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DEVELOPMENTAL CHANGES IN METABOLISM AND TRANSPORT PROPERTIES OF CAPILLARIES ISOLATED FROM RAT BRAIN

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SUMMARY

1. Capillaries were isolated from the brains of 1- to 45-day-old rats in order to study the development of metabolic and transport aspects of the blood–brain barrier.

2. The hydroxyproline content of capillary hydrolysates increased nearly threefold between 5 and 45 days of age. This finding is consistent with histological studies showing thickening of capillary basement membrane during development.

3. The activities of L-DOPA decarboxylase and monoamine oxidase were greatest in capillaries from 10-day-old rat brain. Thus, the metabolic blood–brain barrier for amine precursors is present during early development.

4. Capillaries from all ages were able to metabolize glucose, β-hydroxybutyrate and palmitate. The rate of glucose oxidation more than doubled between 21 and 30 days of age but subsequently decreased. In contrast, β-hydroxybutyrate and palmitate oxidation increased throughout development. These data suggest a sparing effect by alternate fuels on glucose metabolism.

5. Capillary glucose uptake was similar at 10 and 30 days of age and activity of the ouabain-sensitive K^+ pump (measured using $^{86}Rb^+$) was relatively constant at all ages. In contrast, Na^+-dependent neutral amino acid transport was not present until after 21 days of age. Since this transport system may be responsible for the active efflux of neutral amino acids from brain to blood, it is likely that this process does not occur at the immature blood–brain barrier.

6. We conclude that various aspects of brain capillary functions show distinct developmental patterns which may be related to changes in blood–brain barrier permeability during development.

INTRODUCTION

The brain capillary endothelial cell is generally acknowledged to be the anatomical site of the blood–brain barrier (BBB) (Reese & Karnovsky, 1967; Brightman & Reese, 1969; Davson, 1976; Rapoport, 1976; Bradbury, 1979). In recent years, several laboratories have developed methods for isolation of brain capillaries. These preparations are enriched in enzymatic markers for brain capillary endothelial cells (Orlowski, Sessa & Green, 1974; Goldstein, Wolinsky, Csejtey & Diamond, 1975; Lai, Udenfriend & Spector, 1975; Betz, Firth & Goldstein, 1980), are metabolically active

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(Brendel, Meezan & Carlson, 1974; Goldstein, 1979*a*) and exhibit transport properties similar to those described at the BBB *in vivo* (Betz & Goldstein, 1978; Hjelle, Baird-Lambert, Cardinale, Spector & Udenfriend, 1978; Betz, Csejtey & Goldstein, 1979; Goldstein, 1979*a*; Eisenberg & Suddith, 1979). Thus, they provide a convenient model system for studying transport and metabolic aspects of BBB function.

Although developmental changes in BBB permeability have been of interest for a number of years, there have been no studies on the development of specialized BBB functions using a purified preparation of isolated brain capillaries. For the present report, we prepared isolated capillaries from the brains of rats that were between 1 and 45 days of age and used these capillaries to study the development of enzyme activities, metabolic patterns and transport systems that contribute to BBB function. An abstract detailing some of these findings has been published (Betz & Goldstein, 1979).

METHODS

Preparation of capillaries. Capillaries were isolated from the cerebral cortices of Sprague–Dawley rats that were 1, 5, 10, 15, 21, 30 and 45 days of age by a method that has been previously described (Betz *et al.* 1979). The preparative buffer (CPB) was oxygen-saturated and contained 147 mM-NaCl, 4 mM-KCl, 3 mM-$CaCl_2$, 1·2 mM-$MgCl_2$, 15 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), pH 7·4, 5 mM-glucose, and 1 % fraction V bovine serum albumin. The quality of each preparation was monitored by phase contrast microscopy and there were no apparent differences in the purity or morphology of preparations from different ages.

Chemical and enzyme assays. Total capillary protein content was determined by the method of Lowry, Rosebrough, Fara & Randall (1951) after overnight solubilization in 1 % sodium dodecyl sulphate. The spectrophotometric method of Prockop & Udenfriend (1960) as modified by Galasinski, Gadek, Ratkiewicz & Rzeczycki (1978) was used to determine the hydroxyproline content of the capillaries. Alkaline phosphatase (Lindhardt & Walter, 1965), γ-glutamyl transpeptidase (Orlowski & Meister, 1965), cytochrome c oxidase (Cooperstein & Lazarow, 1951), and monoamine oxidase activities (Goridis & Neff, 1971) were determined as previously described.

L-3,4-dihydroxyphenylalanine (L-DOPA) decarboxylase activity was determined by measuring the rate of $^{14}CO_2$ production from L-3,4-dihydroxyphenyl[1-^{14}C]alanine. Incubations were carried out for 30 min at 37 °C in sealed 25 ml. flasks containing 3 ml. CPB, 1 μc[^{14}C]-L-DOPA (0·3 mc/mole) and 0·5 to 1 mg of capillary cell protein. Assays for background activity contained no capillaries. Filter paper saturated with 10 % KOH was used to trap $^{14}CO_2$ that was released. The reaction was stopped and the remaining $^{14}CO_2$ released from solution by the addition of 1 ml. of 1 *N*-H_2SO_4 to the incubation medium and 30 min later the filters were removed and prepared for liquid scintillation counting.

Assay of substrate oxidation. The ability of isolated capillaries to metabolize glucose, palmitate or β-hydroxybutyrate was determined as previously described (Goldstein, 1979*a*). Incubations were for 1 hr in CPB containing varying concentrations of the ^{14}C-labelled substrate at 0·33 to 0·67 μc/ml. and a fixed concentration of an alternate substrate as follows: (1) D-[U-^{14}C]glucose with 1 mM DL-β-hydroxybutyrate, (2) [U-^{14}C]palmitate with 5 mM-glucose or (3) D-[3-^{14}C]β-hydroxybutyrate with 5 mM-glucose. Preliminary experiments indicated that the rate of $^{14}CO_2$ production from each substrate was linear for at least 90 min.

Assay of solute uptake. Sodium-dependent neutral amino acid transport was determined as previously described (Betz & Goldstein, 1978) using α-methylaminoisobutyric acid (αMeAIB) as a non-metabolizable substrate for this transport system (Christensen, 1973). 3-*O*-methyl-D-glucose (3MG) uptake was used as a measure of D-glucose transport by capillaries as previously described (Betz *et al.* 1979).

Potassium transport was studied using $^{86}Rb^+$ since this isotope has essentially the same transport properties as K^+ in mammalian cells (Vaughan & Cook, 1972). The uptake of $^{86}Rb^+$ was determined in the presence or absence of the Na^+, K^+-ATPase inhibitor, ouabain, as described elsewhere (Goldstein, 1979*a*). The rate of $^{86}Rb^+$ uptake was linear for at least 15 min.

Materials. [U-^{14}C]palmitate, D-[3-^{14}C]β-hydroxybutyrate, [3-*O*-methyl-^{3}H]methyl-D-glucose,

[1-^{14}C]α-methylaminoisobutyric acid, [1-^{14}C]tyramine and ^{86}RbCl were obtained from New England Nuclear Corp. (Boston, MA) and L-3,4-dihydroxyphenyl-[1-^{14}C]alanine from Amersham Corp. (Arlington Heights, Ill.). All other chemicals were purchased from Sigma Chemical Co. (St Louis, Mo.) or were analytical grade.

RESULTS

Hydroxyproline content. Hydroxyproline is an amino acid that is found almost exclusively in collagen. Since collagen is an important constituent of basement membrane, the hydroxyproline content of capillary protein hydrolysates can be used as an estimate of the amount of basement membrane collagen in isolated capillaries.

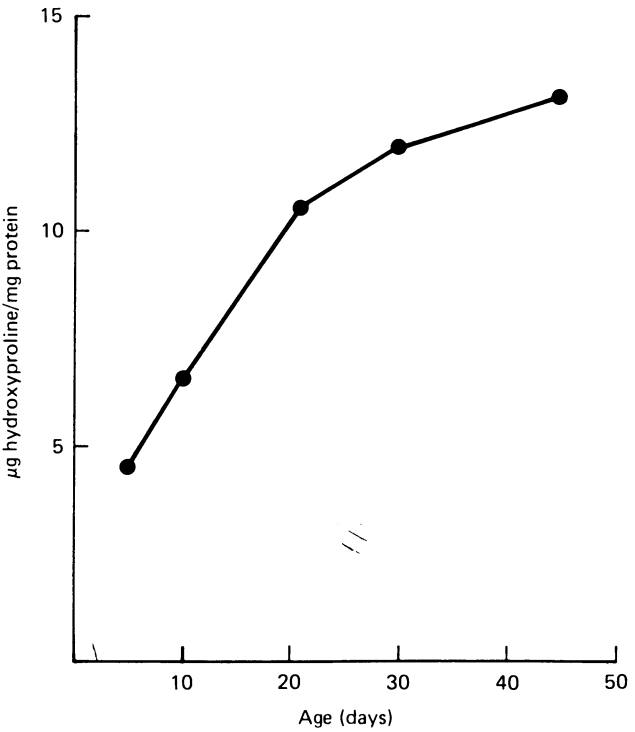

Fig. 1. Hydroxyproline content of brain capillaries at different ages. The amount of hydroxyproline was determined on samples of isolated brain capillaries that had been hydrolysed for 2 hr in 72% perchloric acid at 100 °C. Data are expressed as μg hydroxyproline per mg of capillary protein and are the averages of two determinations.

We found that the hydroxyproline content of isolated brain capillaries increased nearly three-fold between 5 and 45 days of age with the most rapid increase occurring before 30 days (Fig. 1). This finding agrees with histological studies showing a rapid development of basement membrane in rat brain capillaries during the same period of time (Donahue & Pappas, 1961; Bär & Wolff, 1972).

Activities of marker enzymes. Alkaline phosphatase and γ-glutamyl transpeptidase are frequently used as specific markers for brain capillaries (Orlowski *et al.* 1974; Goldstein *et al.* 1975). Sessa & Perez (1975) have shown a large increase in the

γ-glutamyl transpeptidase activity of crude brain capillary preparations from 10- to 21-day-old rats compared to adults. We observed a similar trend (Fig. 2); however, the increase in activity began at between 10 and 15 days. In contrast, alkaline phosphatase activity showed a slight increase in activity up to 15 days of age and a decline thereafter (Fig. 2).

The enzymes L-DOPA decarboxylase and monoamine oxidase are also known to be enriched in brain capillaries (Lai *et al.* 1975). These enzymes are considered responsible

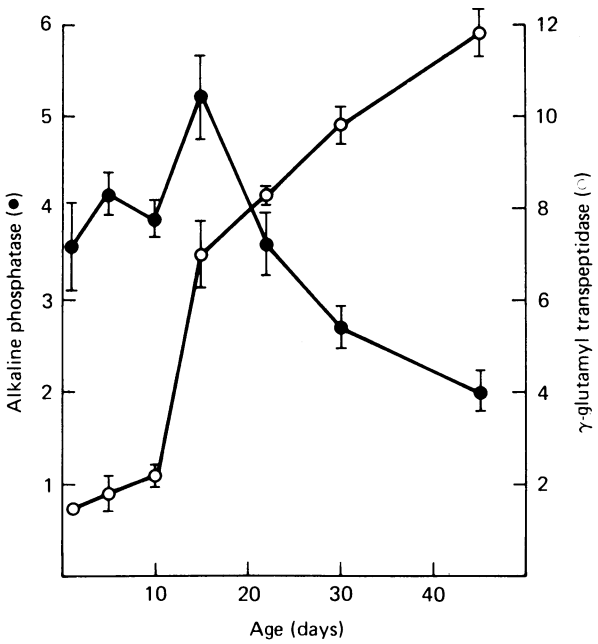

Fig. 2. Enzyme activities in brain capillaries at different ages. The activities of alkaline phosphatase (●) and γ-glutamyl transpeptidase (○) are expressed as μmole product/mg capillary protein per hr at 37 °C. Each point is the average of three determinations ± S.D.

for a metabolic BBB since they limit brain capillary permeability to substrates like L-DOPA (Bertler, Falck, Owman & Rosengren, 1966). Developmentally, L-DOPA decarboxylase in brain capillaries decreased with age (Fig. 3) as suggested by a previous study (Kellogg, Lundborg & Ramstedt, 1973). Monoamine oxidase activity showed a pronounced dip at 21 days of age (Fig. 3). A similar pattern was observed by Sessa & Perez (1975) for whole brain monoamine oxidase activity in rat. Since monoamine oxidase occurs in the outer mitochondrial membrane we also determined the activity of cytochrome c oxidase, an inner mitochondrial membrane marker. This enzyme did not show the same developmental pattern as monoamine oxidase, but rather was nearly constant in activity from 10 to 45 days (Fig. 3).

Oxidative metabolism. The effect of varying substrate concentration upon the oxidation of glucose, β-hydroxybutyrate and palmitate was examined using isolated brain capillaries from 10-, 21-, 30- and 45-day-old rats (data not shown). The concentration of each substrate that gave a half-maximal rate of metabolism did

not change with age (approximate values were 0·1 mM-D-glucose, 0·2 mM-DL-β-hydroxybutyrate, and 0·05 mM-palmitate). Thus, the rate-limiting step for oxidative metabolism of each substrate was probably the same at different ages. Fig. 4 summarizes the developmental changes in oxidative metabolism for substrate concentrations in the physiological range (5 mM-glucose, 1 mM-DL-β-hydroxybutyrate,

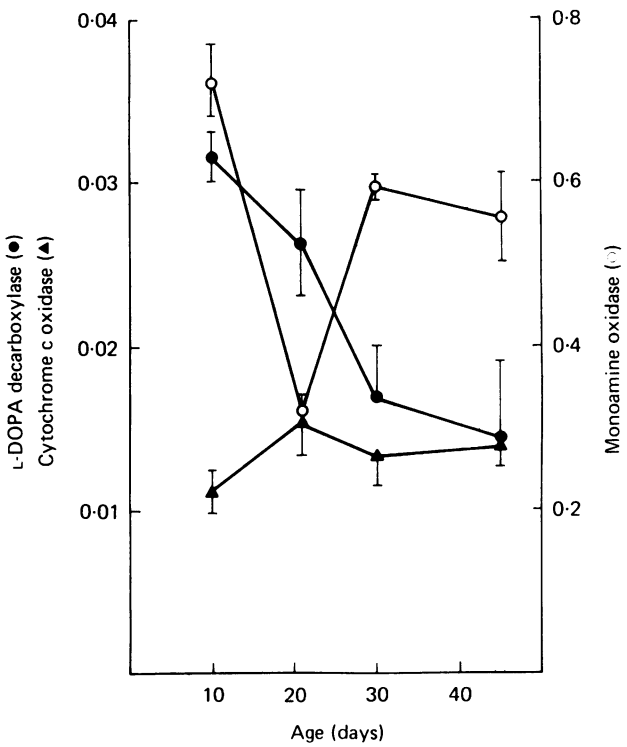

Fig.3. Enzyme activities in brain capillaries at different ages. The activities of L-DOPA decarboxylase (●) and monoamine oxidase (○) are expressed as μmole product/mg of capillary protein per hr at 37 °C. Cytochrome c oxidase activity (▲) is in μmole product/mg of capillary protein per min at 25 °C. Each point is the average of three determinations ± S.D.

and 0·25 mM-palmitate). Up to 30 days of age, there was an increase in the oxidative metabolism of all substrates. After 30 days, the metabolism of palmitate and β-hydroxybutyrate continued to increase while metabolism of glucose decreased. This may be the result of alternate metabolic fuels sparing glucose metabolism in brain capillaries from animals over 30 days of age. In contrast, developmental changes in brain metabolism are quite different since ketone body metabolism is most active between 16 and 21 days of age (Hawkins, Williamson & Krebs, 1971), while the adult uses glucose as an energy substrate almost exclusively. The large increase in glucose oxidation at 30 days and continued high rate of total oxidative metabolism may be related to the development of new energy requiring processes such as active amino acid transport (see below).

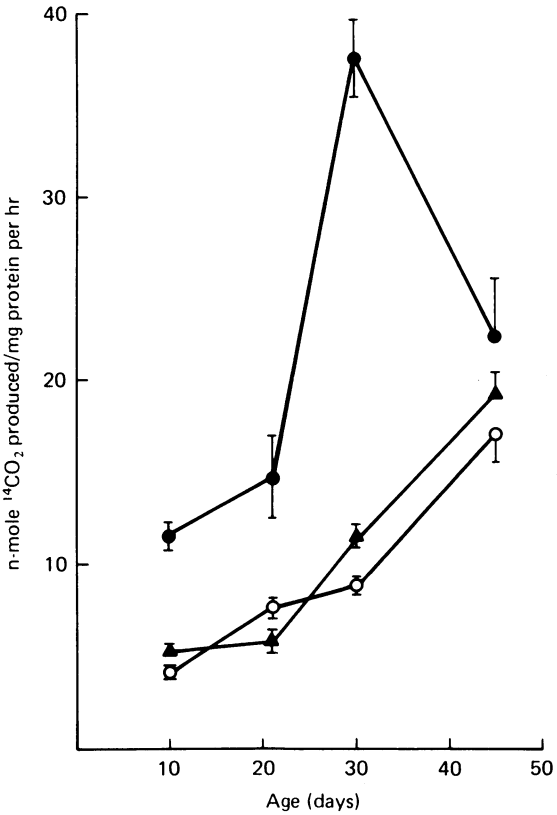

Fig. 4. Developmental changes in substrate oxidation by brain capillaries. The rates of CO_2 production are shown at different ages for (●) 5 mM-D-[^{14}C]glucose in the presence of 1 mM-DL-β-hydroxybutyrate, (▲) 1 mM-DL-[^{14}C]β-hydroxybutyrate in the presence of 5 mM-D-glucose or (△) 0·25 mM [^{14}C]palmitate in the presence of 5 mM-D-glucose. Each point is the average of three determinations ± S.D.

TABLE 1. 3-*O*-methyl-D-glucose uptake by brain capillaries from 10-day-old rats

Incubation conditions	Uptake (n-mole/mg per 30 sec)
Control	5·27 ± 0·40
Cytochalasin B	2·74 ± 0·10
Ouabain	5·22 ± 0·11
Insulin	5·26 ± 0·17

Capillaries isolated from 10-day-old rats were incubated for 30 min in buffer or with added cytochalasin B (50 μM), ouabain (1 mM) or insulin (0·1 u./ml.). 3MG uptake was then measured during a 30 sec incubation at 37 °C in the presence of 5 mM [^{3}H]3MG (5 μc/μmole). Each value is the average of three determinations ± S.D.

Solute transport. We have previously shown (Betz *et al.* 1979) that hexose transport into capillaries from 30-day-old rat brains is rapid, is not rate-limiting for capillary metabolism and can be inhibited by cytochalasin B. It is not inhibited by ouabain or uncouplers of oxidative metabolism (2,4-dinitrophenol). We obtained similar results using brain capillaries from 10-day-old rats. The rate of 3MG transport and equilibrium distribution space ($2{\cdot}1 \pm 0{\cdot}2$ μl./mg cell protein) were similar to our

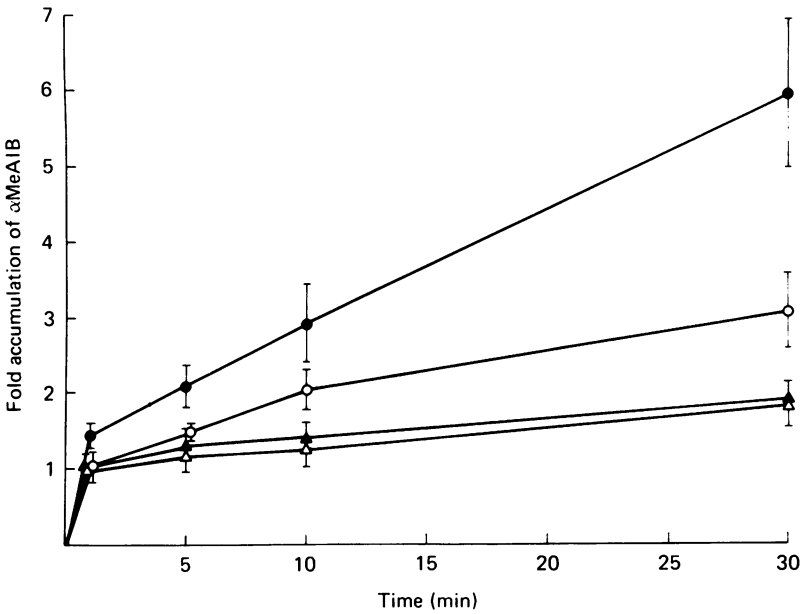

Fig. 5. Accumulation of α-methylaminoisobutyric acid by brain capillaries at different ages. The time course for αMeAIB uptake at 37 °C was determined after a 20 min pre-incubation with or without 1 mM-ouabain. The ratio between αMeAIB uptake in the absence and presence of ouabain is used as an estimate of the tissue to medium ratio of αMeAIB concentration. This measure of accumulation has been plotted against time of incubation for capillaries from 10- (△), 21- (▲), 30- (○) or 45- (●) day-old rats. All incubations contained 0·28 mM-[^{14}C]αMeAIB (8·9 $\mu c/\mu$mole). Each point is the average of three determinations ± S.D.

previous study. In addition, 3MG uptake was inhibited by cytochalasin B and unaffected by ouabain or insulin (Table 1). We conclude that the properties of glucose transport into brain capillaries do not show a marked change between 10 and 30 days of age.

Sodium-dependent neutral amino acid transport has been previously demonstrated in isolated rat brain capillaries using αMeAIB (Betz & Goldstein, 1978). This system is capable of concentrative amino acid transport into cells since it is coupled to the trans-cellular Na^+ gradient (Christensen, 1973). When the Na^+ gradient is eliminated by the Na^+, K^+-ATPase inhibitor, ouabain, concentrative uptake no longer occurs. Thus, neutral amino acid uptake in the presence and absence of ouabain provides a measure of the ability of this transport system to promote transport against a

gradient (accumulation). The developmental change in the ratio between αMeAIB uptake in the absence of ouabain and its uptake after pre-incubation with ouabain is shown in Fig. 5. There was minimal accumulation of αMeAIB in brain capillaries isolated from animals less than 21 days of age. In a previous report, we proposed that Na^+-dependent transport of neutral amino acids by brain capillaries was responsible

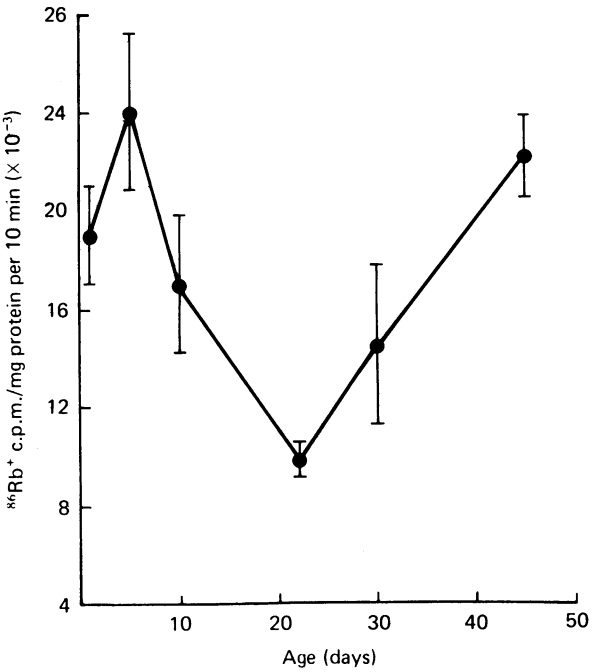

Fig. 6. Uptake of $^{86}Rb^+$ by brain capillaries at different ages. Capillaries were incubated for 10 min at 37 °C with $^{86}Rb^+$ ($2{\cdot}5 \times 10^6$ c.p.m.) in 0·4 ml. with or without 1 mM-ouabain. Data shown are the ouabain-inhibitable fraction of $^{86}Rb^+$ uptake. Each point is the average of four determinations ± S.D.

for active efflux of amino acids from brain to blood (Betz & Goldstein, 1978). The developmental changes presented here suggest that active efflux of amino acids across the BBB may not occur until after 21 days of age.

One possible explanation for decreased Na^+-dependent amino acid uptake by capillaries from young animals would be low activity of Na^+, K^+-ATPase since this is the pump responsible for maintaining a Na^+ gradient in brain capillaries (Betz & Goldstein, 1978). Activity of this pump can be estimated by measuring ouabain-sensitive uptake of $^{86}Rb^+$ (Goldstein, 1979*a*; Eisenberg & Suddith, 1979). In contrast to αMeAIB uptake, the $^{86}Rb^+$ pump was as active in capillaries from young animals as it was in older animals (Fig. 6).

DISCUSSION

Many investigators have noted that the BBB of immature animals is more permeable than that of adults (see Saunders, 1977 for review). The basis for this increased permeability is not presently known. Blood–brain barrier permeability in the adult has been extensively studied and it is generally agreed that the brain capillary endothelial cell is the anatomical site of the BBB. No single property of these cells accounts for all aspects of the BBB, but rather, there are at least four features that must be considered. (1) Low passive permeability to large molecular weight solutes is due to the low level of pinocytosis and the presence of tight junctions between brain capillary endothelial cells. (2) The permeability of low molecular weight solutes depends upon their lipid solubility and the presence of specific carrier-mediated transport systems present in the endothelial cell membranes. For example, D-glucose can readily penetrate the BBB but L-glucose cannot because of a stereospecific D-glucose transport system. (3) The presence of a 'metabolic' BBB is also well established. This aspect of the BBB depends upon the presence of specific enzymes within the brain capillary endothelial cell which rapidly metabolize certain solutes instead of permitting them to cross the capillary wall. (4) Finally, brain capillaries have been shown to promote active efflux of solutes such as amino acids and K^+ from brain to blood. This active efflux component of the BBB probably provides an important means of controlling the interstitial environment in the central nervous system (Davson, 1976; Bradbury, 1979). We will consider developmental changes in each of these four components of the BBB in the following discussion.

Low permeability to proteins. The BBB permeability to lipid-insoluble materials that penetrate passively, such as albumin, is enhanced in immature rats and fetal lambs (Saunders, 1977; Dziegielewska, Evans, Malinowska, Møllgård, Reynolds, Reynolds & Saunders, 1979). Although the reason for this enhanced permeability is unknown, possible mechanisms include leaky tight junctions, increased pinocytosis or transendothelial channels. The reader is referred to Saunders (1977) for a review of this subject since it was not investigated in our study.

Membrane transport of low molecular weight solutes. Blood to brain transport of D-glucose, certain amino acids, pyruvate and lactate occur by carrier-mediated BBB transport systems. We have found that the mechanism of D-glucose uptake by rat brain capillaries is similar at 10 and 30 days of age. This agrees with developmental studies of D-glucose uptake across the BBB of intact rats (Moore, Lione, Regen, Tarpley & Raines, 1971; Daniel, Love & Pratt, 1978; Cremer, Cunningham, Pardridge, Braun & Oldendorf, 1979) and rabbits (Braun, Cornford & Oldendorf, 1980). Likewise, large neutral amino acid uptake from blood to brain is qualitatively similar although quantitatively greater in immature compared to adult rats (Sershen & Lajtha, 1976; Banos, Daniel & Pratt, 1978). Blood to brain uptake of pyruvate, lactate (Cremer *et al.* 1979) and β-hydroxybutyrate (Cremer, Braun & Oldendorf, 1976; Moore, Lione, Sugden & Regen, 1976) is also greater in suckling rats compared to adults due to enhanced carrier-mediated transport.

A frequent finding in many *in vivo* studies of BBB permeability to low molecular weight solutes is the presence of a higher rate of non-saturable uptake in the immature

animal (Moore *et al.* 1971; Sershen & Lajtha, 1976; Cremer *et al.* 1979). This is true regardless of changes in carrier-mediated transport. A likely explanation is that whatever is responsible for the increased permeability to proteins discussed previously also permits increased passive permeability to small solutes.

Metabolic blood–brain barrier. The presence of L-DOPA decarboxylase and monoamine oxidase within brain capillary endothelial cells is thought to result in restricted BBB passage of L-DOPA (Bertler *et al.* 1966). Our data show an age-dependent decrease in L-DOPA decarboxylase activity suggesting that this aspect of the metabolic BBB is most active in young animals. Similar results have been obtained in histochemical studies (Kellogg *et al.* 1973).

Loizou (1970) found that monoamines could easily cross the BBB of rats up to 21 days of age. This would appear to contradict our finding of high monoamine oxidase activity in capillaries from 10-day-old rat brain; however, it is possible that the general increase in diffusion across the BBB discussed above could permit brain uptake of monoamines in spite of active degradative pathways within the capillary endothelium.

Active efflux systems. In previous communications, we proposed that brain capillaries promote active efflux from brain to blood of small neutral amono acids (Betz & Goldstein, 1978) and K^+ (Goldstein, 1979*a*). Similar systems probably exist for iodide, prostaglandins and some organic anions (Davson, 1976; Bradbury, 1979). Our present results suggest that active efflux of neutral amino acids may not occur in the BBB of young animals. Thus, an important homeostatic mechanism for maintaining low levels of amino acids in brain extracellular fluids does not develop until after weaning. This lack of an active efflux mechanism for amino acids at the immature BBB was predicted from the results of *in vivo* studies (Seta, Sershen & Lajtha, 1972).

Not all active efflux systems show this developmental change, since K^+ transport by brain capillaries is at least as active in 5-day as it is in 45-day-old rats. This may be necessary to maintain normal K^+ homeostasis in the face of a relatively large passive leak of plasma solutes from blood to brain.

Conclusions. It is apparent that the BBB is more than a selective sieve. Active substrate efflux and metabolism by brain capillaries are also important in BBB function and these aspects of the BBB do show distinct developmental changes. Since brain capillaries are involved in the formation of brain oedema (Goldstein, 1979*b*), developmental changes in the active properties of brain capillaries may explain why children are more likely than adults to develop brain oedema following toxic and metabolic injuries. Studies with isolated brain capillaries should provide new insights into the pathogenesis of these disorders.

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