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EFFECTS OF CEREBRO-PROTECTIVE AGENTS ON ENZYME ACTIVITIES OF RAT PRIMARY GLIAL CULTURES AND RAT CEREBRAL CORTEX

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Abstract—The effects of different cerebro-protective agents on selected key enzymes of the energy metabolism of rat primary glial cultures and rat cerebral cortex were studied. As indicators for the capacity of the most important pathways of energy metabolism the following enzyme activities were determined: hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G-6-P-DH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), and cytochrome-*c*-reductase (CCR). After a one week growth period, rat glial cultures were incubated for 3 or 4 weeks with the substances to be tested. Bencyclane (5×10^{-5} mol/l) increased the activities of HK, G-6-P-DH, and LDH, whereas PFK and CCR were reduced. Pyritinol (10^{-4} mol/l) led to a higher G-6-P-DH activity, simultaneously lowering the values for PFK, CCR, PK, LDH, and MDH. Under the influence of an extract of the leaves of Ginkgo bilobae (EGB; 100 mg/l) PFK, LDH, and MDH activities were reduced. All these alterations in enzyme activities went along with simultaneous reductions in protein content, therefore not allowing to exclude toxic effects with regard to the doses used. Moreover, direct interference with the analytical procedure was demonstrable for bencyclane and EGB. Piracetam (10^{-3} mol/l), flunarizine (10^{-6} mol/l), dihydroergocristine (5×10^{-6} mol/l), and nicergoline (5×10^{-6} mol/l) failed to induce any alteration in the employed doses. The most striking effects were obtained with meclofenoxate which was tested at 10^{-3} and 10^{-4} mol/l. The higher dose caused an elevation of HK, PFK, CCR, G-6-P-DH, GDH and MDH activities, while slightly reducing PK. With the lower dose of meclofenoxate CCR and G-6-P-DH activities were increased. Short-term incubation of the cultures with 10^{-3} mol/l meclofenoxate for 24 hr led to an increase in LDH, G-6-P-DH, and GDH activities. Chronic incubation with meclofenoxate (10^{-3} mol/l) followed by 48 hr deprivation of the drug resulted in elevated HK, PFK, CCR, G-6-P-DH, GDH, and MDH activities. These changes were accompanied by alterations in related metabolite levels. These include elevations in the concentration of creatine phosphate and fructose-1,6-bisphosphate, whereas glucose-6-phosphate levels were reduced. After one week of meclofenoxate deprivation the activities of CCR and G-6-P-DH were still elevated. The metabolites of meclofenoxate dimethylaminoethanol (DMAE; 10^{-3} mol/l) and *p*-chlorophenoxyacetic acid (10^{-3} mol/l) were also investigated. The latter led to similar changes in the enzyme activity pattern as meclofenoxate itself with the exception of unchanged values for HK, whereas DMAE induced increases in CCR and GDH activities. Male rats were chronically treated for four weeks with either meclofenoxate (200 mg/kg daily) or DMAE (60 mg/kg daily). With both groups CCR activities in brain cortex were increased compared to saline treated animals. It was concluded that rat primary glial cultures could be suitable for the *in vitro* assay of enzyme activity alterations induced by cerebro-protective agents, to some degree allowing to predict *in vivo* changes.

Cerebro-protective agents have been introduced into the treatment of different pathophysiological states, e.g. senile dementia, brain organic syndrome, ischemic attacks, or learning deficits, with contradictory clinical results. The claimed mechanisms of action for this class of drugs are varying widely. In clinical studies beneficial effects have only been seen after long-term treatment (for reviews see [1–3]). With regard to this time span alterations in the enzymatic pattern might occur, which could account for the improvements in cerebral function. Various changes in the enzymatic pattern have been demonstrated under the influence of different cerebro-protective agents [4–11]. In the present study, enzymes of the different pathways of energy metabolism were investigated, because the relation between energy supply and energy demand seems to play an important role in the expression of an ischemic or hypoxic insult. Since chemical properties, pharmacological profiles, and suggested uses differ widely within the class of cerebro-protective agents, no unique mechanism of action can be expected. Therefore, a number of representatives of this class of drugs had to be investigated. A model was chosen, which is easy to handle, with which a number of experiments can be conducted, and which is susceptible for enzyme induction, thus meeting our requirements of a screening model. For reasons of similarities in their physiological and biochemical properties, primary cultures seemed to be favourable over cell lines. Neuronal primary cultures from embryonic rat brain, however, have proved to maintain in culture only in the presence of other cell types. They are obtainable only with minor purity and higher variability with respect to their content of neurones. The other well established

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neuronal primary culture, neurones derived from embryonic chick brain, exhibits degeneration processes as early as 10 days after the onset of cultivation [12]. Since this time span is too short for their use in chronic incubation experiments, cultures exhibiting a longer survival time had to be favoured. Rat primary glial cultures seemed to fulfil these demands, since these cells can be derived with a highly reproducible purity and they survive for 4 to 6 weeks in culture. Enzyme inductivity has already been shown [13]. Similarities in the behaviour of astrozytes *in vivo* and *in vitro* have been demonstrated as well [14–16].

The aim of the present investigation was to elucidate whether cerebro-protective agents could lead to changes in the enzymatic pattern of rat primary glial cultures, whether these changes are attributable to long-term treatment, and whether they are related to possible energy metabolism shifts. It was also intended to evaluate, whether the alterations observed in the cultured cells were comparable to *in vivo* results. Therefore, experiments first were conducted with cultured cells, and then the obtained data had to be confirmed *in vivo*.

MATERIALS AND METHODS

Materials. Enzymes, coenzymes, and substrates were obtained from Boehringer (Mannheim, F.R.G.). P-chlorophenoxyacetic acid, cytochrome *c*, dimethylaminoethanol (DMAE), DL-dithiothreitol, HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid), imidazole, 2-mercaptoethanol, and α-thioglycerol were purchased from Sigma Chemical Co (St. Louis, MO). Bovine serum albumin was from Behringwerke (Marburg, F.R.G.). Cell culture media were delivered by Gibco Europe (Karlsruhe, F.R.G.). Bencyclane was obtained from Thiemann (Lünen, F.R.G.), dihydroergocristine (DHEC) from Spitzner (Ettlingen, F.R.G.), extract of Ginkgo biloba (EGB) from Schwabe (Karlsruhe, F.R.G.), flunarizine from Janssen (Neuss, F.R.G.), meclofenoxate from Promonta (Hamburg, F.R.G.), nicergoline from Farmitalia (Freiburg, F.R.G.), piracetam from UCB-Chemie (Kerpen, F.R.G.), and pyritinol from Merck (Darmstadt, F.R.G.). All other chemicals were of reagent grade.

Male Sprague–Dawley rats (Ivanovas, Kisslegg, F.R.G.), weighing 100–150 g, were used for the *in vivo* experiments, neonatal rats of both sexes, 12–48 hr old, for cultivation of glial cells. The animals were kept on standard diet (Altromin, Lage, F.R.G.) and tap water *ad libitum* under controlled environmental conditions (22°, 55% relative humidity, 12 hr cycle).

Cell cultivation. Rat glial cells were cultivated according to the method described by Booher and Sensenbrenner [17]. All work with cells and nutrient media was performed under sterile conditions under laminar flow (model C424H, Ceag Shirp Reinraumtechnik, Bork, F.R.G.). After disinfection the neonates were decapitated, and the brain was transferred into 20 ml of nutrient medium. The medium consisted of Dulbecco's modified minimum essential medium, fortified with 10% fetal bovine serum. Meningeal tissue was removed under a stereo-zoom microscope, and the brain tissue was forced gently through a nylon mesh (82 μpore: Tripette et Renaud, Paris) according to Sensenbrenner *et al.* [18]. In this primary suspension vitality was determined by nigrosin staining (0.5% in 0.9% NaCl solution). Only preparations with more than 50% vitality were used. The suspension was diluted with nutrient medium to a final volume of 80 ml. Fifteen ml of this dilution, containing $6–7 \times 10^5$ living cells per ml, were seeded on each plastic flask (75 cm^2 Lux tissue culture flask, Flow, Meckenheim, F.R.G.). The cells were cultivated under an atmosphere of 95% air and 5% CO_2 at 37° (B 5060 EK/2, Heraeus, Hanau, F.R.G.). One week after the onset of the cultivation and then twice weekly nutrient medium was exchanged. The last exchange was performed 40 hr before analysis. Chronic incubation with the drugs lasted 3 or 4 weeks and started 1 week after the onset of cultivation. Drugs were added to the nutrient medium before medium exchange. For short-term experiments 4-week-old untreated cultures were incubated for 24 hr with meclofenoxate. In this series of experiments the drug was applied directly to the existing nutrient medium in order to prevent artifacts from medium exchange. Possible interference with the enzyme determinations were tested by incubating an extract of 4–5 weeks old untreated cultures with the respective compounds for 30 min at room temperature, drugs being dissolved in 1 mmol/l EDTA.

Enzyme extraction. The extraction was performed according to the method of Roth-Schechter *et al.* [13]. The cover of the culture flask was cracked and removed, the culture medium was decanted, and the cells were washed three times with ice-cold modified Dulbecco's salt solution (Gibco Europe, Karlsruhe, F.R.G.). The cells were covered with 4 ml ice-cold EDTA solution (1 mmol/l; pH 7.0), scraped off, and homogenized in a Potter-Elvehjem homogenizer for 30 sec at 200 cycles/min (Braun, Melsungen, F.R.G.). Homogenizing was completed by ultrasonic treatment in 3 periods of 20 sec each, the intervals lasting 20 sec (Branson sonifier B12, Branson, Heusenstamm, F.R.G.). One ml of the raw homogenate was removed for protein determination. The rest was centrifuged for 15 min at 33,000 *g*/0° (B10A, Damon/IEC, Biotronic, Frankfurt, F.R.G.). Enzyme activities were determined in the supernatant photometrically at 25° (model 25, Beckman Instruments, Munich, F.R.G.).

In vivo *experiments*. Rats were treated 4 weeks by daily intraperitoneal injection of meclofenoxate, DMAE, or saline solution alone in a volume of 2 ml/kg between 9.00 and 10.00 a.m. The animals were killed the day following the last treatment between 8.00 and 9.00 a.m. Cerebral cortex was removed and placed in a precooled potter, weighed, diluted 1:20 with EDTA solution (1 mmol/l; pH 7.0), and homogenized in a Potter-Elvehjem homogenizer by 20 strokes within 1 min. Ultrasonic treatment was performed as described for the cultured cells. The raw homogenate was centrifuged for 15 min at 40,000 *g* (model J2-21, Beckman Instruments, Munich, F.R.G.), and the supernatant was diluted 1:10 with EDTA solution for final enzyme assay.

Enzyme assay. Cytochrome-c-reductase (CCR; EC 1.6.99.3) was determined at 550 nm according to

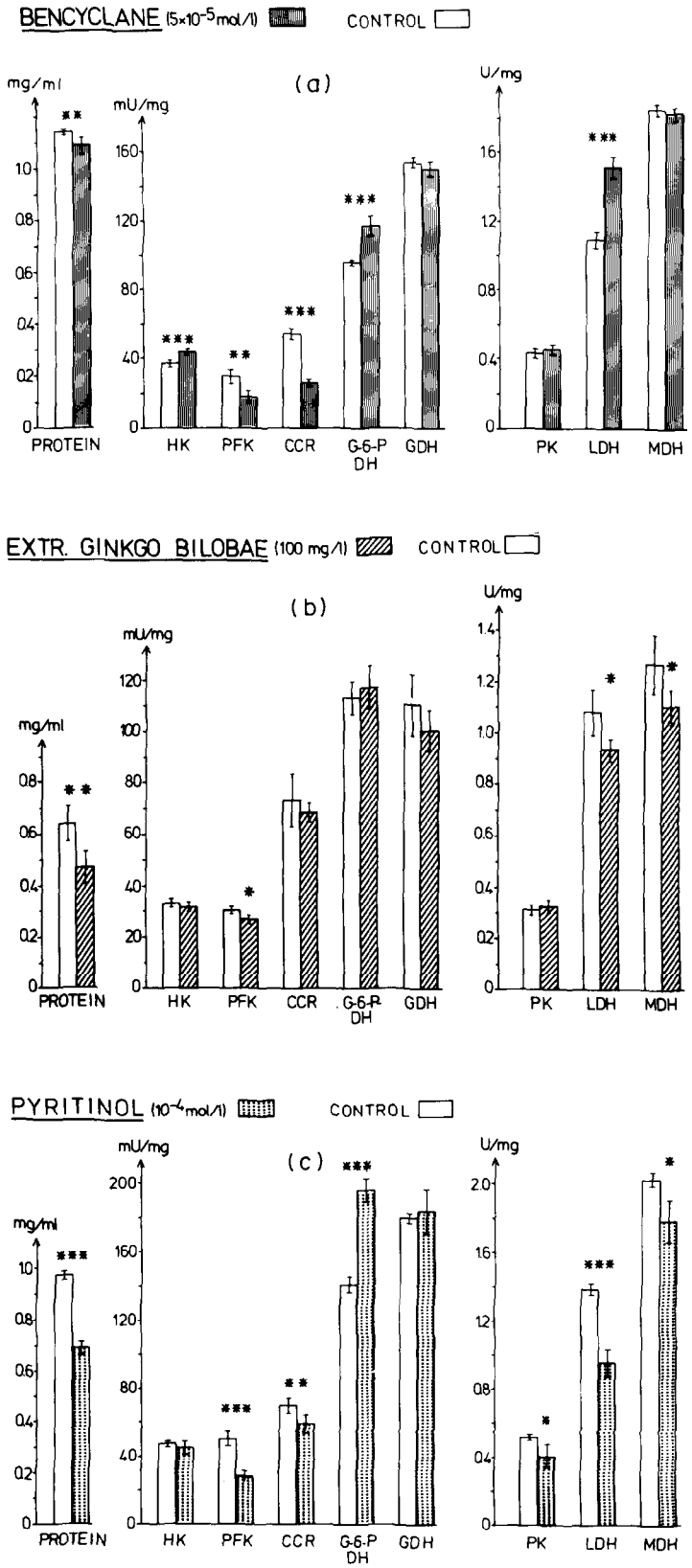

Fig. 1(a–c). The influence of a 3 week (bencyclane and EGB) or 4 week (pyritinol) incubation of primary rat astrocytes with cerebro-protective agents on selected enzyme activities of brain energy metabolism and on protein content. The values are given as means ± S.D. of 5 experiments. For statistical comparison, Student's *t*-test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 1. Incubation of an extract from glial cells with 0.05 mmol/l bencyclane

	Control	Bencyclane
Protein (mg/ml extract)	0.77	0.78
PFK (mU/mg protein)	55.04 ± 2.15	46.88 ± 1.29**
CCR (mU/mg protein)	48.74 ± 1.09	43.40 ± 1.53**

Extraction was performed on the 30th day of growth. The extract was incubated with bencyclane for 30 min. Values are given as means ± S.D. of 8 experiments. For statistical comparison, Student's *t* test was used.
** $P < 0.001$.

Nason and Vasington [19] using a molar extinction coefficient of 18.5 $cm^2/\mu mol$. All other activities were measured in NAD(P)/NAD(P)H-dependent reactions at 340 nm using a molar extinction coefficient of 6.22 $cm^2/\mu mol$. Hexokinase (HK; EC 2.7.1.1) was assayed according to the method of Hernandez and Crane [20], phosphofructokinase (PFK; EC 2.7.1.11) using the method of Wu and Racker [21]. Glucose-6-phosphate dehydrogenase (G-6-P-DH; EC 1.1.1.49) was determined according to the procedure described by Kornberg and Horecker [22]. Measurements of pyruvate kinase (PK; EC 2.7.1.40), lactate dehydrogenase (LDH; EC 1.1.1.27), malate dehydrogenase (MDH; EC 1.1.1.37), and glutamate dehydrogenase (GDH; EC 1.4.1.3) were performed according to Bergmeyer [23]. Protein content was determined according to Lowry *et al.* [24]. One unit of enzymatic activity was defined as the amount of enzyme which transformed 1 μmol substrate/min at 25°.

Determination of substrates. The following substrates were measured enzymatically according to procedures given by Bergmeyer [23]: creatine phosphate, ATP, ADP, AMP, glucose, gluconate-6-phosphate, pyruvate, and lactate. Glucose-6-phosphate and fructose-1,6-biphosphate were analyzed by fluorometric methods reported by Lowry and Passonneau [25].

Statistics. The results were evaluated statistically using Student's *t*-test [26].

RESULTS

Rat primary glial cells were incubated with bencyclane (5×10^{-5} mol/l) for three weeks. The cells exhibited a different morphology compared to control cultures, cell aggregations being more prominent and alternating with cell free areas. Additionally, the characteristic processes of the astroglia were more distinct. The drug markedly increased the activities of HK, G-6-P-DH, and LDH (Fig. 1a). The values for PFK and CCR as well as protein content were lowered significantly. Direct incubation of a cell extract for 30 min with bencyclane in the same concentration also reduced PFK and CCR activities (Table 1). The other calcium entry blocker flunarizine was not capable of inducing any change in enzyme activities or protein content at a concentration of 10^{-6} mol/l (data not presented). A higher concentration (5×10^{-5} mol/l) led to degeneration of the cultures within one week. Under the influence of 5×10^{-6} mol/l dihydroergocristine enzyme activities and protein content of the glial cultures were not altered. The same holds true for nicergoline (5×10^{-6} mol/l), the other ergot alkaloid derivative tested in this study, and for 10^{-3} mol/l piracetam (data not presented). Under the influence of a 3 week incubation with EGB (100 mg/l) PFK, LDH, and MDH activities were slightly reduced (Fig. 1b). Similar to the results derived with bencyclane these alterations went along with simultaneous reductions in protein content. Incubation of an extract of 4-week-old glial cells for 30 min with 100 mg/l EGB also lowered the values for LDH and MDH, PFK activity not being affected (Table 2). Pyritinol (10^{-4} mol/l) led to a higher G-6-P-DH activity, simultaneously lowering the values for PFK,

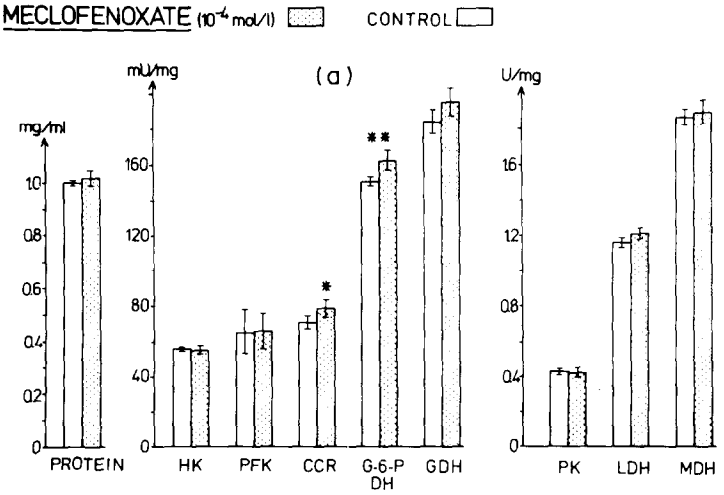

Fig. 2(a)

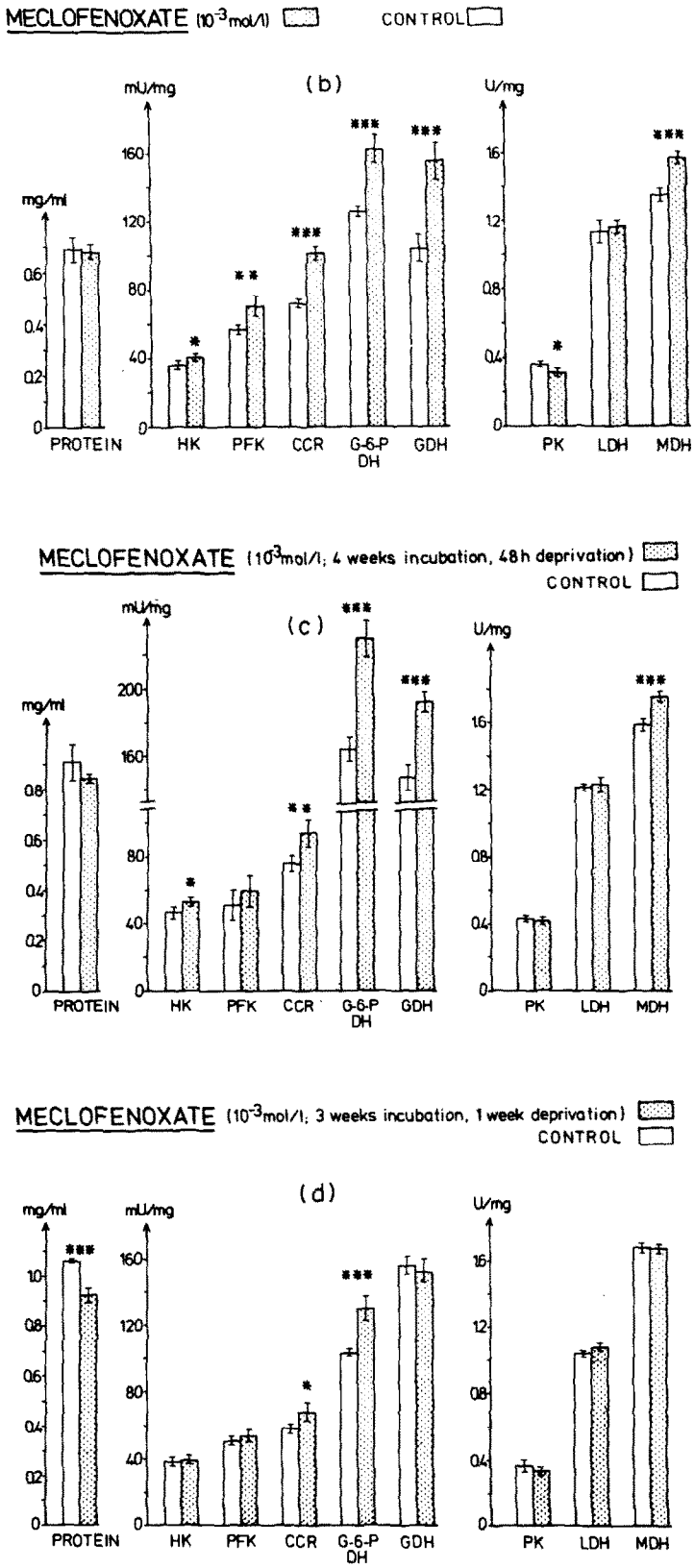

Fig. 2(a–d). The influence of an incubation of primary rat glial cultures with meclofenoxate as well as subsequent withdrawal of the drug for 48 hr or 1 week on enzyme activities related to energy metabolism. Values are given as means ± S.D. of 5 experiments. For statistical comparison, Student's *t*-test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2. Incubation of an extract from glial cells with 100 mg/l EGB

	Control	EGB
Protein (mg/ml extract)	0.59	0.53
PFK (mU/mg protein)	31.67 ± 3.00	28.24 ± 2.41
LDH (U/mg protein)	1.32 ± 0.08	1.11 ± 0.06*
MDH (U/mg protein)	1.22 ± 0.07	1.02 ± 0.04†

Extract was performed on the 30th day of growth. The extract was incubated with EGB for 30 min. Values are given as means ± S.D. of 5 experiments. For statistical comparison, Student's t test was used.
* $P < 0.01$, † $P < 0.001$.

CCR, LDH, PK, and MDH. These alterations were accompanied by a distinct decrease in protein content (Fig. 1c). The incubation time was 4 weeks in this series. Meclofenoxate was applied to the cultures for 4 weeks at 10^{-3} and 10^{-4} mol/l. The higher concentration caused an elevation of HK, PFK, CCR, G-6-P-DH, GDH, and MDH activities, while slightly reducing PK. With the lower concentration CCR and G-6-P-DH levels were increased, both concentrations not influencing protein content (Figs. 2a and b).

To evaluate direct interference of the compound with enzyme assaying, incubation of a cell extract with 1 mmol/l meclofenoxate was performed for 30 min. No differences compared to drug-free extracts could be observed when analysed for PK, G-6-P-DH, and CCR (Table 3).

In order to compare chronic incubation with effects of short-term exposition to meclofenoxate cells were treated for 24 hr with the drug before enzyme assessment. Incubation of glial cultures with 10^{-3} mol/l meclofenoxate for 24 hr led to an increase in LDH, G-6-P-DH, and GDH levels (Table 4).

In another series of experiments the influence of depriving the drug after chronic treatment was tested. The cultured cells were incubated for 4 weeks with meclofenoxate at a concentration of 10^{-3} mol/l followed by a deprivation period of 48 hr. Compared with untreated cultures, the activities of HK, CCR, G-6-P-DH, GDH, and MDH were elevated (Fig. 2c).

Under the same experimental conditions metabolite levels were altered, too (Table 5). Creatine phosphate and fructose-1,6-biphosphate levels were increased, whereas the concentration of glucose-6-phosphate was reduced compared with untreated cultures.

Withdrawing the drug for 1 week elicited still higher values for CCR and G-6-P-DH (Fig. 2d). The incubation period was 3 weeks in this series.

In biological systems meclofenoxate easily splits off to the components DMAE and *p*-chlorophenoxyacetic acid. For this reason these metabolites were tested on the same model to discriminate the effects of either substance. *P*-chlorophenoxyacetic acid (10^{-3} mol/l) after a 4-week incubation period was capable of inducing similar effects as meclofenoxate itself (Fig. 3a). Compared to untreated cells, higher activities for HK, PFK, CCR, G-6-P-DH, GDH, and MDH were registered. In contrast to meclofenoxate, PK activity was unaltered and protein content was somewhat reduced in this series. Elevated values for CCR and GDH were assayed following a 4 week incubation period with DMAE

Table 3. Incubation of an extract from glial cells with 1 mmol/l meclofenoxate

	Control	Meclofenoxate
Protein (mg/ml extract)	0.88	0.88
PK (mU/mg protein)	391.57 ± 4.38	394.89 ± 5.25
G-6-P-DH (mU/mg protein)	108.48 ± 4.25	112.64 ± 4.21
CCR (mU/mg protein)	51.82 ± 4.08	54.54 ± 3.46

Extraction was performed on the 30th day of growth. The extract was incubated with meclofenoxate for 30 min. Values are given as means ± S.D. of 8 experiments.

Table 4. 24 hr incubation of glial cells with 1 mmol/l meclofenoxate

	Control	Meclofenoxate
Protein (mg/ml extract)	1.10 ± 0.05	1.10 ± 0.03
HK (mU/mg protein)	46.76 ± 1.70	48.07 ± 0.69
PFK (mU/mg protein)	66.60 ± 5.96	70.71 ± 4.49
PK (mU/mg protein)	461.83 ± 26.96	505.23 ± 29.31
LDH (U/mg protein)	1.19 ± 0.03	1.27 ± 0.04*
G-6-P-DH (mU/mg protein)	148.00 ± 5.96	164.16 ± 3.05†
MDH (U/mg protein)	1.75 ± 0.16	1.87 ± 0.01
GDH (mU/mg protein)	183.16 ± 8.98	197.68 ± 5.60‡
CCR (mU/mg protein)	60.09 ± 11.76	65.59 ± 4.22

Primary rat glial cultures were incubated for 24 hr with meclofenoxate on the 35th day of growth. Values are given as means ± S.D. of 5 experiments. For statistical comparison, Student's t test was used.
* $P < 0.01$, † $P < 0.001$, ‡ $P < 0.05$.

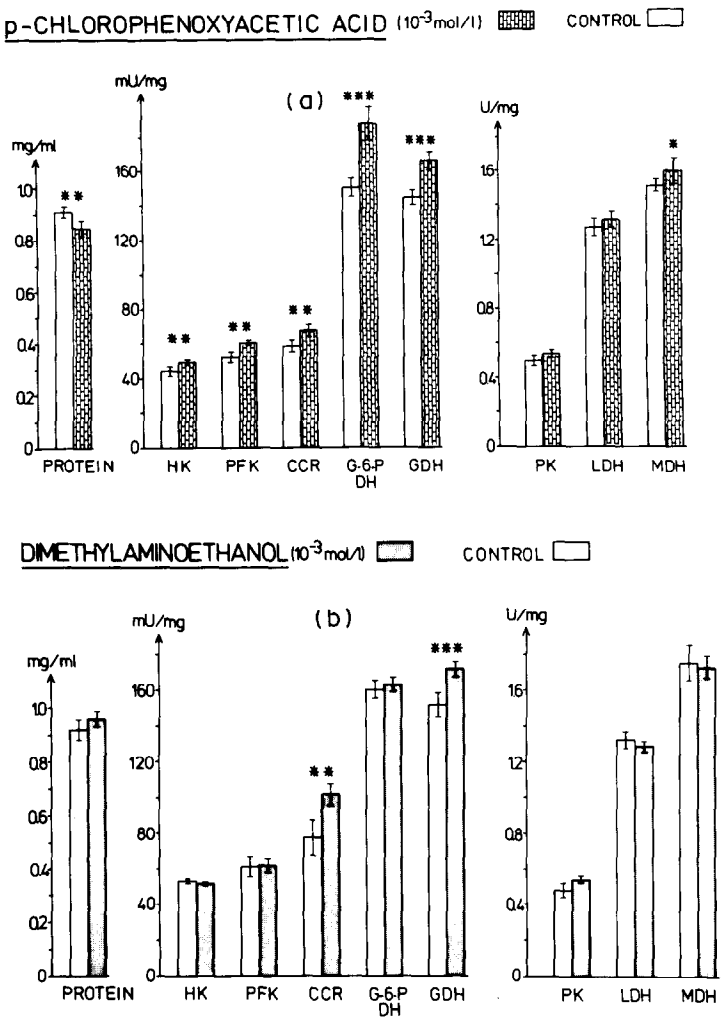

Fig. 3(a–b). The influence of a 4 week incubation period with dimethylaminoethanol and *p*-chlorophenoxyacetic acid on enzyme activities related to energy metabolism of primary rat glial cultures. Values are given as means ± S.D. of 5 experiments. For statistical comparison, Student's *t*-test was used: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5. The influence of meclofenoxate on substrates and metabolites of energy metabolism in glial cultures

	Control	Meclofenoxate
Creatine phosphate	36.06 ± 1.23	50.53 ± 2.52*
ATP	33.38 ± 1.32	34.24 ± 1.31
ADP	2.95 ± 0.42	3.15 ± 0.56
AMP	4.67 ± 1.20	4.92 ± 0.74
Glucose	8.21 ± 1.46	7.44 ± 3.16
Glucose-6-phosphate	1.01 ± 0.07	0.78 ± 0.10†
Fructose-1,6-bisphosphate	1.16 ± 0.08	1.67 ± 0.08*
Lactate	41.76 ± 5.63	38.08 ± 7.83
Pyruvate	1.00 ± 0.12	0.90 ± 0.13
Protein	3.49 ± 0.13	2.87 ± 0.13*

Primary rat glial cultures were incubated for 4 weeks with 1 mmol/l meclofenoxate, and subsequently the drug was withdrawn for 48 hr. The values are given in nmol/mg protein as means ± S.D. of 5 experiments. For statistical comparison, Student's *t*-test was used.
* $P < 0.001$, † $P < 0.01$.

(10^{-3} mol/l), this substance not inducing changes in protein content (Fig. 3b).

In order to compare the results obtained with the cultured cells to *in vivo* effects, rats were treated for 4 weeks with either meclofenoxate (200 mg/kg daily) or DMAE (60 mg/kg daily), no apparent alterations in behaviour being observable with either group. Chronic treatment with both meclofenoxate or DMAE led to significant increases in CCR activity (Fig. 4a and 4b).

DISCUSSION

In the past various cell culture techniques have been employed in order to either replace or supplement *in vivo* investigations. The aim of the present study was to show whether rat primary glial culture cells are capable of reacting to pharmacologically active compounds with reproducible alterations in their enzymatic pattern, and whether these changes resemble the results of *in vivo* testing. Cultures were incubated with different cerebro-pro-

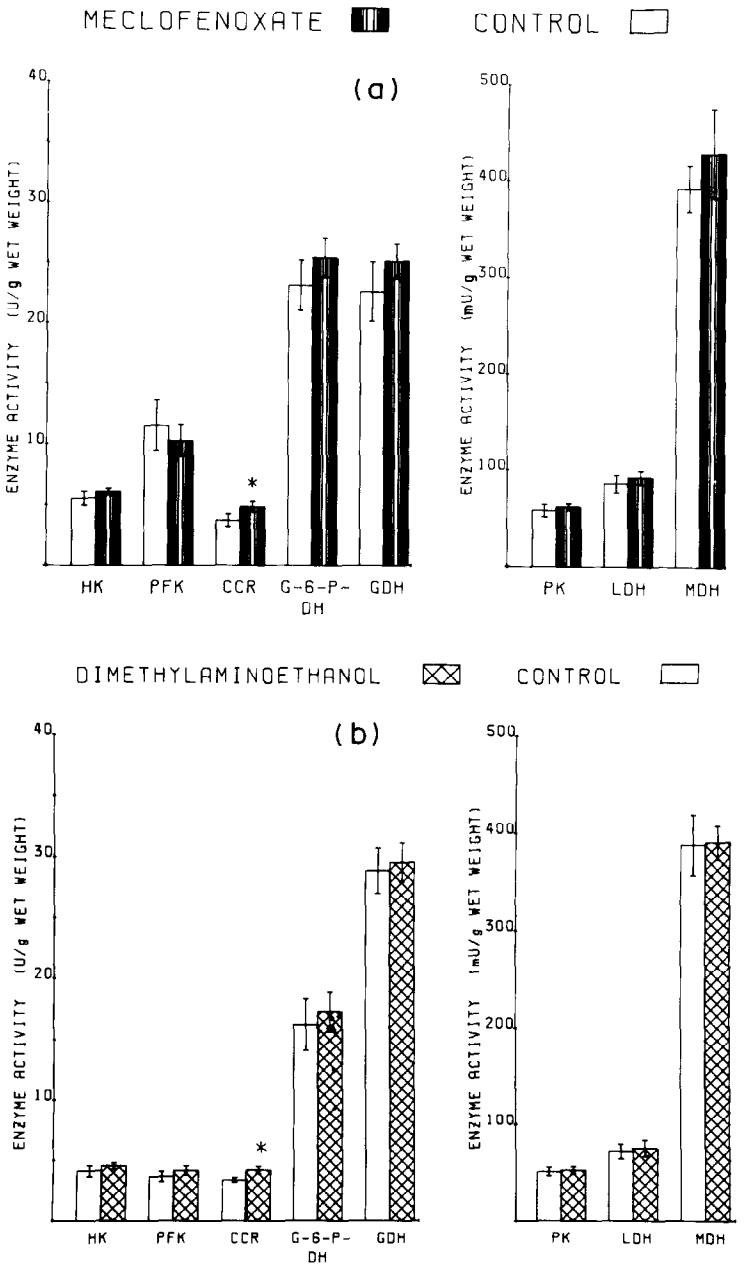

Fig. 4(a–b). The influence of a 4 week chronic treatment of male rats with meclofenoxate (200 mg/kg daily intraperitoneal dose) or dimethylaminoethanol (60 mg/kg daily intraperitoneal dose) on cortical enzyme activities related to energy metabolism. Values are given as means ± S.D. of 6 experiments. For statistical comparison, Student's *t*-test was used: * $P < 0.05$.

tective agents for 3 to 4 weeks, and thereafter the activities of different key enzymes involved in the regulation of brain energy metabolism were studied. No alterations were observed with the calcium entry blocker flunarizine this result being in accordance with the proposed mechanism of action for this class of drugs and with the results hitherto obtained. Except for Ca-ATPase and Na-K-ATPase, no direct influence on enzymes involved in cerebral energy metabolism could be shown so far [27].

The other calcium entry blocker tested here, bencyclane, brought about various changes in the enzymatic pattern, these being accompanied by a reduction in protein content as well as by morphological alterations. Because of their magnitude the reduced PFK and CCR values were verified for direct interference with the determination procedure. The analogous diminution under these conditions make the results obtained with bencyclane at least in part unlikely to be correlated to the chronic incubation. Moreover, since cell adhesion is Ca-dependent, interference of bencyclane with this ion might account for the observed morphological differences [28, 29]. On the other hand, elevated levels

of G-6-P-DH were also assayed following chronic treatment with pyritinol and *p*-chlorophenoxyacetic acid, both compounds leading to reductions in protein content. Elevated G-6-P-DH activity could reflect a shift from glycolysis towards the pentose phosphate cycle, indicating increased demands for either NADPH or ribose-5-phosphate. As the model used consists of actively growing cells thereby being dependent on sufficient pentose phosphate supply for cell development and division, relatively high G-6-P-DH levels might reflect the basic requirements for the pentose phosphate cycle under circumstances of a reduced growth rate [30].

The investigated ergot alkaloid derivatives DHEC and nicergoline produced no significant changes in the enzymatic pattern. These observations are in contrast to *in vivo* experiments, which reveal various alterations in the enzymatic equipment after chronic treatment with ergot alkaloids, including changes in enzymes tested in this study [4, 7, 10, 11]. The fact that the modifications reported by these authors were quite variable with the employed doses and to some degree age dependent might account for the differences.

The lack of changes in brain energy metabolites under various experimental conditions following piracetam administration is indicative of an unaltered energy metabolism [31–33]. These observations are consistent with failure of piracetam to induce changes in the enzymatic pattern of rat glial cultures.

Only reductions in enzyme activities were effected by EGB, these being accompanied by a somewhat lowered protein content. The results after incubation of a cell extract with the drug hint at least partly at a direct influence on enzyme activities.

Several enzymes show reduced activities following chronic pyritinol application, only G-6-P-DH being elevated. For reasons described above, the observed effects may be due to unspecific toxicity of the applied dose.

The most impressive enzyme inductions were obtained with meclofenoxate, this effect being dose dependent with regard to the intensity of induction and the number of enzymes involved. The only enzyme not affected by the higher dosage of meclofenoxate was LDH. Concerning this enzyme however one has to keep in mind that modifications of LDH in primary cultures of rat glial cells can also affect its isoenzymatic profile [16]. The alterations in enzyme activities were accompanied by related changes in metabolite levels. The relevance of the increase in PFK activity for instance, is well documented by the simultaneous decrease of glucose-6-phosphate and the increase of fructose-1,6-bisphosphate levels. The elevated G-6-P-DH activity might as well contribute to the reduction of the glucose-6-phosphate concentration. The fact that hexokinase activity is albeit marginally increased and that on the other hand glucose-6-phosphate levels are decreased lends further support to the hypothesis of an increased glycolytic flow at the site of PFK and a shift towards the pentose phosphate cycle. The elevated creatine phosphate levels indicate an increase in high energy phosphate stores. An increased capacity of the respiratory chain might well be responsible for this elevation.

Most of the observed alterations in enzyme activities can be attributed to *p*-chlorophenoxyacetic acid since this metabolite of meclofenoxate led to almost the same effects as meclofenoxate itself. *In vivo* however this metabolite is lost quite rapidly by urinary excretion [34, 35]. Therefore, attention had to be drawn towards DMAE with regard to possible *in vivo* effects. Putting together the facts that DMAE induces CCR and GDH activities in the glial cells after chronic treatment and that on the other hand GDH activity is also elevated after a 24 hr incubation period with meclofenoxate, only the CCR elevation is undoubtedly attributable to the chronic incubation with DMAE. The relevance of CCR induction is confirmed by the fact that withdrawal of meclofenoxate for 1 week after chronic treatment resulted in higher activities of CCR and G-6-P-DH. Therefore, with regard to the pharmacokinetics of meclofenoxate the *in vivo* elevation of CCR activity following chronic treatment with this drug as well as with DMAE is in good agreement with the *in vitro* results.

The increased CCR activity in rat cerebral cortex is in accordance with increased oxygen and glucose consumption and CO_2-delivery following meclofenoxate application [36–38]. An increased glucose consumption accompanied by a reduced CO_2 production has been observed in human glial cells in a stationary cell culture system and has been discussed as a shift from glycolysis towards the pentose phosphate cycle [39]. Our results confirm this hypothesis, but as discussed above, the elevation of G-6-P-DH may be due to nonspecific toxicity and was not observable in chronically treated rats. On the other hand, meclofenoxate has been shown to increase RNA and protein synthesis in human glial cultures [39]. Higher rates of mRNA synthesis have been shown in old rats as well [40]. Though not observed in that study, mRNA and protein synthesis might also be affected in young animals, leading to more selective variations in the enzymatic pattern. Moreover, CCR is reported to be strongly reduced with increasing age [7]. As these authors state a progressive narrowing of enzyme inductivity with increasing age including effects on CCR and as they relate this effect to a slowing of protein turnover in old animals, it would be of considerable interest to investigate the age dependency of CCR induction using a drug like meclofenoxate which is capable of enhancing protein turnover preferentially in old age. On the relevance of this enzyme induction with respect to the cerebro-protective action of meclofenoxate, however, one cannot judge from the data presented in this study.

In conclusion, cerebro-protective agents could induce enzymes related to energy metabolism in rat primary glial cells. With respect to meclofenoxate the data obtained with cultured cells were in good agreement with *in vivo* results. Thus, in this first attempt rat primary glial cultures seem to be suitable for evaluating *in vitro* effects of cerebro-protective agents on enzyme activities, to some degree allowing to predict in vivo changes.

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REFERENCES

1. D. E. Hyams in *Textbook of Geriatric Medicine and Gerontology* (Ed. J. C. Brocklehurst), p. 671. Churchill, Livingstone, Edinburgh (1978).
2. A. Spagnoli and G. Tognoni, *Drugs* **26**, 44 (1983).
3. J. A. Yesavage, J. R. Trinklenberg, L. E. Hollister and P. A. Berger, *Archs gen. Psychiat.* **36**, 220 (1979).
4. E. Arrigoni, G. Benzi, F. Dagani, G. Falconi, V. Mandelli, R. Moretti, R. Scelsi and F. Villa, *Arzneim. Forsch.* **29**, 1231 (1979).
5. D. Atanackovic, A. Simonic and B. Jamnicky, *Archs int. Pharmacodyn.* **250**, 266 (1981).
6. G. Benzi, E. Arrigoni, E. Dagani, F. Marzatico, D. Curti, A. Manzini and R. F. Villa, *Biochem. Pharmac.* **28**, 2703 (1980).
7. G. Benzi, E. Arrigoni, E. Dagani, F. Marzatico, D. Curti, S. Raimondo, M. Dossena, M. Polygatti and R. F. Villa, *Exp. Geront.* **15**, 593 (1980).
8. G. Benzi, E. Arrigoni, O. Pastoris, F. Marzatico, D. Curti, G. Piacenza and R. F. Villa, *Il Farmaco* **36**, 811 (1981).
9. F. Dagani, A. Gorini, M. Polgatti, R. F. Villa and G. Benzi, *J. Neurosci. Res.* **10**, 135 (1983).
10. D. M. Djuricic and B. B. Mrsulja, *Gerontology* **26**, 99 (1980).
11. A. Gorini and R. F. Villa, *Il Farmaco* **38**, 191 (1983).
12. B. Pettman, J. C. Louis and M. Sensenbrenner, *Nature, Lond.* **281**, 378 (1979).
13. B. F. Roth-Schechter, M. Laluet, G. Tholey and P. Mandel, *Biochem. Pharmac.* **26**, 1307 (1977).
14. L. Hertz, B. H. J. Juurlink, H. Fosmark and A. Schousboe, in *Neuroscience Approached Through Cell Culture*, Vol. 1 (Ed. S. E. Pfeiffer), p. 175, CRC-Press, Boca Raton (1982).
15. H. K. Kimelberg, S. Narumi and R. S. Bourke, *Brain Res.* **153**, 55 (1978).
16. G. Tholey, B. F. Roth-Schechter and P. Mandel, *Neurochem. Res.* **5**, 847 (1980).
17. J. Booher and M. Sensenbrenner, *Neurobiology* **2**, 97 (1972).
18. M. Sensenbrenner, J. Booher and P. Mandel, *Z. Zellforsch.* **117**, 559 (1971).
19. A. Nason and F. D. Vasington in *Methods in Enzymology*, Vol. 6 (Eds. S. P. Colowick, N. O. Kaplan), p. 409. Academic Press, New York (1963).
20. A. Hernandez and R. K. Crane, *Archs Biochem. Biophys.* **113**, 223 (1966).
21. R. Wu and E. Racker, *J. biol. Chem.* **234**, 1029 (1959).
22. A. Kornberg and B. L. Horecker, in *Methods in Enzymology*, Vol. 1 (Eds. S. P. Colowick, N. O. Kaplan), p. 323, Academic Press, New York (1955).
23. H. U. Bergmeyer (Ed.), *Methoden der enzymatischen Analyse*. Verlag Chemie, Weinheim (1970).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. O. H. Lowry and J. V. Passonneau, *A Flexible System of Enzymatic Analysis*. Academic Press, New York (1972).
26. L. Sachs, *Angewandte Statistik*. Springer Verlag, Berlin (1974).
27. ThT. Zsoter and J. G. Church, *Drugs* **25**, 93 (1983).
28. D. Barnes and G. Sato, *Analyt. Biochem.* **102**, 255 (1980).
29. R. G. Rahwan, *Medical Research Reviews* **3**, 21 (1983).
30. M. J. Morgan and P. Faik, *Bioscience Rep.* **1**, 669 (1981).
31. B. Dirks, A. Seibert, G. Sperling and J. Krieglstein, *Arzneim. Forsch.* **34**, 258 (1984).
32. L. Kellman, Thesis, Philipps-Universität Marburg (1983).
33. V. J. Nickolson and O. L. Wolthuis, *Biochem. Pharmac.* **25**, 2241 (1976).
34. C. Guili, C. Bertoni-Freddari and C. Pieri, *Mech. Ageing Dev.* **14**, 265 (1980).
35. H. Miyazaki, K. Nambu, Y. Minaki, M. Haschimoto and K. Nakamura, *Chem. Pharm. Bull.* **24**, 763 (1976).
36. H. D. Herrmann and J. Dittmann, *Arzneim. Forsch.* **21**, 984 (1971).
37. J. Nickel, K. Breyer, B. Claver and G. Quadbeck, *Arzneim. Forsch.* **13**, 881 (1963).
38. S. Oeriu, D. Winter, V. Dobre and S. Bruchis, *J. Pharmac.* **4**, 497 (1973).
39. M. Ludwig-Festl, B. Gräter and K. Bayreuther, *Arzneim. Forsch.* **33**, 495 (1983).
40. I. Zs.-Nagy and I. Semsei, *Exp. Geront.* **19**, 171 (1984).