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THE EFFECT OF PENTOBARBITAL ON THE CARBOHYDRATE METABOLISM OF GLIAL CELLS IN CULTURE

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Abstract—Hamster astroblast glial cells (clone NN) and dissociated glial cells from newborn rat brains in primary culture were exposed to from 5×10^{-5} to 1×10^{-3} M pentobarbital-Na and 1×10^{-4} M or 2.5×10^{-4} M morphine hydrochloride. After various periods of time the following enzymatic activities were measured in the cell culture: fructose-diphosphate-aldolase (ALD), pyruvate kinase (PK), lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and NADH cytochrome *c* reductase (CCR). The long-term exposure to pentobarbital resulted in increases in LDH, MDH and GDH activities and a consistent decrease in PK activity. Continuous exposure to morphine resulted in minor, uniformly depressive changes. When neuroblastoma cells (clone M1) or rat fibroblasts in primary culture were exposed to pentobarbital no specific changes could be detected. The results are interpreted to suggest that barbiturates affect the carbohydrate metabolism of glial cells in a drug- and cell-dependent fashion.

An *in vitro* system of homogeneous brain cells which reflects some known *in vivo* actions of barbiturates in a reproducible manner would prove extremely useful in the study of the biochemical mechanism of action of these drugs.

Previous work from this laboratory has shown that certain glial cells in culture provide such a cellular system reflecting some aspects of barbiturate-induced dependence [1]. Further, it was shown that during continuous exposure to pentobarbital, tolerance to this drug can be induced [2]. This tolerance was accompanied by a drastic increase in oxygen and glucose consumption of the cells, despite the fact that direct addition of the barbiturate to normal respiring cells was found to result in the well-known depression of oxygen uptake [3].

Based on these observations a systematic study was undertaken to investigate the effects of long-term exposure of glial cells to pentobarbital on carbohydrate metabolism. The following enzymes were studied: fructose-diphosphate-aldolase (ALD), pyruvate kinase (PK), lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), and NADH cytochrome *c* reductase (CCR). These enzymes can be considered to be indicative of the action of the Embden-Meyerhof pathway, pyruvate synthesis, anaerobic metabolism, the tricarboxylic acid cycle and the cytochrome transport chain, respectively. In order to gain some insight into the specificity of barbiturate action the effect of pento bital was studied as a function of the cell type involved and was compared to that of morphine.

METHODS

Cell culture conditions. Several cell types were cultured either in continuous line or in primary culture. For the glial cells in continuous line hamster astroblast glial cells (clone NN) were used. These cells were initially isolated and subsequently characterized by Shein and collaborators [4, 5]. They were obtained from North-American Biologicals, Inc. The neuroblastoma cell line was isolated and characterized in this laboratory [6]. The glial cells in primary culture were prepared from newborn rats according to the method of Booher and Sensenbrenner [7]. Primary cultures of rat fibroblasts were prepared by the same technique using rat embryos at 13 days of gestation. Embryos were removed from the uterus under sterile conditions and only the body (without head or extremities) was used.

For the continuous cell lines (M1 and NN) the cells were grown under conditions identical to those described [2]. The primary cultures were grown in plastic Falcon flasks (75 cm^2) at 37° in 5% CO_2–95% air atmosphere with saturated humidity. The cultures were maintained in Pasteur modified Eagle medium supplemented with 20% fetal calf serum. The medium was changed every fourth day.

Pentobarbital-Na (Abbott) was added to the culture media in final concentrations ranging from 5×10^{-5} to 1×10^{-3} M for various periods of time up to a total of 4 weeks. Morphine–HCl (Merck) was added to result in the final concentrations of 1×10^{-4} M or 2.5×10^{-4} M. The drugs were initially added at the time of subculturing for the continuous lines and one week after the start of the culture in the case of the primary cultures. The pH of the culture medium was kept constant at 6.7–7.3 for the continuous lines and between 6.9 and 7.3 for the primary cultures. Untreated cells of the same subculture and the same cell density served as controls. The cultures were periodically observed and photographed under an inverse phase contrast microscope plus camera assembly (Leitz, Wetzlar, Illford PANF black/white film).

Enzymatic activities. With the exception of the results contained in Fig. 1 (comparison of the effect

during logarithmic and stationary phase of growth) all enzyme determinations were done during the stationary phase of growth.

Cells were rinsed 2 times with isotonic saline and then harvested into 10 ml of saline with the aid of a rubber spatula. They were centrifuged at 500 rpm for 5 min, washed again and then homogenized in a Potter–Elvehjem glass homogenizer for 2 min in 1 mM EDTA pH 7.0. This was followed by three times 20 sec sonications separated by 20 sec cooling periods in an ultrasonic disintegrator (power 150 W). The sonicate was then centrifuged at 27,000 *g* for 15 min. The supernatant was used as crude extract. All assays were prepared at 25° and followed spectrophotometrically.

ALD was measured in Tris (50 mM, pH 7.6), 10 mM EDTA, 1.8 mM fructose diphosphate, 0.15 mM NADH with 10 μg/ml of a glycerophosphate dehydrogenase/triose phosphate isomerase mixture according to Kowal *et al.* [8]. LDH activity was measured by using the method of Bergmeyer *et al.* [9]. GDH was determined by the method of Schmidt [10], MDH according to the method of Bergmeyer and Bernt [11], PK by the method of Bergmeyer [12]. CCR was assayed spectrophotometrically at pH 7.4 by following the reduction of cytochrome *c* at 550 nm in 1 ml 0.025 M phosphate buffer, pH 7.4, containing 0.5 mg cytochrome *c* (horse heart, Boehringer), 1.3×10^{-4} M KCN, 50 mM NADH. One unit of enzymatic activity was defined as the amount of enzyme which transforms 1 μM substrate per min at 25°. Specific activity is expressed as unit per mg of protein, which was determined by the method of Lowry *et al.* [13].

For the cross-incubation experiments crude extracts were prepared as described above. Thereafter, respective extracts were mixed in a 50:50 (v/v) ratio and incubated at room temperature for 30 min. This was followed by the determination of the enzymatic activities as described above.

NADH was procured from Sigma, all substrates from Boehringer, all other chemicals used were of highest laboratory grade (Merck).

The data were analysed by the Student *t*-test (2-tailed).

RESULTS

The effect of pentobarbital on the carbohydrate metabolism of NN glial cells. Based on the dose–response curve for morphological and biochemical results [2] a dose of 5×10^{-4} M Pentobarbital was chosen and tested for its effect during the logarithmic (3 days) and stationary (7 days) phase of growth of the NN glial cells (Table 1). The presence of pentobarbital during 3 days of logarithmic growth results in a significant reduction of the activities of LDH and significant increases in the activities of GDH and CCR. At confluence and after 7 days of exposure to the drug, the activity of LDH of barbiturate treated cells was found to be higher than that in untreated cells and the initial increase in CCR activity to be diminished to non-significant levels. The effect on the GDH activity remained unchanged. In addition, after 7 days of growth in the presence of pentobarbital, the activity of PK was significantly reduced when compared to untreated cells of the same passage and same cell density.

The enzymatic activities of MDH, PK and GDH were studied in NN glial cells exposed to various doses of pentobarbital for a period of 2 weeks. The results obtained are summarized in Table 2. Over the dose range and time period studied it is apparent that the activity of MDH is not greatly affected whereas the activities of PK and GDH are respectively decreased or increased as a function of the dose in the incubation medium.

Figure 1 summarizes the changes observed in all enzymatic activities measured as a function of time of culturing in a medium containing the intermediate

Table 1. The effect of pentobarbital on carbohydrate metabolism of NN glial cells in culture during different growth phases

Enzyme	Culture medium	3 Days in culture (Logarithmic)	7 Days in culture (Stationary)
		μmole substrate used per mg protein per min	
LDH	Control	1.38 ± 0.11	1.33 ± 0.11
	Barbiturate†	0.77 ± 0.06*	2.13 ± 0.06*
MDH	Control	1.18 ± 0.09	0.95 ± 0.05
	Barbiturate	1.05 ± 0.08	1.03 ± 0.05
PK	Control	1.57 ± 0.17	1.47 ± 0.07
	Barbiturate	1.49 ± 0.16	0.54 ± 0.05*
ALD	Control	0.026 ± 0.003	0.034 ± 0.003
	Barbiturate	0.027 ± 0.002	0.037 ± 0.004
GDH	Control	0.017 ± 0.002	0.018 ± 0.002
	Barbiturate	0.032 ± 0.002*	0.025 ± 0.002*
CCR	Control	2.18 ± 0.23	1.70 ± 0.16
	Barbiturate	2.77 ± 0.26*	1.98 ± 0.13

All values represent means of 4 plastic (Falcon) tissue flasks ± S.E.M.
* $P < 0.05$ compared to respective value in drug-free medium.
† 5×10^{-4} M pentobarbitol-Na.

Table 2. The effect of various concentrations of pentobarbital-Na in the culture medium on the activities of MDH, PK and GDH of NN glial cells (2 weeks)

Culture medium	MDH	PK	GDH
	μmole substrate used per mg protein per min		
Drug-free	0.99 ± 0.08	1.05 ± 0.12	0.024 ± 0.001
5×10^{-5} M Pentobarbital	1.05 ± 0.13	0.92 ± 0.09	0.025 ± 0.003
1×10^{-4} M Pentobarbital	1.09 ± 0.11	1.04 ± 0.03	0.036 ± 0.002*
2.5×10^{-4} M Pentobarbital	1.04 ± 0.11	0.82 ± 0.03*	0.034 ± 0.004*
5×10^{-4} M Pentobarbital	1.17 ± 0.05*	0.65 ± 0.04*	0.037 ± 0.003*
1×10^{-3} M Pentobarbital	1.13 ± 0.09	0.052 ± 0.03*	0.032 ± 0.001*

All values represent means of at least 3 plastic Falcon flasks ± S.E.M.
* $P < 0.05$ compared to drug-free medium.

dose of 5×10^{-4} M pentobarbital. As can be seen over a period of 4 weeks, the initially observed increase in LDH activity progressively declines, and that of GDH activity progressively increases. PK remains at the same level of reduced activity throughout the entire period. CCR never changes significantly from control levels. The increase in ALD and MDH activities reaches levels of statistical significance at 2 and 4 weeks, respectively.

The direct effect of pentobarbital on PK and LDH and the effect of cross-incubation. The effect of pentobarbital-Na when added to the crude extract of untreated cells was measured on the activities of LDH and PK. The results are presented in Fig. 2. The enzymatic activities measured in extracts from untreated cells are depicted in columns 1A and 1B. The direct addition of 5×10^{-4} M pentobarbital for an incubation period of 30 min does not affect either enzyme (columns 2A and 2B). Furthermore, from the temperature control it is apparent that both enzymatic activities are stable over this incubation period (columns 3A and 3B). Columns 4A and 4B represent the enzymatic activities measured in extracts of cells that had been cultured in the presence of pentobarbital-Na for 2 weeks. In columns 5A and 5B are shown the results of a cross-incubation between one half of the extract from untreated cells and one half of that from the barbiturate-treated cells. These "mixed" activities should be compared to the theoretical value (columns 6A and 6B) which would be obtained if the activities from untreated and those from barbiturate-treated cells were simply additive. It can be seen that the increase of LDH activity after cross-incubation is not different from that seen in barbiturate-treated cells, that is the activity observed after cross-incubation is higher than that of the theoretical value. In the case of PK, there is a greater reduction of activity after cross-incubation than that observed in the

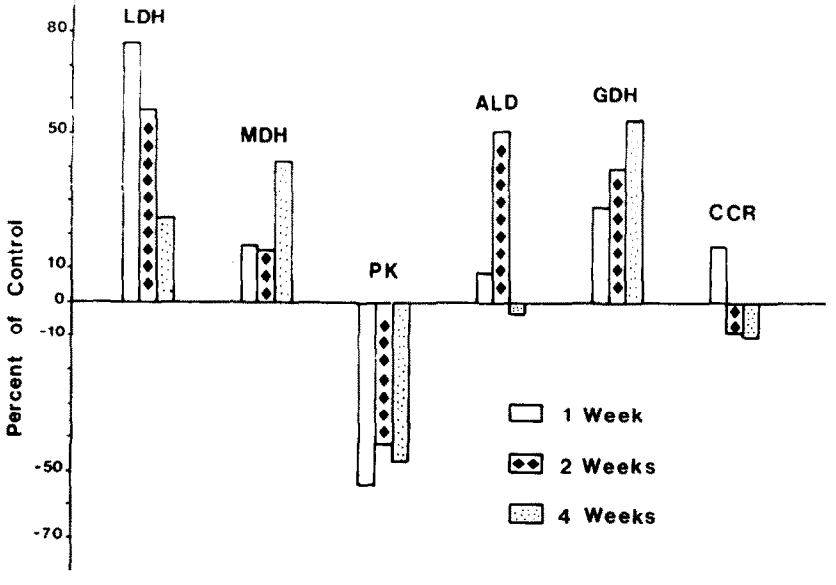

Fig. 1. The effect of continued exposure to pentobarbital-Na on carbohydrate metabolism of glial cells (NN) in culture. Control and barbiturate-treated cultures were subcultured once weekly, the medium was changed every 2nd day. The barbiturate-treated cells were maintained in a medium containing 5×10^{-4} M pentobarbital-Na. All determinations were done after a 7-day growth period. The control values were (μmole of substrate per min per mg protein ± S.E.M.): LDH—1.27 ± 0.05; MDH—1.02 ± 0.07; PK—1.37 ± 0.07; ALD—0.029 ± 0.002; GDH—0.021 ± 0.002; CCR—1.74 ± 0.09.

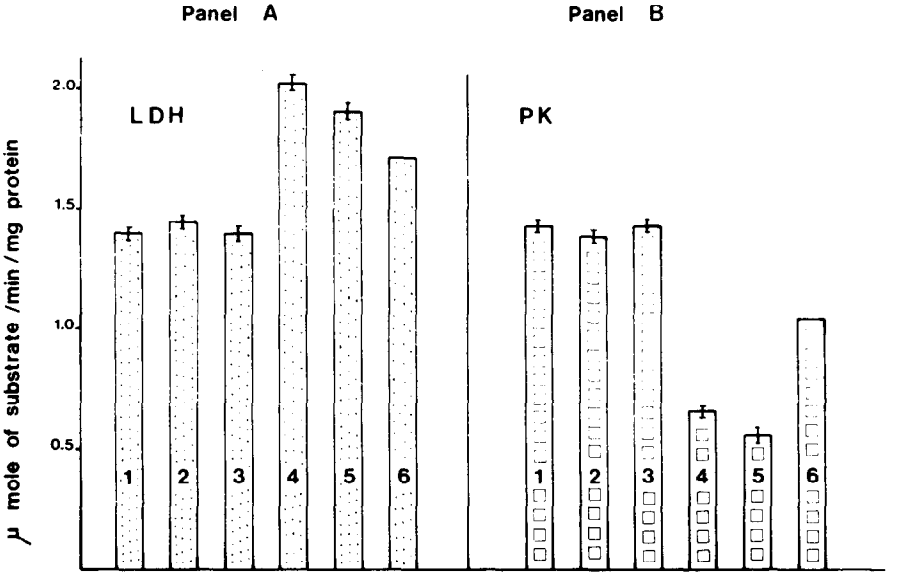

Fig. 2. The direct effect of pentobarbital-Na on LDH and PK activities and the effect of cross-incubation. (1) Enzymatic activity of cells cultured in drug-free medium. (2) Enzymatic activity of *1* after incubation for 30 min in the presence of 5×10^{-4} M pentobarbital-Na at 25°. (3) Enzymatic activity of *1* after drug-free incubation for 30 min at 25°. (4) Enzymatic activity of cells cultured for 2 weeks in the presence of 5×10^{-4} M pentobarbital-Na. (5) Enzymatic activity of extracts *1* and *4* in a 50:50 (v/v) mixture. (6) Theoretical value obtained if extracts *1* and *4* were additive after a 50:50 (v/v) mixture. All values represent means of 4 Falcon flasks determined in duplicates ± S.E.M.

extract of barbiturate-treated cells alone, i.e. the actual activity measured is much lower than the theoretical value of dilution.

The effect of morphine on the carbohydrate metabolism of NN glial cells in culture. The effect of a 2-week exposure period to morphine-HCl on the carbohydrate metabolism of NN glial cells is shown in Fig. 3. It is apparent that with the increase in dose there is a tendency towards a uniform depression of all enzymatic activities measured. Only two points reach the level of statistical significance (PK at 2.5×10^{-4} M and GDH at 1×10^{-4} M). When NN glial cells were exposed to 1×10^{-4} M morphine for up to 4 weeks (data not included here) no statistically significant change in the activity of any enzyme could be detected.

The effect of pentobarbital on the carbohydrate metabolism of neuroblastoma cells (M1) in culture. Three different dosages of pentobarbital-Na were tested in the neuroblastoma cells over a period of

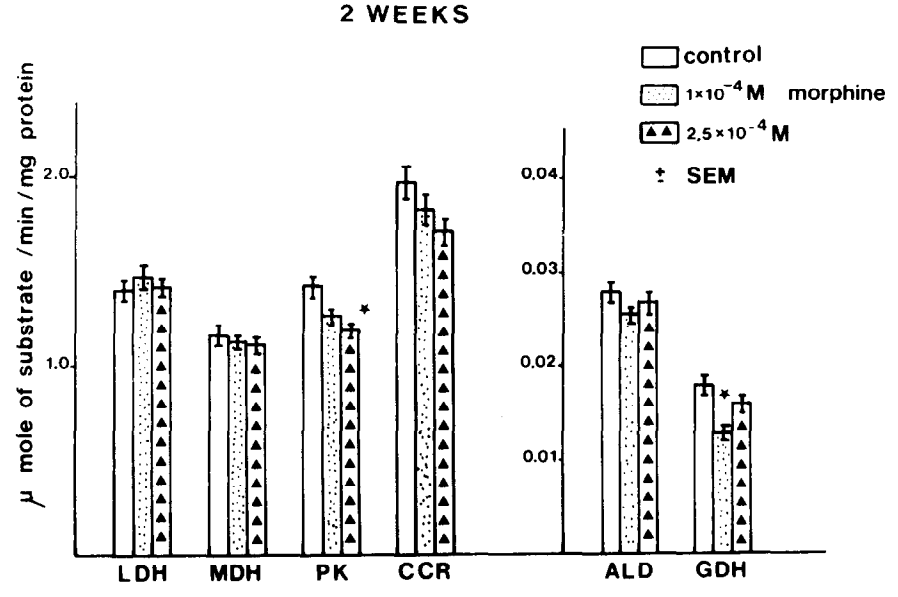

Fig. 3. The effect of morphine-HCl on carbohydrate metabolism of glial cells (NN) in culture. All values represent means of 4 Falcon flasks ± S.E.M. The culture period was 2 weeks. *$P < 0.05$.

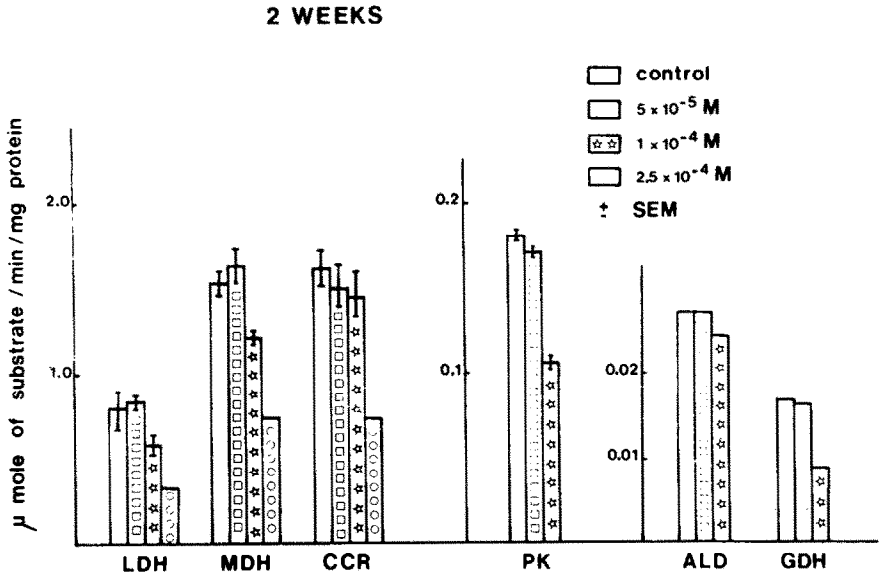

Fig. 4. The effect of pentobarbital on carbohydrate metabolism of neuroblastoma (M1) cells in culture. The values represent either means of 4 Falcon flasks ± S.E.M. or less than 4 flasks (no S.E.M.). The culture period was 2 weeks.

2 weeks (Fig. 4). With the increase in dose a progressive decrease in all enzymatic activities measured was found. No enzyme appeared particularly sensitive when compared to the others at the same dose.

The effect of pentobarbital on the carbohydrate metabolism of rat glial cells in primary culture. In Fig. 5 are summarized the results obtained in astroblasts from newborn rats in primary culture. The growth of these glial cells for 4 weeks in a culture medium containing 5×10^{-4} M pentobarbital resulted in the same qualitative changes in carbohydrate metabolism as in NN cells. The activities of LDH, MDH and GDH are significantly increased, with GDH being the most responsive. PK is significantly reduced, whereas aldolase and CCR remained unchanged.

The effect of pentobarbital on the carbohydrate metabolism of rat fibroblasts in primary culture. Three different doses of pentobarbital were tested for their effect on the various enzymatic activities of cultured rat fibroblasts (Fig. 6). It is readily apparent that the effect of the barbiturate is uniformly depressive in fibroblasts over this time period. The activities of LDH and GDH appear particularly sensitive whereas those of MDH and CCR are more resistant. It should

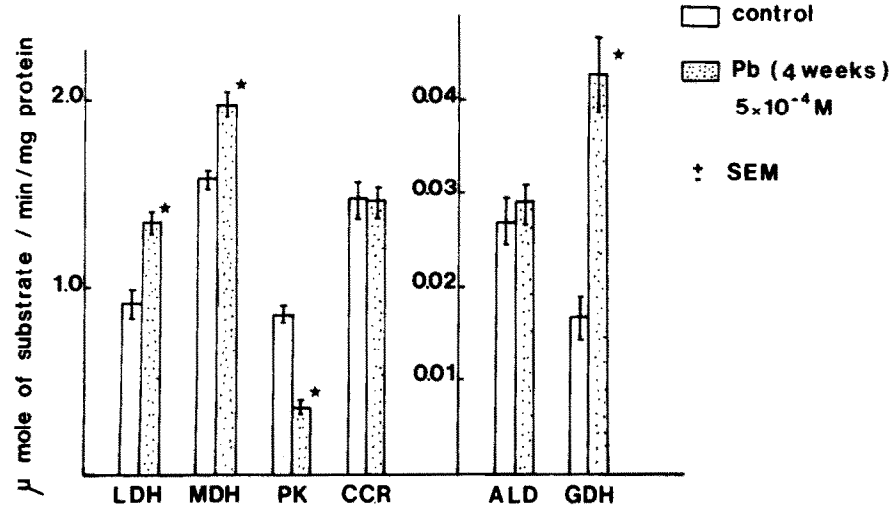

Fig. 5. The effect of pentobarbital-Na(Pb) on carbohydrate metabolism of glial cells in primary culture. Glial cells were prepared from brains of newborn rats. Each value represents a mean of 4 Falcon flasks ± S.E.M. *P < 0.05. The culture period was 4 weeks.

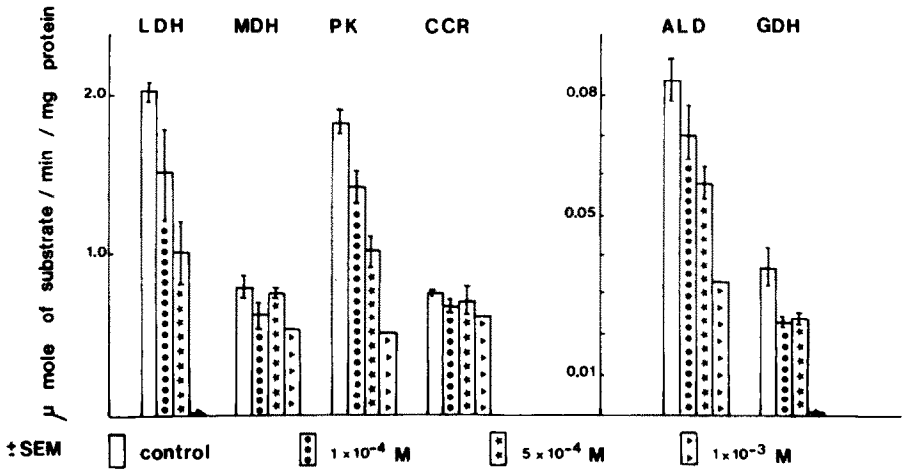

Fig. 6. The effect of pentobarbital-Na on carbohydrate metabolism of rat fibroblasts in primary culture. Cells were prepared from 13-day-old rat embryos. Each value represents a mean of 4 Falcon flasks ± S.E.M. The culture period was 4 weeks. *$P < 0.05$.

be pointed out that cultures grown in the presence of the highest concentration of the drug (1×10^{-3} M) had protein contents reduced by approximately 50 per cent at the end of the 4 week culture period.

DISCUSSION

As has been reported previously [2] growth of glial cells in culture in the presence of pentobarbital induces an increase in their oxygen and glucose consumption. In the present communication we report on the effect of pentobarbital on the activities of several enzymes of the carbohydrate metabolism in different cell types in culture. It was found that the initial effect of the drug in glial cells in continuous line was different during the logarithmic or the stationary phase of growth (Table 1). Over a 2 week growth period in the presence of concentrations ranging from 5×10^{-5} M to 1×10^{-3} M pentobarbital-Na some enzymes exhibited a graded response depending on the dose (Table 2). When NN glial cells were exposed over a time period of 4 weeks to a single dose of 5×10^{-4} M pentobarbital-Na certain time-dependent changes in enzymatic activities were observed. Of primary interest are those changes observed in glial cells only, and not in neuroblastoma cells or in fibroblasts in primary culture.

The most striking effects in glial cells were on the activities of GDH and LDH. Whereas the former showed a progressive increase over the entire culture period studied, LDH was depressed during the initial exposure period, but then remained elevated throughout the 4 weeks. Whether that initial depression represents the direct effect of pentobarbital, as could be speculated based on results obtained *in vivo* [14], or is due to the fact that the cells were in the logarithmic phase of growth can not be determined. The important fact is that the stimulatory responses only occurred in the glial cell population, and were not reproduced in neuroblastoma cells nor in fibroblasts. The somewhat sporadic changes of ALD and MDH activities in glial cells, although statistically significant, may not be of functional significance. Pyruvate kinase activity of glial cells was reduced throughout the entire culture period in a dose-dependent fashion, but the same effect was seen in the fibroblasts and neuroblastoma cells. The other enzymatic activities of these two cell types were either not affected by any of the doses of pentobarbital employed or depressed with increasing doses of the drug (Figs 4 and 6).

The purpose of the cross-incubation experiments was to gain some insight into the mechanism of the barbiturate effect in NN glial cells. It was found that the stimulatory or inhibitory action of continuous culture in pentobarbital on LDH or PK activities, respectively, remained the same whether the extract was assayed alone or whether the extract from barbiturate treated cells was diluted to one half by extract from control cells. These results could be interpreted to indicate the excessive presence of activating or inhibiting factors in barbiturate-treated cultures. It does not seem likely that the actual rates of enzyme synthesis were affected by the drug treatment.

The prolonged exposure of the same glial cells to morphine was undertaken since it has been shown that morphine, like pentobarbital, reduces growth rate of the cells [2]. It was found that culturing of NN glial cells in a medium containing morphine-HCl did not affect the enzymatic activities in the same way as pentobarbital. If any effect was observed, it was a reduction of activity (Fig. 3). It may be concluded, therefore, that the enzymatic changes induced by the barbiturate are not related to its growth inhibitory effect.

Since the NN glial cells constitute a continuous line, it was of primary importance to study the response of non-transformed glial cells in primary culture to barbiturates. It was found that cultures of astroblasts from newborn rats responded to the drug in a qualitatively similar fashion as the glial cells of the NN line. In fact, it was surprising to find that the actual activities were of comparable magnitude. The stimulatory effect on GDH and LDH activities was considerably greater in the primary cultures than in the glial cells of the continuous line. The other enzymes were affected to about the same extent. The

significance of that differential response is not apparent. It should be appreciated that the growth rate of primary cultures is considerably less than that of the continuous line and this may partially account for the differences observed. Nevertheless, it may be concluded that the continuous line glial cells seem to be representative for the action of barbiturates on glial cells, since their response to the drug is reproduced by normal glial cells in primary culture. It is tempting to speculate that the unique changes seen in glial cells represent cell specific responses and that the time-dependent alterations signify some process of biochemical adaptation, the mechanism of which, however, remains unclear. Similarly, to what extent these changes may or may not be related to the tolerance to barbiturate observed [2] is impossible to ascertain.

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