respect. The parallel increases in estimated amounts of the catalytic unit and in Na^+-dependent steady-state phosphorylation allow us to conclude that increments in specific activity are due to incre-

ments in the amount of enzyme protein per mg of microsomal membrane protein.

Prior studies of (Na^+-K^+)-ATPase in rat hindbrain have failed to agree on the timetable of development and their end points have varied from 21 to 50 days. Even at 60 days, the hindbrain wet weights and enzyme activities in our series continue to rise, as opposed to the relative plateaus seen in the cerebrum at this age (BERTONI & SIEGEL, 1978). If due attention is given to mode of expression of enzyme activity, the sole prior study with a comparable developmental schedule is that of KISSANE & HAWRYLEWICZ (1975). All other studies show apparently slower development of this enzyme system in rat hindbrain.

The brainstem was included in these hindbrain preparations as it was impractical to try to separate fetal cerebellum from brainstem. By wet weight, the cerebellum comprises nearly 50% of the hindbrain in the strain of adult rats used in these experiments and similar values have been recorded (SUGITA, 1917). According to the single published photograph of rat hindbrain sections stained histochemically by the *p*NPPase method of GUTH & ALBERS (1974), most of the product, by far, is in the cerebellum and very little is present in the brainstem at pontine levels. We are unaware of any biochemical study comparing (Na^+-K^+)-ATPase activity of the cerebellum with that of the entire brainstem. Although certain portions of the brainstem, such as the superior and inferior colliculi, have specific activities comparable with or greater than those of the entire cerebellum (MEISAMI & TIMERAS, 1974), such fragmentary data cannot be extrapolated to the brainstem as a whole.

Prior studies of (Na^+-K^+)-ATPase in the rat find from 1 to 1.2 times as much specific activity in the whole cerebellum compared to the whole cerebrum (AKERA *et al.*, 1973; KISSANE & HAWRYLEWICZ, 1975). Comparisons of whole cerebellum to portions of cerebral cortex show from 0.76 to 1.9 as much specific activity in the cerebellum (GARCIA ARGIZ *et al.*, 1967; VALCANA & TIMERAS, 1969; MEISAMI & TIMERAS, 1974; MEISAMI & MANOOCHEHRI, 1977). In comparison to values obtained with NaI-treated microsomes from whole rat cerebrum reported from this laboratory (BERTONI & SIEGEL, 1978) the present values obtained with hindbrain microsomes prepared simultaneously at each age are (mean $\pm$ S.E.M. for all the ages in Fig. 1) 2.37 ± 0.60 times as great for (Na^+-K^+)-ATPase, 2.28 ± 0.31 times as great for K^+-*p*NPPase, and 2.36 ± 0.38 times as great for Na^+-dependent phosphorylation levels. This difference for all three enzyme measurements combined is statistically significant ($P < 0.0005$, one-tailed test).

In order to view the increases in (Na^+-K^+)-ATPase with an anatomic perspective, an analysis of available information regarding cerebellar and brainstem development in the rat is worthwhile. The major changes in (Na^+-K^+)-ATPase specific activity in the rat hindbrain succeed the time of proliferation of Purkinje cells (gestational days 15 and 16) and Golgi cells (gestational day 17 to postnatal day 1). Multiplication of basket cells (postnatal days 5–10) and stellate cells (postnatal days 7–12) is just ending as (Na^+-K^+)-ATPase attains half-maximal values. Granule cells multiply especially between postnatal days 7 and 15 (DAS & NORNES, 1972; ALTMAN & BAYER, 1978*a*). The most active phase of proliferation of glial cells in the rat cerebellum occurs between the 7th and 13th postnatal days (MITROVA, 1967). Inputs to the rat cerebellum arrive at different times during development (ALTMAN & BAYER, 1978*b*) depending on the site of origin within the brainstem. Mossy fibers approach and make synapses with granule cells on postnatal days 10–12 (from lateral reticular nucleus) on postnatal days 12–15 (from nucleus reticularis tegmenti pontis) and on postnatal days 15–25 (from the pontine gray). Climbing fibers arrive from postnatal day 8 well to the end of the third postnatal week. Much less is known at this time about input arrival to the deep cerebellar nuclei or about cellular and fiber tract development in the rat brain stem. Other measures that change mainly during the period of rapid changes in (Na^+-K^+)-ATPase include the following: the thickness of the molecular layer, days 12–14 (ALTMAN & WINFREE, 1977; HEINSEN, 1977); the mean length of parallel fibers, day 15 (LAUDER, 1978); the thickness of the granule cell layer, days 10–14 (ADDISON, 1911; HEINSEN, 1977); and the surface area of Purkinje cell perikarya, day 13 (ALTMAN & WINFREE, 1977).

Myelin deposition in the rat hindbrain begins, according to histologic and biochemical criteria, near the 9th postnatal day and is most active between days 13 and 16, but continues into adult life (TILNEY, 1933; ROBINS & LOWE, 1961; MITROVA, 1967). Cyclic nucleotide phosphohydrolase, useful as a marker for myelin, reaches half of adult specific activity at postnatal day 12 in the rat cerebellum (SPRINKLE *et al.*, 1978). These myelin changes are most rapid near or shortly after the time the half-adult value for (Na^+-K^+)-ATPase is attained.

Although these correlations may sort out those anatomic parameters that might be candidates for the site of (Na^+-K^+)-ATPase protein deposition from those that might not, such as thickness of the medullary layer with a half adult time near 18 days, these correlations do not specify only one candidate. Also, the relationship between the length of processes or the thickness of a particular layer of cells may not be strictly linear with amount of plasma membranes and/or the number of (Na^+-K^+)-ATPase molecules present.

Besides such morphometric data, there is additional information concerning (Na^+-K^+)-ATPase from an histochemical technique for K^+-*p*NPPase which indicates that the formation of product in the rat hindbrain is mainly in the molecular layer of the cerebellum. There is a faint staining of the Purkinje cell layer, intermediate staining in the granule cell layer, and little staining in the brain stem itself at pontine levels (GUTH & ALBERS, 1974). An immunocytochemi-

cal method utilizing rabbit antibodies raised against purified *Electrophorus electricus* electroplax (Na^+–K^+)-ATPase shows localization on both astrocytic and neuronal membranes in the neuropil of the cerebellum of the knifefish, *Sternarchus albifrons* (WOOD *et al.*, 1977).

The regulatory factors responsible in the rat hindbrain for the increases in the mean density of (Na^+–K^+)-ATPase per mg microsomal protein and for the concurrent migration and interactions of cells cited above are unknown. If the density of (Na^+–K^+)-ATPase is indeed related to the amount of specialized plasma membrane present, then the deposition of enzyme molecules and the elongation of such differentiated membranes are necessarily interrelated. Recent studies have demonstrated that chemical factors appear during early hindbrain development which may serve as signals for intercellular recognition and growth of cell processes. ZANETTA *et al.* (1978) have reported a transient massive accumulation of mannose-containing glycoproteins demonstrated by fluorescent and horseradish peroxidase coupled Concanavalin A in growing parallel fibers in the rat cerebellum. TRENKNER & SARKAR (1977) have shown that antibodies raised in rabbits against various glycoproteins bind reversibly to mouse cerebellar tissue cultures and inhibit fiber formation and cell migrations.

Regulatory factors leading to the increase in average number of (Na^+–K^+)-ATPase molecules per mg of microsomal membrane protein may exert their effects at any point from synthesis to deposition of membrane constituents. Microsomal fractions contain both old and newly synthesized membrane particles. The characteristics of new membranes, such as in elongating cell processes, are necessarily diluted by the previously synthesized membranes, such as those on cell bodies. It is possible that increments in the average density of (Na^+–K^+)-ATPase per mg of microsomal protein are due to the production of very specialized membranes highly saturated with (Na^+–K^+)-ATPase molecules or to the addition of (Na^+–K^+)-ATPase to existing membranes, or both. Newly synthesized membranes may be relatively richer in (Na^+–K^+)-ATPase per mg microsomal protein by virtue of more enzyme molecules and/or by virtue of fewer unrelated (e.g. perikaryal) protein molecules per unit area of membrane.

Further studies relating critical cellular interactions to the growth of neurites and the concomitant proliferation of plasma membranes enriched in (Na^+–K^+)-ATPase in cerebellum are required.

REFERENCES

ADDISON W. H. F. (1911) The development of the Purkinje cells and of the cortical layers in the cerebellum of the albino rat. *J. comp. Neurol.* **21,** 459–481.

AKERA T., RECH R. H., MARQUIS W. J., TOBIN T. & BRODY T. M. (1973) Lack of relationship between brain (Na^+–K^+)-activated adenosine triphosphatase and the development of tolerance to ethanol in rats. *J. Pharmac. exp. Ther.* **185,** 594–601.

ALTMAN J. & BAYER S. A. (1978*a*) Prenatal development of the cerebellar system in the rat—I. Cytogenesis and histogenesis of the deep nuclei and the cortex of the cerebellum. *J. comp. Neurol.* **179,** 23–48.

ALTMAN J. & BAYER S. A. (1978*b*) Prenatal development of the cerebellar system in the rat—II. Cytogenesis and histogenesis of the inferior olive, ponine gray, and the precerebellar reticular nuclei. *J. comp. Neurol.* **179,** 49–76.

ALTMAN J. & WINFREE A. T. (1977) Postnatal development of cerebellar cortex in rat—V. Spatial organization of Purkinje cell perikarya. *J. comp. Neurol.* **171,** 1–16.

BANIK J. A. & DAVISON A. N. (1969) Enzyme activity and composition of myelin and subcellular fractions in the developing rat brain. *Biochem. J.* **115,** 1051–1062.

BERTONI J. M. & SIEGEL G. J. (1978) Development of (Na^+–K^+)-ATPase in rat cerebrum: correlation with Na^+-dependent phosphorylation and K^+-*para*-nitrophenylphosphatase. *J. Neurochem.* **31,** 1501–1511.

DAHL J. L. & HOKIN L. E. (1974) The sodium-potassium adenosinetriphosphatase. *A. Rev. Biochem.* **43,** 327–356.

DAS G. D. & NORNES H. O. (1972) Neurogenesis in the cerebellum of the rat: an autoradiographic study. *Z. Anat. EntwGesch.* **138,** 155–165.

FAIRBANKS G., STECK T. L. & WALLACH D. F. H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10,** 2606–2617.

GARCIA ARGIZ C. A., PASQUINI J. M., KAPLUN B. & GOMEZ C. J. (1967) Hormonal regulation of brain development—II. Effect of neonatal thyroidectomy on succinate dehydrogenase and other enzymes in developing cerebral cortex and cerebellum of the rat. *Brain Res.* **6,** 635–646.

GUTH L. & ALBERS R. W. (1974) Histological demonstration of (Na^+ + K^+)-activated adenosine triphosphatase. *J. Histochem. Cytochem.* **22,** 320–326.

HEINSEN H. (1977) Quantitative anatomical studies on the postnatal development of the cerebellum of the albino rat. *Anat. Embryol.* **151,** 201–218.

HOKIN L. E., DAHL J. L., DEUPREE J. D., DIXON J. F., HACKNEY J. F. & PERDUE J. F. (1973) Studies on the characterization of the sodium-potassium transport adenosine triphosphatase—X. Purification of the enzyme from the rectal gland of *Squalus acanthias*. *J. biol. Chem.* **248,** 2593–2605.

KISSANE J. Q. & HAWRYLEWICZ E. J. (1975) Development of Na^+–K^+-ATPase in neonatal rat brain synaptosomes after perinatal protein malnutrition. *Pediat. Res.* **9,** 146–150.

LAUDER J. M. (1978) Effects of early hypo- and hyperthyroidism on development of rat cerebellar cortex—IV. The parallel fibers. *Brain Res.* **142,** 25–39.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L. & RANDALL R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265–275.

MEISAMI E. & MANOOCHEHRI S. (1977) Effects of early bilateral chemical destruction of olfactory receptors on postnatal growth, Mg-ATPase and Na K-ATPase activity of olfactory and non-olfactory structures of the rat brain. *Brain Res.* **128,** 170–175.

MEISAMI E. & TIMERAS P. S. (1974) Influence of early visual deprivation on regional activity of brain ATPases in developing rats. *J. Neurochem.* **22,** 725–729.

MITROVA E. (1967) Karyometric investigation of glial cells

in the cerebellum in the course of myelination. *Z. mikrosk.-anat. Forsch.* **77,** 304–312.

NAKAO T., TASHIMA Y., NAGANO K. & NAKAO M. (1965) Highly specific sodium–potassium-activated adenosine triphosphatase from various tissues of rabbit. *Biochem. biophys. Res. Commun.* **19,** 755–758.

ROBINS E. & LOWE I. P. (1961) Quantitative histochemical studies of the morphogenesis of the cerebellum—I. Total lipid and four enzymes. *J. Neurochem.* **8,** 81–95.

SKOU J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. biophys. Acta* **23,** 394–401.

SPRINKLE T. J., ZARUBA M. E. & MCKHANN G. M. (1978) Activity of 2′,3′-cyclic-nucleotide 3′-phosphodiesterase in regions of rat brain during development: quantitative relationship to myelin basic protein. *J. Neurochem.* **30,** 309–314.

SUGITA N. (1917) Comparative studies on the growth of the cerebral cortex.—I. On the changes in the size and shape of the cerebrum during the postnatal growth of the brain. Albino rat. *J. comp. Neurol.* **28,** 495–510.

TILNEY F. (1933) Behavior in its relation to development of brain: correlation between development of brain and behavior in albino rat from embryonic states to maturity. *Bull. neurol. Inst. N.Y.* **3,** 252–358.

TRENKNER E. & SARKAR S. (1977) Carbohydrate-specific antibodies distinguish between mouse cerebellar cells of different developmental stages. *J. supramol. Struct.* **6,** 465–472.

VALCANA T. & TIMERAS P. S. (1969) Effect of hypothyroidism on ionic metabolism and Na–K activated ATP phosphohydrolase activity in the developing rat brain. *J. Neurochem.* **16,** 935–943.

WOOD J. G., JEAN D. H., WHITAKER J. N., MCLAUGHLIN B. J. & ALBERS R. W. (1977) Immunocytochemical localization of the sodium, potassium activated ATPase in knifefish brain. *J. Neurocytol.* **6,** 571–581.

ZANETTA J. P., ROUSSEL G., GHANDOUR M. S., VINCENDON G. & GOMBOS G. (1978) Postnatal development of rat cerebellum: massive and transient accumulation of Conconavalin A binding glycoproteins in parallel fiber axolemma. *Brain Res.* **142,** 301–319.