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Postmortem Activity of the Key Enzymes of Glycolysis

In Rat Brain Regions in Relation to Time After Death

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Summary. The activities of the rate-limiting enzymes of glycolytic pathway were measured in various areas of rat brains kept at a temperature of +25°C for various intervals after death by cervical dislocation. Hexokinase shows a substantial decline in activity over a period of 24 h, reaching 41%, 57%, 44%, and 51% of the controls in cerebellum, medulla oblongata and pons, cerebral cortex, and diencephalon, respectively. In the same areas the phosphofructokinase reached 28%, 61%, 60%, and 40% of the zero-time activity, respectively. Lactate dehydrogenase behaves differently in the four areas, with an increase in cerebral cortex and diencephalon and a decrease in cerebellum and medulla oblongata and pons. Pyruvate kinase activity was quite stable over the 24 h period studied. Therefore, the activities of hexokinase and phosphofructokinase in brain tissue were of little value for diagnosis of the time of death.

Key words: Postmortem enzymes – Data of death – Glycolytic enzymes – Rat brain enzymes

Zusammenfassung. Es wird über die Aktivität der für die Glykolyse notwendigen Schlüsselenzyme in verschiedenen Hirngebieten der Ratte berichtet. Nach Tötung der Tiere mittels Zervikalluxation wurden die Gehirne bei +25°C verwahrt. Die Bestimmungen wurden zu verschiedenen Zeitpunkten durchgeführt. Die Hexokinase zeigte eine deutliche Aktivitätsverminderung nach den ersten 24 h: 44% der Kontrollaktivität in der Großhirnrinde, 51% im Diencephalon, 57% in der Medulla oblongata, im Pons und 41% im Cerebellum. In denselben Zonen zeigte die Phosphofructokinase ein 60%, 40%, 61% und 28% der Aktivität im Verhältnis mit der Kontrolle. Die Werte für die Laktatdehydrogenase waren verschieden. Zwar zeigten sich auch eine Abnahme im Pons und in der Medulla oblongata und im Cerebellum, dagegen eine Zunahme in der Cortex und im Diencephalon. Die Aktivität der Pyruvatkinase war ziemlich beständig. Trotzdem erscheint es, daß das Studium der Aktivität der Hexokinase und Phosphofructokinase von geringem Wert für die Bestimmung der Todeszeit ist.

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Schlüsselwörter: Postmortale Enzyme – Todeszeitbestimmung – Enzyme der Glykolyse

Introduction

The postmortem activity of enzymes in nervous tissue rises considerable interest for several reasons. One is the possible application of the postmortem enzymic activity in forensic medicine. The determination of the postmortem time is always a problem, and at present there is no reliable method of estimating the time of death. One approach to the problem has been the biochemical analysis of various body fluids from corpses. This involved the quantification of many of the constituents in body fluids including serum, vitreous humor, cerebrospinal fluid (CSF), and pericardial fluid [1–4]. Although the changes in these parameters follow a certain pattern, they are unpredictable and subject to a great deal of individual variation.

The study of biochemical changes in tissues provides an alternative to the body fluids, with additional information for certain diagnostic and forensic questions. Several studies on the activity of various enzymes in different types of tissue during the postmortal interval have been published [3, 6–8], but only a few are available on the postmortal activity of enzymes in nerve tissue. Oehmichen [9] reviewed the literature on the activity of various enzymes in the central nervous system (CNS) during the postmortal interval up to 48 h. These studies were performed on whole brain or cerebellum, and the enzyme activities were evaluated by histochemical and biochemical methods.

There are some regional studies on brain enzymes [10–12] and we found previously (results unpublished) a heterogeneous distribution of energy-requiring enzymes in rat brains. Other authors have made regional studies on postmortal enzyme activities in rat brain [13, 14].

The brain is metabolically one of the most active of all the organs in the body. Glucose serves as the main fuel for energy metabolism in the adult mammalian brain, being metabolized almost entirely on the Embden-Meyerhoff pathway. The rate of glycolysis in the brain appears to be regulated by the coordinated action of a few rate-limiting enzymes (hexokinase, phosphofructokinase, and pyruvate kinase) in this pathway [15].

In this investigation, at various times after death, four rat brain areas were analyzed (cerebellum, medulla oblongata and pons, cerebral cortex, and diencephalon) for the evolution in the activities of the following enzymes: hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40), and lactate dehydrogenase (EC 1.1.1.27). As there are considerable problems associated with obtaining human postmortem tissue at short intervals after death, this study was limited to rat brain.

Materials and Methods

Male and female Wistar rats (180–200 g b.wt.) were used during the experiments. Five rats each were kept in separate cages and allowed free access to food and water. The animals were

Table 1. Postmortem changes in hexokinase activity in rat brain areas

Region	Death time (h)					
	0	1	3	6	12	24
Cerebellum	100[a] ± 5.66	88.89 ± 3.14	61.75 ± 7.03***	45.10 ± 2.77***	44.36 ± 2.91***	41.24 ± 5.06***
M. oblongata and pons	100[b] ± 8.39	101.37 ± 28.26	49.51 ± 8.28**	65.05 ± 9.91	49.83 ± 6.87**	57.15 ± 4.09
C. cortex	100[c] ± 6.10	84.50 ± 20.89	51.69 ± 3.95	97.71 ± 11.26	74.05 ± 19.65	44.19 ± 7.53*
Diencephalon	100[d] ± 7.57	121.08 ± 13.74	68.26 ± 5.33	95.32 ± 9.90	66.28 ± 7.08	51.33 ± 6.93*

Each value is the mean of five animals ± SEM expressed as percentage of control (0 h) except in the case of 0 h ($n = 10$)
* $P < 0.05$, ** $P < 0.02$, and *** $P < 0.001$ as compared to control in all areas. Actual values: [a] 19.12 mU/mg protein; [b] 16.01 mU/mg protein; [c] 31.11 mU/mg protein; [d] 25.07 mU/mg protein

kept under regular dark-light conditions (light period 8 a.m. to 8 p.m.). Six groups of five animals were employed. Rats were killed by cervical dislocation and their brains kept with the rest of the body at room temperature (average $t^a = 25°C$). At 0, 1, 3, 6, 12, and 24 h after death brains were removed from the skull, placed in liquid nitrogen at −196°C and dissected in four different regions according to the method described by Glowinski and Iversen [16]: cerebellum, medulla oblongata and pons, cerebral cortex, and diencephalon. All dissected regions were homogenized with 5 vol. of ice-cold medium containing 0.25 *M* sucrose, 1 m*M* EDTA (ethylenediamine-tetra acetic acid disodium salt), 0.1 m*M* DL-dithiothreitol and 20 m*M* triethanolamine, pH 7.4, in an all-glass Potter-Elvehjen homogenizer. The homogenate obtained was centrifuged at 2,060 x*g* (10 min) at 4°C (Beckman, model J2–21), and one aliquot of the supernatant was used for hexokinase assay. Then, the rest of the supernatant was centrifuged again at 12,900 x*g* for 10 min, and this supernatant was employed for the determination of phosphofructokinase, pyruvate kinase, and lactate dehydrogenase activities.

Hexokinase was measured as described by Hernandez and Crane [17] modified by others [10, 18]. Phosphofructokinase was assayed essentially by the method of Racker [19] modified by Johnson [20] and Bielicki [21]. Pyruvate kinase was determined as described by Bücher and Pfleiderer [22] and Johnson [20], and lactate dehydrogenase was assayed as reported by Bergmeyer and Bernt [23]. Protein was determined by the method of Lowry et al. [24] with bovine serum albumin as the standard. All enzyme assays were carried out at 25°C, and measures were made with a Beckman 25 Spectrofotometer-recorder following the rate of change of extinction at 340 nm. Results were expressed as specific activities (nmol · min^{-1} · mg $protein^{-1}$), and statistical analysis was performed using a one-way analysis of variance (ANOVA I) and Dunnet's test.

Results

The activity of hexokinase decreased significantly during the period studied after death (Table 1). The activity at 24 h was markedly reduced as compared with that found immediately after cervical dislocation (0 h). Figure 1 shows the profile of hexokinase activity between 0 and 24 h, with an irregular behavior in

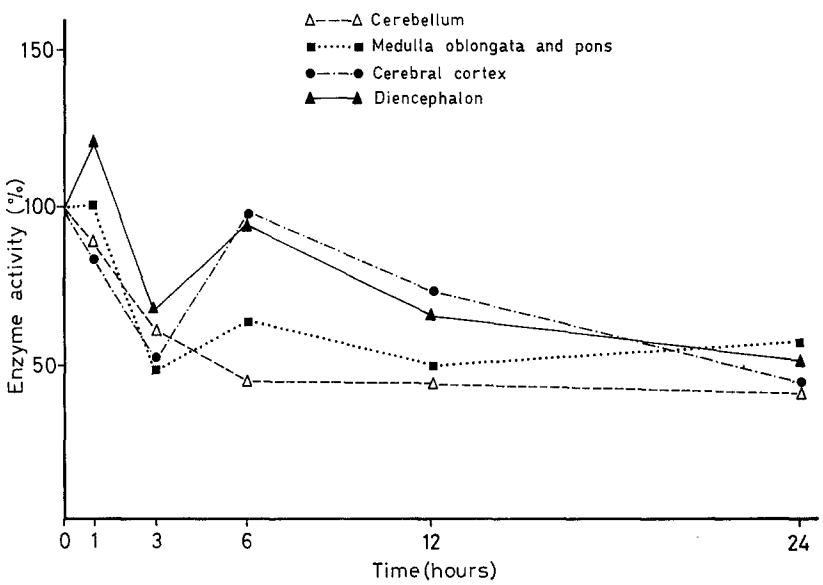

Fig. 1. Hexokinase activity (%) plotted against time (h) after death

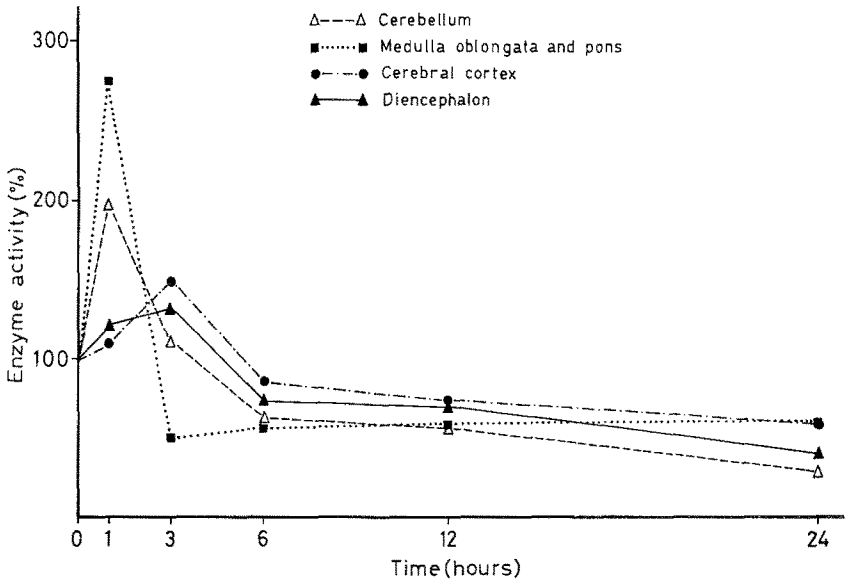

Fig. 2. Phosphofructokinase activity (%) plotted against time (h) after death

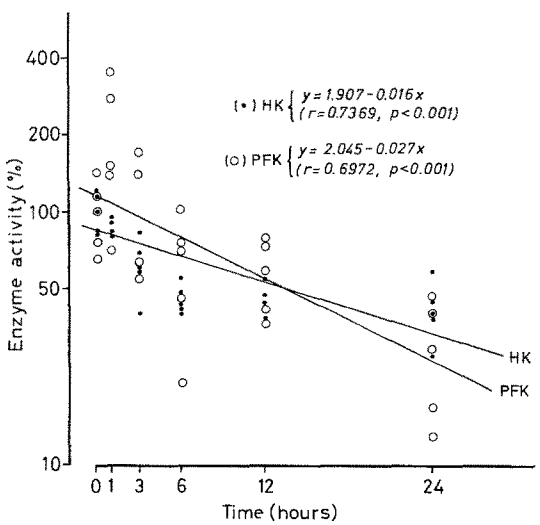

Fig. 3. The significant exponential correlation between the specific activity of hexokinase and phosphofructokinase (% of controls) in cerebellum and the time after death (enzyme activity on exponential paper)

the first 6 h. Thereafter, the activity decreased slowly until 24 h, reaching percentages of about 50% in the four areas assayed.

Significant exponential correlation values between the enzyme activity, and the postmortem time were 0.737, 0.394, 0.519, and 0.665 in the four areas studied (cerebellum, medulla oblongata and pons, cerebral cortex, and diencephalon, respectively). In particular, the hexokinase activity in cerebellum and the postmortem time is shown in Figure 3 ($y = 1.907 - 0.016x$; $P < 0.001$).

Phosphofructokinase, the rate-limiting enzyme in the glycolysis also exhibits a decrease in its activity with a reduction of 39%–72% relative to the controls

Table 2. Postmortem changes in phosphofructokinase activity in rat brain areas

Region	Death time (h)					
	0	1	3	6	12	24
Cerebellum	100[a] ± 12.59	198.37 ± 51.04*	114.04 ± 22.88	63.49 ± 13.71	57.43 ± 7.89	28.56 ± 6.22
M. oblongata and pons	100[b] ± 10.55	276.29 ± 31.97**	50.16 ± 4.78	57.83 ± 10.11	59.54 ± 8.28	61.29 ± 11.51
C. cortex	100[c] ± 9.79	116.66 ± 11.09	151.71 ± 13.12*	86.50 ± 7.53	74.76 ± 10.36	59.77 ± 20.84
Diencephalon	100[d] ± 9.83	123.63 ± 13.25	133.93 ± 25.51	75.35 ± 1.67	65.39 ± 9.26	39.83 ± 12.24*

Each value is the mean of five animals ± SEM expressed as percentage of control (0 h) except in the case of 0 h ($n = 10$)
* $P < 0.05$ and ** $P < 0.001$ as compared to control in all areas. Actual values: [a] 17.02 mU/mg protein; [b] 15.57 mU/mg protein; [c] 35.80 mU/mg protein; [d] 40.58 mU/mg protein

Table 3. Postmortem changes in pyruvate kinase activity in rat brain areas

Region	Death time (h)					
	0	1	3	6	12	24
Cerebellum	100[a] ± 5.54	95.87 ± 3.79	74.79 ± 7.65	68.80 ± 9.74*	66.76 ± 8.45*	67.40 ± 6.62*
M. oblongata and pons	100[b] ± 7.80	108.30 ± 13.04	65.74 ± 9.63	76.85 ± 12.52	70.95 ± 7.75	76.04 ± 11.77
C. cortex	100[c] ± 6.40	129.76 ± 10.15	112.74 ± 8.00	100.31 ± 4.90	104.52 ± 7.84	113.56 ± 7.77
Diencephalon	100[d] ± 5.43	100.30 ± 11.44	117.62 ± 6.96	93.86 ± 2.82	93.52 ± 2.47	108.21 ± 3.95

Each value is the mean of five animals ± SEM expressed as percentage of control (0 h) except in the case of 0 h ($n = 10$)
* $P < 0.05$ as compared to control in all areas. Actual values: [a] 537.53 mU/mg protein; [b] 488.93 mU/mg protein; [c] 594.41 mU/mg protein; [d] 695.59 mU/mg protein

Table 4. Postmortem changes in lactate dehydrogenase in rat brain areas

Region	Death time (h)					
	0	1	3	6	12	24
Cerebellum	100[a] ± 6.47	87.16 ± 8.11	62.19 ± 7.91***	93.61 ± 4.25	89.01 ± 5.96	68.99 ± 3.74*
M. oblongata and pons	100[b] ± 6.39	102.52 ± 10.31	63.16 ± 5.56*	89.79 ± 6.48	82.78 ± 7.65	80.40 ± 10.81
C. cortex	100[c] ± 3.78	150.17 ± 10.09****	128.26 ± 4.57**	115.79 ± 7.80	123.80 ± 4.50	145.09 ± 12.67***
Diencephalon	100[d] ± 4.33	122.62 ± 15.38	136.11 ± 11.10*	100.38 ± 6.17	94.65 ± 3.40	146.74 ± 5.27***

Each value is the mean of five animals ± SEM expressed as percentage of control (0 h) except in the case of 0 h ($n = 10$)
* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, and **** $P < 0.001$ as compared to control in all areas. Actual values: [a] 1482.77 mU/mg protein; [b] 1313.59 mU/mg protein; [c] 1401.83 mU/mg protein; [d] 1595.43 mU/mg protein

after 24 h (Table 2). However, there was considerable variation in the activity of this enzyme and the SEM's were higher as compared with other enzymes studied. Figure 2 presents the change of phosphofructokinase activity with a marked increase after 1 h (especially in cerebellum and medulla oblongata and pons) and a progressive diminution up to the 24 h. The significant exponential correlation values for this enzyme in the four areas pointed out previously were $r = 0.697$, 0.405, 0.603, and 0.728, respectively. The exponential correlation for the activity in cerebellum is shown in Figure 3 ($y = 2.045 - 0.027x$; $P < 0.001$).

Tables 3 and 4 illustrate the variation in the activity of pyruvate kinase and lactate dehydrogenase in the postmortem interval (0–24 h). Pyruvate kinase presents only a significant decrease in cerebellum and medulla oblongata and pons, with activities quite similar to the controls in the other areas.

Lactate dehydrogenase shows a small decrease in cerebellum and medulla oblongata and pons and a more pronounced variation in cerebral cortex and diencephalon with increases of 45% and 46% as compared with the controls (0 h). In general, all the enzymes studied show considerable fluctuations between 0 and 6 h after death.

Discussion

Several studies on the activity of various enzymes in the brain during postmortem intervals have been published [13, 14, 25–29], but to our knowledge there are very few investigations on the relationships between the postmortem evolution and the enzyme activities of oxidative metabolism, and even fewer on the regional changes. The principal aim of this work was to study the postmortem regional changes of the glycolytic enzymes in rat brain. The evaluation of the extent to which autolysis of cell constituents may have occurred after death could be very interesting in various aspects and especially in the forensic field. These results could be applied to estimate the time after death if we assume that the enzymes are affected similarly in the animal model and human brain.

Of the enzymes studied, those that show a substantial decline of activity over a period of 24 h postmortem are phosphofructokinase and hexokinase (Tables 1,2, Figs. 1,2). These activities are initially increased but subsequently decline, whereas pyruvate kinase and lactate dehydrogenase exhibit a different pattern (Tables 3,4). The pyruvate kinase activity shows a small increase only in two regions (cerebellum and medulla oblongata and pons) with significant differences in the first area only. The other areas (cerebral cortex and diencephalon) remain unaltered over the period assayed. Lactate dehydrogenase also presents a different behavior in the four areas studied.

Phosphofructokinase and hexokinase showed a significant exponential correlation especially in the cerebellum (Fig. 3) and diencephalon. In general, there are not great differences in the results obtained for the four areas. The time course of enzyme activity for each area assayed was broadly similar to that obtained for the others. However, it appears that cerebellum exhibits the most constant behavior. In practice, the use of a particular brain region would not be decisive.

With respect to the use of these enzymes as a tool to establish the time after death, as can be seen from Figure 3, the scatter of points covered a large area. Although we found a gradual decrease in activities of phosphofructokinase and hexokinase with time after death, the decrease in activity would not give a very reliable estimation of time of death because of the spread of points.

Only one investigation [29] has been reported on postmortem changes in the activities of hexokinase, phosphofructokinase, and pyruvate kinase. It was carried out over a period of 145 h on human cerebellum and revealed variable changes in cytoplasmic enzymes: phosphofructokinase activity rapidly decreased and hexokinase was unaltered. It is difficult to compare these results with ours, mainly because of the different experimental conditions, but broadly speaking the behavior of phosphofructokinase is similar, reaching 37% of activity as compared with controls at 24 h (28.56% in our study). The results of hexokinase and pyruvate kinase are in contrast with those found by us.

Lactate dehydrogenase has been studied in rabbit cerebellum [30], rat brain [28], and human cerebellum [29] showing a similar profile in all cases. The variations of this enzyme over a period of time are not important.

As for the difference in the stability of the enzymes studied in this work, we must consider the autolytic process after death. Because of the respiratory and circulatory failure, the brain only has a small amount of locally stored energy available. These stores are rapidly depleted in the mouse [31] and consequently new enzyme synthesis ceases. Therefore, postmortem changes must represent loss of total activity present at the time of death. Autolysis begins shortly after death, apparently with continuous temperature-dependent release of lysosomal contents [27, 31]. Lysosomes release a variety of proteases that might contribute to the postmortem decline in some enzyme activities (Tables 1–4). Mann et al. [29] proposed that the remarkable stability of some enzymes may be due to their protection by the membranes in the organelles that would preserve against the lysosomal attack. The enzymes studied by us, however, are cytoplasmic (not protected by membranes) and this suggests that factors other than lysosomal release of proteases may contribute to change their activity. The decrease in enzyme activity can be due to alterations resulting from proteolysis, inactivation by inhibitors, or a combination of both [29].

Although the changes of phosphofructokinase and hexokinase in cerebellum are important (Fig. 3), the profile of these enzymatic activities would not give a very reliable estimation of time of death because of the spread of points and the special environmental conditions (average $t^a = 25°C$). From the standpoint of their practical value in forensic medicine it seems that assay of these enzymes in cerebral tissue is of limited value for the diagnosis of time after death.

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