Subcellular fractions were prepared from sucrose homogenates of rat cerebrum, according to the procedure summarized in the Figure. P_2, the crude mitochondrial and synaptosome (pinched-off nerve ending) pellet, was resuspended in either 0.32 *M* sucrose or H_2O; in the latter case it was designated P_2W. The fractions obtained by this procedure have been characterized by TUČEK[7]. Subfraction B (obtained from P_2) contains synaptosomes, while O (obtained from P_2W) is soluble material; D contains synaptic vesicles while F, G and H are nerve ending membranes and I, mitochondria.

Each fraction and subfraction was examined for GPC diesterase and choline acetyltransferase activity. The choline acetyltransferase was assayed using the method described by HEBB et al.[8]. The results obtained were expressed as nmols/g/h and are given in Table II. A considerable proportion of GPC diesterase activity was bound to membranes and in particular to nerve-ending membranes; 38% of the total was present in the P_2 fraction. Subfractionation of this showed that 24% (i.e. 63% of P_2) was in the synaptosomes. Fractionation of P_2W showed that much of the activity in the synaptosome was in, or associated with, the nerve-ending membrane. It has already been shown that GPC diesterase in the liver is associated with plasma membranes[9].

GPC diesterase was also incubated in the presence of 10 m*M* hemicholinium-3, an inhibitor of ACh synthesis in vivo, but no inhibition of GPC diesterase was observed even when the substrate concentration was lowered.

Discussion. The assay of GPC diesterase described here is simple and reliable and gives values for brain close to those previously reported. There is, however, a significant difference between the optimum pH and substrate concentration used here and those reported by other authors[1-3]. It has not been possible to establish the reason for this but it may be associated with the Mg^{++} concentration of the original tissue, a question that can only be resolved by using purified enzyme preparations in place of the crude homogenates used in the present work.

The distribution of GPC diesterase in the rat brain is very uniform and is not specifically localized in any area, thus differing from many of the enzymes which are associated with neurotransmitter synthesis. It is, therefore, somewhat surprizing to find that its subcellular distribution is associated with nerve-ending particles, and in this respect its distribution closely resembles that of choline acetyltransferase. This would tend to suggest that the enzyme is associated with the transmitter function of the nerve ending and possibly the nerve-ending membrane. The amount of the enzyme in the brain leaves no doubt that it could be a major contributor to the pool of free choline in the brain. But it could be argued that this pool would then be evenly distributed through the brain like the GPC diesterase. Where this enzyme occurs in cholinergic neurons the choline released could well be utilized for the synthesis of ACh, although some, at least, is likely to be used to form choline phosphate[10], which is a precursor of phosphatidylcholine. The uniform distribution in the brain would tend to indicate that it is present in most if not all nerve endings. In addition to releasing choline this enzyme also releases energy in the form of glycerol-3-phosphate, and this could be of importance in membrane function. The work reported here does not indicate which of these possibilities is the most likely and it may be that the release of choline and a source of energy have an equal importance in the transmitter function of the nerve ending.

Summary. The distribution of glycerylphosphorylcholine diesterase in the rat brain has been examined. The enzyme was evenly distributed throughout the brain but was localized in the synaptosome (nerve ending) fraction which was prepared by ultracentrifugation.

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Regional Distribution of Lactate Dehydrogenase Isoenzymes in Adult Rat Brain

The special functions of different brain parts must be based on specific biochemical differences. Recently[1] we were able to show that there are significant regional differences in levels of substrates and adenine nucleotides in adult rat brain. These differences may be related to different enzymatic patterns of the glycolytic sequence. We have now determined the activity of lactate dehydrogenase (EC 1.1.1.27; LDH) and its isoenzymes in cerebral cortex, thalamus, cerebellar cortex and pons, because the total activity, and especially isoenzymatic pattern of this enzyme, can give useful information about major pathways of glucose utilization[2-4].

Experimental. Pyruvate-Na, α-oxobutyrate and NADH were obtained from Boehringer Mannheim GmbH. DEAE Sephadex A-50 (3.5 mEq/g) was purchased from Pharmacia (Uppsala, Sweden).

The investigations were carried out on adult male Louis rats. Non-anesthetized animals were decapitated, their heads were opened and the brains removed. After weighing, tissue samples were homogenized in ice-cold hypotonic (10 m*M*) potassium phosphate buffer (pH 7.0); the tissue/buffer ratio was 1:50 (w/v). Homogenates were centrifuged at 25,000 × *g* at 0°–4 °C for 1 h. Supernatant solutions were divided into 2 parts: one part was used for the measurement of the total and LDH_1 activity and the other part (0.5 ml) was mixed with 1 ml DEAE Sephadex A-50 (20 mg/ml phosphate buffer, pH 6.0)[5] and allowed to stand for 10 min; resin was removed by centrifugation (4,000 × *g*, 10 min). DEAE Sephadex adsorbs anodic LDH isoenzymes[4,5] i.e. LDH_1 and LDH_2.

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Regional distribution of lactate dehydrogenase isoenzymes in adult rat brain

Brain part	Total LDH activity [a]	LDH_1 activity [a]	Not-adsorbed LDH [a]
Cerebral cortex	1180 ± 20 [b, d]	690 ± 20 [c, d]	270 ± 2 [c, d]
Thalamus	1250 ± 20 [c, d]	670 ± 20 [c, d]	250 ± 10 [c, d]
Cerebellar cortex	1180 ± 20 [d]	500 ± 5 [d]	48 ± 2
Pons	850 ± 20	470 ± 4	60 ± 7

[a] For tissue treatment and assay conditions, see under Experimental. Enzymatic activities are expressed as nmoles substrate converted per min per mg total tissue protein, 25 °C. Values represent mean for 5 animals ± SEM. Student's *t*-test was performed for comparisons. [b] $p < 0.01$ relative to thalamus. [c] $p < 0.01$ relative to cerebellar cortex. [d] $p < 0.01$ relative to pons.

Lactate dehydrogenase activity was determined by following the disappearance of NADH at 365 nm in a reaction mixture that contained 50 m*M* phosphate buffer (pH 7.5), 0.2 m*M* NADH and appropriate substrate. 'Total LDH activity' was determined in $25{,}000 \times g$ supernatant with 0.6 m*M* pyruvate. 'LDH_1 activity' was determined with 3 m*M* oxobutyrate (the other isoenzymes also participate in activity with oxobutyrate, but to a significantly lesser degree than does LDH_1[6, 7]). 'Not adsorbed LDH' was determined with 0.6 m*M* pyruvate in supernatants obtained after the adsorption on DEAE Sephadex A-50, representing basic LDH isoenzymes, i.e. LDH_4 and LDH_5. Temperature of reaction mixture was maintained at 25 °C by means of a thermostated cuvette holder.

Protein concentrations were determined in homogenates according to HARTREE's modification[8] of the Lowry method, using human serum albumin as standard.

Results. The results of our investigations are shown in the Table.

Total LDH activity (0.6 m*M* pyruvate). The highest total LDH activity was found in thalamus and the lowest in pons. There is no significant difference between cerebral and cerebellar cortex in respect to total lactate dehydrogenase activity, but these activities were significantly ($p < 0.01$) lower than the thalamic one, and significantly ($p < 0.01$) higher than that recorded in pons (Table).

LDH_1 activity (3 m*M* oxobutyrate). LDH_1 represents 58% of the total lactate dehydrogenase activity in cerebral cortex, 54% in thalamus, 42% in cerebellar cortex and 55% in pons. If the activities are expressed in terms of mU (Table), the highest LDH_1 activities are found in cerebral cortex and thalamus, significantly lower ($p < 0.01$) in cerebellar cortex and the lowest in pons.

Not-adsorbed LDH (0.6 m*M* pyruvate). After the adsorption on DEAE Sephadex A-50, 23% and 20% of the total lactate dehydrogenase activity were still present in extracts of cerebral cortex and thalamus, while in extracts of cerebellar cortex and pons only 4% and 7% remained. It is clear that the not-adsorbed LDH activities in cerebellar cortex and pons are significantly ($p < 0.001$) lower than those in cerebral cortex and thalamus.

Discussion. The results of our investigations (Table) show that there are significant regional differences in lactate dehydrogenase activity and isoenzymatic pattern in adult rat brain. Our results are in agreement with the results obtained on whole brain homogenates for total LDH activity[9, 10] as well as for general isoenzymatic pattern[4, 11, 12].

The highest lactate dehydrogenase activity found in thalamus (Table) may be due to the fact that this subcortical gray matter consists of tightly packed neuronal cell bodies; in contrast, pons contained proportionately (to a total mass) lesser cell content, consistent with our finding of the lowest total lactate dehydrogenase activity in this brain part. Also, it has been shown[13] that morphologically different types of neurons have different lactate dehydrogenase activities: facial nucleus cells and Deiters nucleus cells (both localized in pons) have considerably lower LDH activity than do pyramidal cells from cerebral cortex or Purkinje cells from cerebellum. Probably both of these factors determine the level of total LDH activity.

LDH_1 is the predominate lactate dehydrogenase isoenzyme in all brain region we investigated (Table), constituting more than 50% (except in cerebellar cortex) of total LDH activity. This is consistent with the fact that in aerobic tissues LDH_1 dominates[2, 3]. We cannot explain the low percentage of LDH_1 in cerebellar cortex, especially since cerebellum has a high energy status[1]. However, determination of LDH_1 activity with 3 m*M* oxobutyrate as a substrate has certain limitations, since it has been shown that the ratio 'activity with 3 m*M* oxobutyrate/activity with 0.6 m*M* pyruvate' is not the same for LDH_1 isoenzymes from different species or even different tissues[4, 6]. It is possible that in Purkinje cells, which have many distinct morphological, functional and biochemical characteristics[13], LDH_1 may have unusual molecular and/or catalytic features. The finding that not-adsorbed LDH represents a minor part of total lactate dehydrogenase activity in cerebellar cortex (Table) is consistent with such a hypothesis, because not-adsorbed isoenzymes are LDH_4 and LDH_5.

The 4- to 5-fold greater not-adsorbed LDH activity in cerebral cortex and thalamus compared with cerebellar cortex and pons (Table) might be interpreted as a sign of the unequal participation of the lactate dehydrogenase system in the production of the oxidized form of NADP, since it has been shown[14] that lactate dehydrogenase isoenzymes from cattle retina are able to use NADP as coenzyme; PASANTES-MORALES et al.[15] suggested that high LDH_5/LDH_1 ratio is indicative of high activity of the hexose monophosphate pathway of glucose utilization. Although KAUFMAN[16] demonstrated regional differences in the activities of the enzymes of hexose monophosphate pathway in rat brain, correlation between lactate dehydrogenase system and hexose monophosphate pathway remains to be elucidated.

Regulation of lactate dehydrogenase activity by pyruvate inhibition, even at the physiological (i.e. cellular) level, is a generally accepted assumption[17]. The

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tighest pyruvate regulation may be expected in tissues with high levels of LDH_1 activities, because this isoenzyme is strongly inhibited with higher pyruvate concentrations. Our findings (Table) suggest that brain has the capability for such a control of LDH activity.

Summary. The highest lactate dehydrogenase (LDH) activity was found in thalamus, statistically significantly less in cerebral and cerebellar cortex and the lowest in pons. LDH_1 and LDH_{4+5} represented 58% and 23% of the total activity in cerebral cortex, 54% and 20% in thalamus, 42% and 4% in cerebellar cortex and 55% and 7% in pons, respectively.

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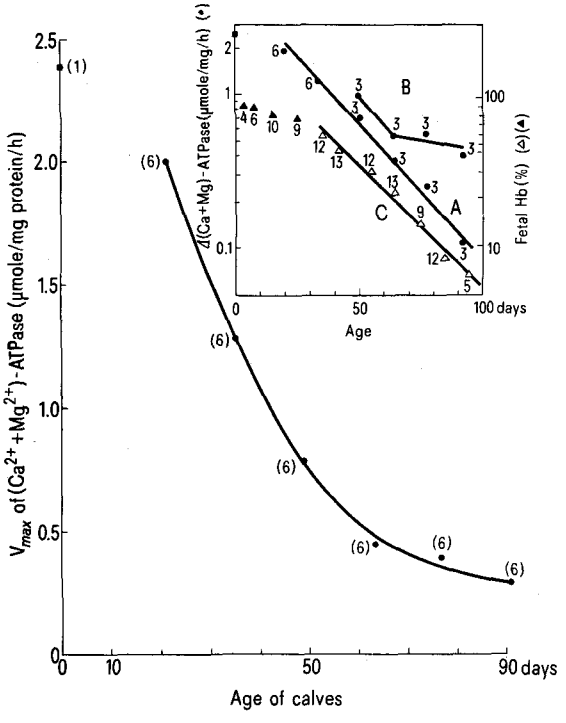
Postnatal Decline of (Ca^{2+} + Mg^{2+})-Activated Membrane ATPase in Cattle Red Cells

(Ca^{2+} + Mg^{2+})-stimulated, membrane bound ATPase ((Ca^{2+} + Mg^{2+})-ATPase) in human red cells is believed to reflect the presence of an active outward Ca transport system[1–5]. In adult cattle erythrocytes, the (Ca^{2+} + Mg^{2+})-ATPase activity is only about 1/50 of that found in human cells[6,7]. However, in young calves the enzyme activity exceeds that of human cells and starts falling after the third week[6]. In the present study we examined the time course of this decline in a group of 6 calves of the Simmenthal breed, fed on artificial milk, supplemented with an oral dose of iron shortly after birth. This is compared with the time course of disappearance of fetal hemoglobin (fHb), measured in a group of 13 calves of the same breed, fed in the same way.



v_{max} of red cell membrane (Ca^{2+} + Mg^{2+})-ATPase, taken from 1/v vs. 1/Ca^{2+}-conc. plots, as function of age of calves. ■, red cells of one case obtained from umbilical vein at birth. Number of animals near points. Inset: Same observations, together with data for fetal hemoglobin from another group of calves. •, ordinate: v_{max} of (Ca^{2+} + Mg^{2+})-ATPase minus 0.015 µmole/mg/h (value for cows). A) fast growing calves; B) slowly growing calves. For the first two points the 6 animals were pooled. Later on the group was divided into fast and slowly growing animals, because a difference in weight gain became apparent after 50 days. ▲△, fetal Hb in percent of total Hb (value at infinity assumed to be zero). Note that disappearance of fetal Hb becomes rapide and exponential (linear in log plot) after 20 days. Therefore only the part of the curve marked by open triangles was used for calculation of regression line. Rate constant for decline of ATP-ase (line A) = 0.041 ± 0.002 d^{-1}, for fetal Hb (line C) = 0.037 ± 0.002 d^{-1}. Number of animals near points.

Equal amounts of fresh, washed red cells from 3 or 6 animals were pooled, membranes were prepared as described before[5] and (Ca + Mg)-ATPase assayed by measuring liberation of inorganic phosphate[5] in the following medium: (m*M*) Choline-Cl 110, imidazole-Cl 30, $MgCl_2$ 4, Na-ATP 2, ouabain 0.17, Ca-EGTA buffer or *tris*-EGTA 1, pH 7.0. Sample volume was 2.5 ml, mean protein concentration 0.64 mg/ml, temperature 37 °C and incubation time 90 min. ATPase requiring Ca alone showed negligible activity. The fraction of fHb was determined in calves with type A adult hemoglobin[8] by electrophoretic separation on cellulose acetate strips at pH 8.6 and scanning the stained strips (Ponceau S) photometrically[9]. Ca influx into intact fresh cells was measured after ATP depletion in the following way: 1 vol of washed cells was preincubated for 1 h at 37 °C in 30 vol of medium ((m*M*) NaCl 120, KCl 5, *tris*-Cl 30, iodoacetamide 5, inosine 5, pH 7.4) or starved for 17 h at 37 °C and preincubated with the metabolic poisons for 20 min. Then 1 m*M* $^{45}CaCl_2$ was added, samples taken at 1 h intervals and cells washed 4 times in 50 vol ice-cold medium without labelled Ca and inhibitors. An aliquot of cells was dried on a planchet and counted in a windowless Geiger-Müller tube. Results were corrected for the difference in self-absorption of medium and cells.

The Figure shows the time course of decay of the (Ca + Mg)-ATPase with age. Maximal rate (v_{max}) was found by plotting activation curves obtained with Ca^{2+}-concentrations between 10^{-6} and 10^{-5} *M* according to Lineweaver-Burk. K_{Ca} values varied between 0.83 and 2.6×10^{-6} *M* and showed no dependence on age. The

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