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A STUDY ON THE DEVELOPMENT OF BARBITURATE TOLERANCE AND DEPENDENCE IN HAMSTER GLIAL CELLS IN CULTURE

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Abstract—Hamster astroblast glial cells (clone NN) in cell culture were exposed to from 1×10^{-5} to 3×10^{-3} M pentobarbital-Na and from 1×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M morphine hydrochloride for various periods of time. Profound morphological changes were induced in a dose/time-related fashion by pentobarbital only. These consisted of growing of cellular expansions, parallel cellular arrangement and increased amounts of intracellular material. Exposure of the cells to morphine up to 6 weeks resulted in a dose-correlated decreased rate of proliferation, but no specific morphological alteration could be observed. The morphological changes induced in cultured glial cells by pentobarbital were accompanied by an increase in oxygen consumption (35–45%) as well as an increase in glucose uptake (90–110%). These effects were compared to those obtained with bromodeoxyuridine which affected glucose metabolism similarly. Furthermore, glial cells that had been treated for 4 weeks with the barbiturate were less sensitive to the depression of oxygen consumption by a challenging dose of pentobarbital-Na signifying the development of cellular tolerance. After having cultured the cells in barbiturate for 4, 9 or 14 weeks, normal medium was substituted. This resulted in severe degenerative changes and cell deaths as well as altered growth characteristics in the surviving cells, demonstrating some degree of cellular dependence on the barbiturate. In addition, cross-tolerance with ethyl alcohol was established since it could be substituted for the barbiturate without the occurrence of any degenerative changes.

It is generally agreed at present that the tolerance observed after prolonged treatment with barbiturate [1, 2] is due to a combination of at least two mechanisms: an increased hepatic metabolism and a central adaptation at the cellular level [3]. In the case of the former it seems that tolerance to the shorter acting barbiturates appears to be mediated to a much larger extent by enzyme induction than for the longer acting members, but in all cases, as the length of barbiturate action increases the extent of central nervous system (CNS) adaptation becomes progressively greater [4]. As far as central cellular adaptation is concerned very little is known as to differences between various barbiturates, and its mechanism is unclear. It is unclear both with respect to the underlying biochemistry as well as to the cell type involved.

Many adaptive biochemical changes have been identified as occurring in brain during barbiturate tolerance and dependence [5, 6] but in view of the complexity of the whole brain on one hand and the many compensatory mechanisms known to modify all drug actions in the whole animal on the other, these results have escaped meaningful interpretation as to the mechanism and specific locus of central cellular adaptation.

Tissue culture of various cerebral cell types offers a novel and unique approach to the problem. It allows the study of many different types of cells in a well-controlled environment, both with respect to the specificity of the cell involved and with respect to drug levels at the proposed site of action. Tissue culture allows the study of pure drug effects, unmodified by secondary and metabolic factors, a condition essential in studying mechanism and locus of adaptation at the cellular level.

In the present study cell culture of hamster glial cells (astroblasts, clone NN) was used. These cells were chosen for two reasons. Firstly, the greater part of brain tissue consists of glial cells, and their function is virtually unknown. Secondly, the cells are considered to represent normal mammalian cells, although they are of embryonic origin. Pentobarbital was chosen as a widely used, representative barbiturate, that has been well studied in other systems [7–9].

The purpose of the investigation was to establish whether dependence on barbiturate can be produced in a homogeneous glial cell population in cell culture, whether tolerance to the depressive action of barbiturates can similarly be produced at the cellular level, and which biochemical changes, if any, accompany these events.

Therefore, we studied the direct as well as the long-term effects of pentobarbital on some morphological and biochemical aspects of normal glial cells in culture.

MATERIALS AND METHODS

Cell culture. Hamster astroblast glial cells (clone NN) were obtained from North American Biologicals, Inc. They were grown in plastic Falcon flasks (75 cm^2 and 25 cm^2) at 37° in 5% CO_2–95% air atmosphere with saturated humidity. The cultures were maintained in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum. The medium was changed every 2 days and subcultures were prepared

by using 0·25% trypsin at a rate of once per week. The cells were subcultured at the approximate concentration of 8×10^6 cells/75 cm^2 flask and $1{\cdot}5 \times 10^6$ cells/25 cm^2 flask. Pentobarbital-Na (Abbott) was added to the culture medium in final concentrations ranging from 1×10^{-5} M to 3×10^{-3} M for various periods of time up to a total of 14 weeks. Pentobarbital was initially added at the time of subculturing, i.e. to dividing cells. Thereafter, barbiturate-treated cultures were subcultured according to the identical schedule as control cells.

The pH of the culture medium was kept constant at 7·0 ± 0·4 units. Unless stated otherwise for each series of experiments untreated glial cells of the same subculture and the same cell density served as controls.

For the biochemical assays the cultures were exposed to 5×10^{-4} M pentobarbital-Na according to three different schedules: short-term exposure was defined as cultivation in pentobarbital-Na for 2 weeks. Long-term exposure was defined to range from 4 to 8 weeks of growth in the presence of the drug. Withdrawal was defined as substituting drug-free culture media in cells previously grown in barbiturate-containing medium for a minimum of 4 weeks.

The cultures were examined for any morphological changes every 2 days, except during withdrawal, when they were followed every hour for the first 12 hr, after that on a daily basis. The cultures were observed through an inverse phase-contrast microscope (Leitz, Wetzlar), and photos were taken at various intervals using Ilford Pan F black/white film.

Biochemical assays. Oxygen uptake of intact glial cells in suspension was measured in a Gilson Oxygraph with a Clark oxygen electrode. They were recovered from the Falcon flasks in the following manner. The cells were rinsed three times with glucose-free buffer and then harvested into 5 ml of buffer by the aid of a rubber spatula. They were centrifuged at 500 rev/min for 5 min and resuspended in 50 to 100 μl of the final incubation buffer which consisted of the following: (pH 7·3), 150 mM NaCl, 3·0 mM KCl, 1·0 mM $CaCl_2$, 0·6 mM $MgCl_2$, 6·0 mM glucose, 9·0 mM Na_2HPO_4, 1·7 mM KH_2PO_4. The final volume of incubation medium was 1·65 ml, and incubation was carried out at 37°. The standard used for calibration was the oxidation of NADH by catalase in the presence of 0·2% phenazine methosulfate.

In the experiments establishing the direct effects of pentobarbital-Na and KCl on the oxygen consumption of normal glial cells, the drugs were added to the incubation chamber in a volume of 50 μl of buffer after oxygen uptake had been linear for at least 10 min. In the experiments establishing the sensitivity of differently pretreated cells to a challenging dose of pentobarbital the barbiturate was also added in a volume of 50 μl of buffer directly into the incubation chamber. The experiments were designed such, that a measurement of normal cells was always followed by a measurement of treated cells and vice versa. After each determination, the cells were removed, the incubation chamber rinsed and the cells washed three times with buffer. Protein content of each incubation sample was determined according to the method of Lowry *et al.* [10]. The cells used in each assay were found to represent an amount of total protein between 0·8 mg and 1·4 mg. Respiratory rates were expressed in μl O_2 consumed/mg protein/min.

Glucose uptake was measured in a Beckman automatic analyzer according to the following procedure. After removal of the culture medium, the cells were detached by the use of a rubber spatula and rinsed twice with glucose-free buffer. At time zero 2·5 ml of glucose-containing buffer was added, a control sample of 50 μl was taken, and the cells were incubated at 37° for 2 hr; at that time the incubation medium was centrifuged at 5000 *g* for 5 min and glucose content of the medium was determined in triplicate of 50 μl each. Protein content of each incubation sample was measured according to the method of Lowry *et al.* [10]. The difference between glucose content at time zero and at 2 hr was calculated, and glucose uptake was expressed as mg glucose taken up from the media/mg protein/hr.

NADH, catalase and phenazine methosulfate were procured from Sigma Chemical Company, 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 morphology of NN glial cells

Dose-response relationship. Normal hamster glial cells (NN clone) are known to retain their astroblast-like appearance (Fig. 1) without any visible extensions and prolongations. Their shape is almost round, and they contain large amounts of plasma with several nucleoli.

Addition of pentobarbital-Na to the tissue culture media induces a characteristic change in the morphology of these hamster glial cells (Fig. 2). The cells are elongated and enlarged with processes extending from the perikaryon. They are oriented in a parallel fashion. The number of nucleoli per cell is increased. Based on a count of 200 cells in each group it was found that control cells contained 2·645 ± 0·075 nucleoli, whereas barbiturate-treated cells (5×10^{-4} M for 4 weeks) contained 3·735 ± 0·118 nuclei ($P < 0{\cdot}001$). The occurrence of this barbiturate-induced appearance was found to be a function of the dose of pentobarbital as well as of the time of exposure. The concentrations of pentobarbital studied ranged from 1×10^{-5} M to 3×10^{-3} M in the culture media. The minimally effective dose was found to be 1×10^{-4} M with a minimal duration of exposure of 2 weeks, and a concentration of 3×10^{-3} M was found to be lethal within 4·5 days. The inverse relationship between concentration of pentobarbital and time of exposure existed throughout the entire dose range studied. The growth characteristics of the most frequently used doses of pentobarbital and morphine hydrochloride are shown in Fig. 3. The growth rate is reduced by pentobarbital as well as by morphine, but the duration of logarithmic phase growth is the same as in untreated cells. At the dose of 5×10^{-4} M pentobarbital the earliest time point of any morphological change was after 3 days of exposure, i.e. after at least one and one half generation time the whole culture being completely changed after

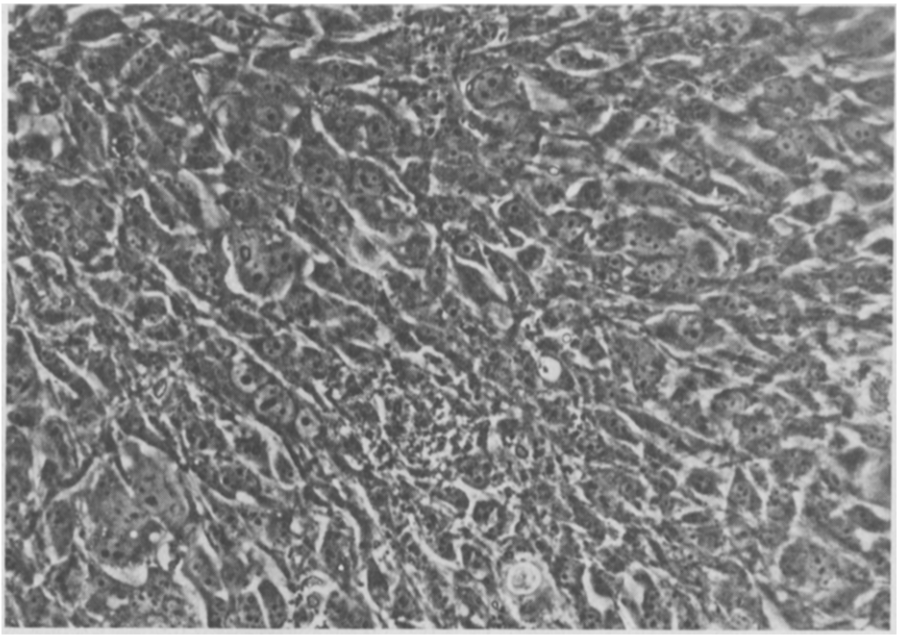

Fig. 1. Hamster astroblast glial cells, clone NN, untreated (Magnif. 200×).

6 days. Thereafter, no further morphological change was evident up to an exposure period of 14 weeks.

Long-term exposure and effect of withdrawal. The results establishing long-term barbiturate exposure and barbiturate dependence were obtained with cultures grown in a pentobarbital concentration of 5×10^{-4} M for a minimum of 4 weeks and a maximum of 14 weeks. No additional morphological changes were seen to occur once the culture had attained the barbiturate-related appearance.

In an attempt to elucidate whether this effect is a general effect of central nervous system depressants, glial cells were likewise exposed to various concentrations of morphine hydrochloride (1×10^{-5} M to $2{\cdot}5 \times 10^{-4}$ M) and ethyl alcohol (0·1 M) for periods up to 6 weeks. In no case was there any change in the morphology of glial cells in culture, although the rate of growth is reduced by both drugs.

In order to evaluate whether glial cells can become dependent on pentobarbital-Na, half of the culture was withdrawn after 4 weeks of exposure, while the other half remained exposed to the drug. The first visible change in glial cells after having replaced the culture medium with drug-free medium occurs as early

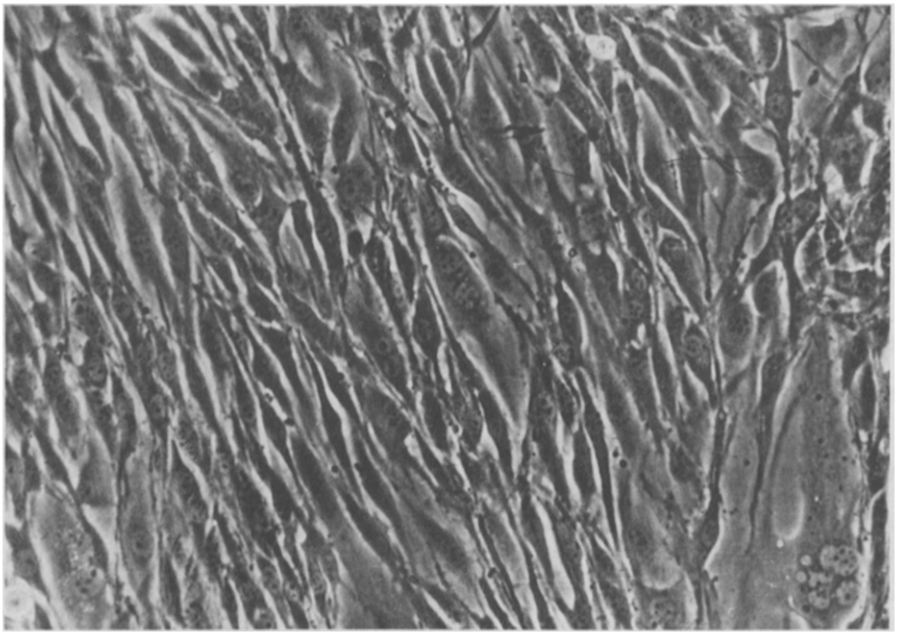

Fig. 2. Hamster astroblast glial cells, clone NN, exposed to 5×10^{-4} M pentobarbital-Na for a period of 2 weeks (Magnif. 200×).

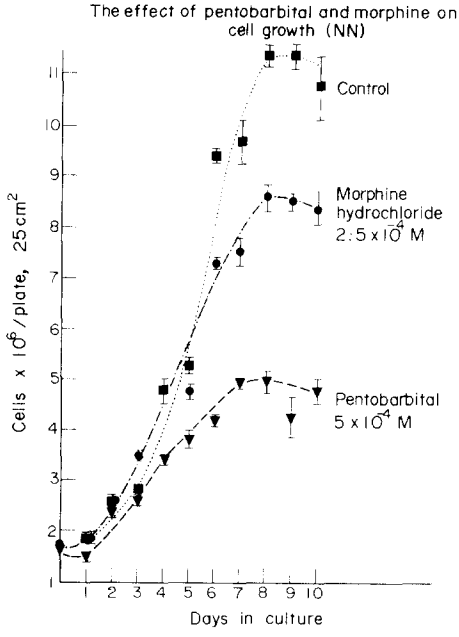

Fig. 3. The effect of pentobarbital-Na and morphine hydrochloride on cell growth of NN glial cells in culture. Cells were subcultured into control medium (■ ■), medium containing $2{\cdot}5 \times 10^{-4}$ M morphine hydrochloride (● ●) and containing 5×10^{-4} M pentobarbital-Na (▼ ▼). Culture medium was changed every second day and each point represents the mean ± S.E.M. of 4 plastic flasks.

as 3 hr after withdrawal and consists mainly of vacuolization. One day after withdrawal (Fig. 4) two additional phenomena are apparent: there is a large number of floating dead cells, and some of the cellular expansions are detached. Between the 4th and 5th day after withdrawal the morphology of the surviving cells begins to resemble control morphology. A quantitation of the barbiturate-related degeneration is presented in Fig. 5. Vacuolization increases drastically during the first 24 hr after withdrawal and then declines slowly. Cell deaths on the other hand peak on day 2. Similar results were obtained with cultures of glial cells exposed for shorter or longer periods of time to pentobarbital, with the withdrawal reaction varying only in the degree of severity and the duration.

In order to establish whether those cells that did survive withdrawal would eventually return to normal growth characteristics, control cells were grown in parallel with those that had been treated with 5×10^{-4} M pentobarbital for 14 weeks as well as those that had been withdrawn into drug-free medium at day zero. Dead cells were identified by staining them with Trypan blue, and only viable cells are plotted in Fig. 6. It is readily apparent that barbiturate-treated and barbiturate-withdrawn cells grow slower than control cells. From a time course of only 12 days it cannot be established whether the barbiturate dependent cells reach stationary phase during this time. The barbiturate-withdrawn cultures reach a plateau in the number of viable cells between days 4 and 6. This was found to correspond to the peak of cell deaths observed. Thereafter, the surviving cells enter their logarithmic phase of growth during which their growth rate is identical to that of untreated cells. However, they do not remain in log phase for the same period of time neither do they reach the same cell density as control cells.

Based on the known existence of cross-tolerance between barbiturates and alcohol *in vivo* [3], barbiturate-treated cultures (4 and 9 weeks) were "withdrawn" into a medium containing 0·1 M ethyl alcohol. In Fig. 7 is shown a representative photo taken

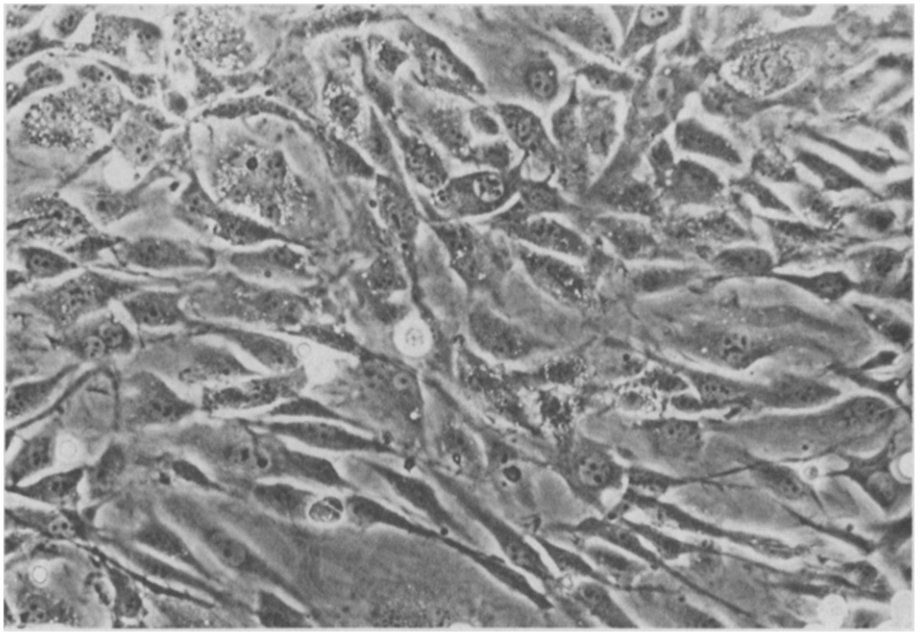

Fig. 4. Hamster astroblast glial cells, exposed to 5×10^{-4} M pentobarbital-Na for 4 weeks, followed by withdrawal into normal medium for 1 day (Magnif. 200×).

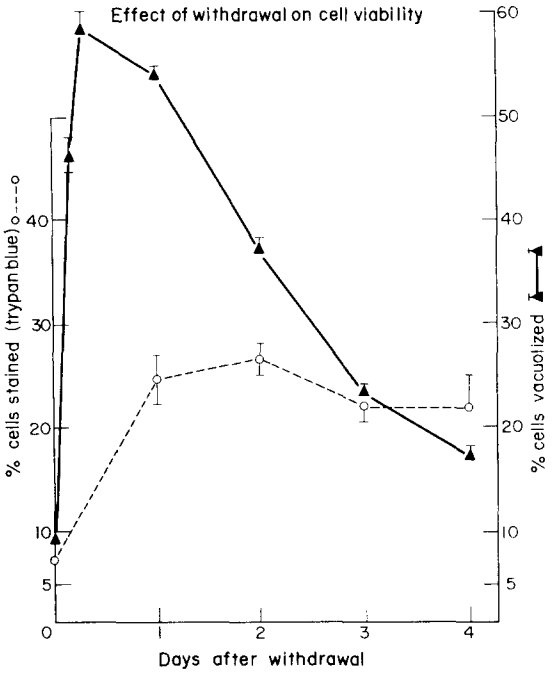

Fig. 5. The effect of withdrawal from pentobarbital-Na on cell viability of NN glial cells in culture. Cells were exposed for 4 weeks to 5×10^{-4} M pentobarbital-Na, followed by withdrawal into drug-free medium at day zero. Dead cells were counted by differential staining with trypan-blue and neutral red. For the dead cells (O—O) each point represents the mean ± S.E.M. of 4 flasks. For the vacuolized cells (▲—▲) each point represents a mean ± S.E.M. count of 4 times 50 cells in a photomicrograph at 320× magnification.

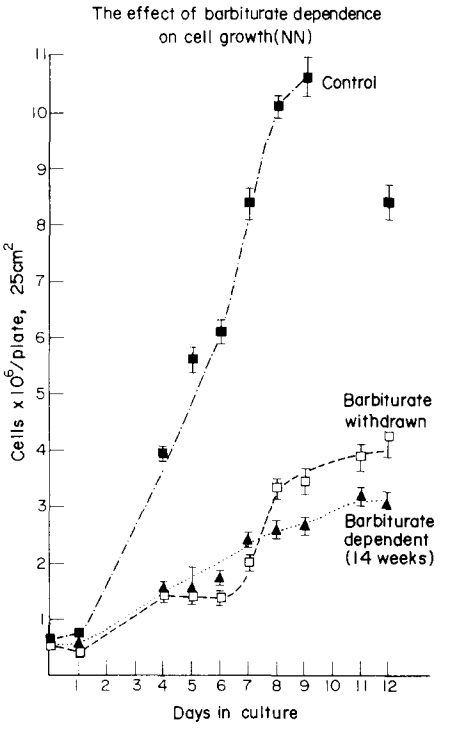

Fig. 6. The effect of barbiturate dependence on cell growth of NN glial cells in culture. Cells were grown for 14 weeks in control medium or in 5×10^{-4} M pentobarbital. At day zero they were subcultured with one half of the barbiturate-treated culture withdrawn into drug-free medium. The media dia were changed every second day and each point represents the mean ± S.E.M. of 4 flasks.

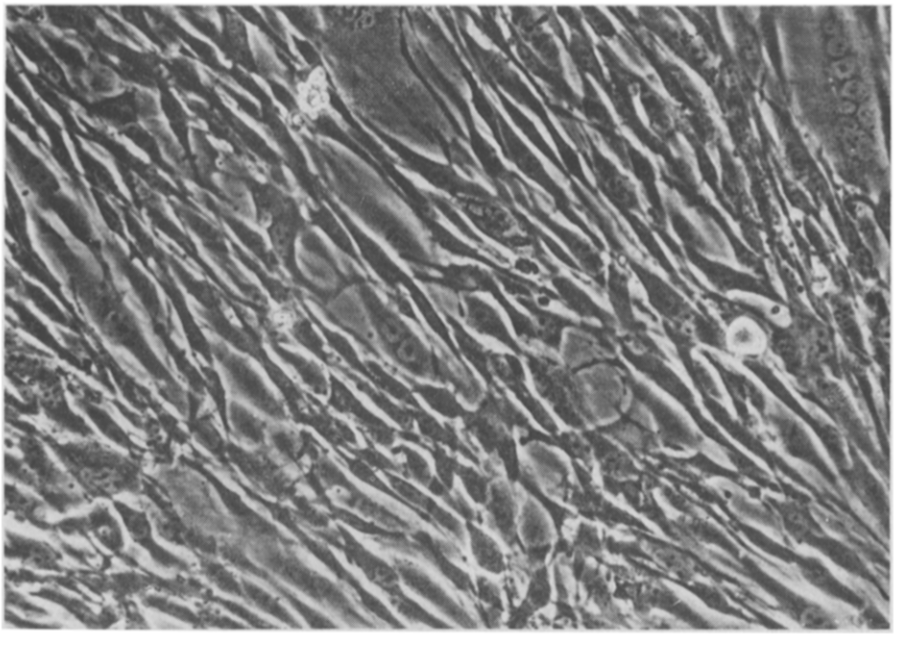

Fig. 7. Hamster astroblast glial cells, exposed to 5×10^{-4} M pentobarbital-Na for 4 weeks, followed by withdrawal into ethyl alcohol containing medium for 4 days (Magnif. 200×).

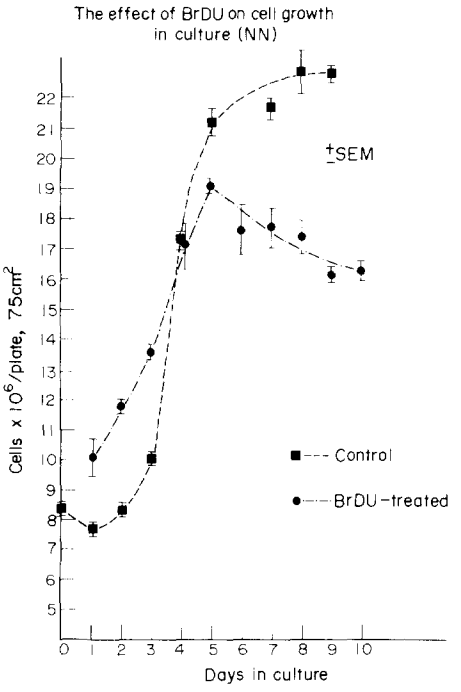

Fig. 8. The effect of 5-bromodeoxyuridine on cell growth of cells in culture. Cells were subcultured into the presence of normal medium (■ ■) and one containing 1×10^{-5} M bromodeoxyuridine (● ●). The culture medium was changed every second day. Each point represents the mean ± S.E.M. of 4 flasks.

4 days after such substitution. As is readily apparent, the barbiturate-induced morphological changes are well maintained throughout this period, and no degeneration or cell deaths were observed under those conditions.

Comparison with the effects of 5-bromodeoxyuridine (BrdU). Since BrdU is a well known differentiation-inducing agent in tissue culture, [11], the effects of pentobarbital-Na on glial cell morphology were compared to those obtained with BrdU in the same cells. A growth curve of NN glial cells in the presence and absence of 1×10^{-5} M BrdU is presented in Fig. 8. Like pentobarbital (see Fig. 3) BrdU also decreases growth rate, but degeneration and cell deaths occur after a short period of exposure. Morphologically, the most striking difference between the changes induced by pentobarbital and those induced by BrdU is the lack of orientation in the cells treated with BrdU. The cells are also enlarged with multiple extensions, but after several passages cell number is considerably less than that of untreated cells. Although it is difficult to ascertain on purely morphological grounds, it appears that the density of intracellular material is less in BrdU-treated cells than in those exposed to pentobarbital. Therefore, the morphological changes induced by BrdU in glial cells appear to be different than those induced by pentobarbital.

In order to establish whether the effects observed after withdrawal of pentobarbital were common to drugs producing differentiation-like morphological changes glial cells were likewise exposed to BrdU for 4 weeks and the effect of withdrawal was tested. At no time point after substituting normal medium were there any degenerative changes. After several days, the culture began to divide again, and eventually attained normal appearance and normal cell number. No cell deaths could be detected in the course of a 4 day withdrawal period.

The effect of pentobarbital on the biochemistry of NN glial cells

Direct effect on respiration. In order to observe the direct effect of pentobarbital on glial cell metabolism, normal glial cells were incubated in the Oxygraph (see Materials and Methods) and their oxygen consumption was measured. After a minimum of 10 min of stable respiration, drug was added in a constant volume. Thus, every drug effect could be expressed as per cent of its own control respiration. The oxygen consumption of glial cells in the absence and in the presence of various doses of pentobarbital-Na as well as of potassium chloride is presented in Table 1. As can be seen, the basal respiration of glial cells is a stable phenomenon, and the rates obtained in this study agree very well with those reported in the literature [12]. The addition of increasing doses of pentobarbital-Na produces a progressive inhibition of glial cell respiration, resulting in a 59% reduction in the presence of 1×10^{-3} M pentobarbital. The rate of respiration stabilized within 2–3 min after the addition of the barbiturate and the new rates are based on a respiratory period of 15 min in the presence of the drug. The addition of 100 mM KCl which is

Table 1. Direct effect of pentobarbital-Na and potassium chloride on the oxygen consumption of glial cells

Treatment*	(μl O_2 consumed/mg protein/min) $\times 10^{-2}$ Control	Drug	% Change
5×10^{-4} M pentobarbital	15·26 ± 1·65 (8)	10·29 ± 0·89† (8)	−34%
$7{\cdot}5 \times 10^{-4}$ M pentobarbital	14·96 ± 0·99 (6)	6·63 ± 0·90‡ (6)	−51%
1×10^{-3} M pentobarbital	13·87 ± 1·37 (9)	5·79 ± 0·87‡ (9)	−59%
100 mM potassium chloride	14·43 ± 2·30 (4)	13·78 ± 2·20 (4)	−6%

All values represent means ± standard error of the mean.
( ) Numbers of plastic (Falcon) tissue flasks (75 cm^2) used for a particular determination.
* Final concentration of pentobarbital-Na or potassium chloride in incubation mixture.
† $P < 0{\cdot}05$ compared to control values.
‡ $P < 0{\cdot}01$ compared to control values.

Table 2. Oxygen consumption and glucose uptake of glial cells in tissue culture

Culture medium*	(μl O_2/mg protein/min) $\times 10^{-2}$	(mg gluc./mg protein/hr) $\times 10^{-2}$
Normal medium	14·97 ± 0·99 (6)	40·67 ± 5·64 (8)
Medium containing 5×10^{-4} M pentobarbital	19·55 ± 0·42† (7)	82·95 ± 17·96† (8)
Medium containing 3×10^{-6} M BrdU	25·00 ± 8·60† (5)	93·76 ± 27·63† (5)

All values represent means ± standard error of the mean.
( ) Numbers of plastic (Falcon) tissue flasks (75 cm^2) used for a particular determination.
* 2 weeks culture period.
† At least $P < 0.05$ compared to control values in normal medium.

known to stimulate respiration in cerebral cortical slices [13] and isolated neurons [14] has no significant effect on NN glial cell respiration.

Effect of short-term exposure to pentobarbital on glial cell metabolism and barbiturate sensitivity. The cultivation of NN glial cells in barbiturate containing medium for the duration of 2 weeks was defined as short-term exposure. Table 2 summarizes the rates of oxygen consumption and glucose uptake of glial cells cultivated in media containing pentobarbital or BrdU compared to those of parallel cultures in control medium. It should be pointed out that the actual measurements were performed in the identical drug-free buffer. As is readily apparent, respiration is significantly increased in those cells that had been exposed for 2 weeks either to pentobarbital or BrdU. Glucose uptake is similarly increased, in the case of the barbiturate-treated cells to a larger extent than the oxygen consumption. The increases due to pentobarbital or BrdU are not significantly different from each other.

In order to test for the possible development of barbiturate tolerance in glial cells in culture, sensitivity of cellular respiration to a challenging dose of pentobarbital added directly to the oxygraph was measured. The addition of 5×10^{-4} M pentobarbital-Na to control cells resulted in a reduction of oxygen consumption by 43% (Table 3). In cells that had been cultured in barbiturate-containing medium for 2 weeks the addition of the same amount of pentobarbital produced only a 13% inhibition of respiration. This proved to be a statistically significant reduction ($P < 0.05$) in respiration, independent of the elevated rate of respiration. Most importantly, however, the extent of this reduction is significantly less than that obtained in control cells.

Effect of long-term exposure to pentobarbital on glial respiration and barbiturate sensitivity. The cultivation of NN glial cells in barbiturate medium for 4 weeks and longer was defined as long-term exposure. As can be seen in Table 4 exposure of hamster glial cells to pentobarbital for a period of 4 weeks induced further changes in the rate of oxygen consumption. One can no longer see an increased rate of respiration in the barbiturate treated culture. Whether this return to control levels was a reflection of true cellular adaptation was tested by measuring the depressive response of O_2 consumption to 1×10^{-3} M pentobarbital. Although these barbiturate-treated cells have a similar rate of O_2 consumption as control cells, they are still less sensitive to this challenging dose of pentobarbital ($P < 0.05$).

The effect of withdrawal from pentobarbital on respiration and pentobarbital sensitivity. The effect of withdrawal on the morphological appearance of barbiturate-dependent cells has been described in section "long-term exposure and effect of withdrawal". As may be recalled, on the 5th day after withdrawal the appearance of the withdrawn culture had recovered normal glial cell morphology. The rates of O_2 consumption at this same time point are presented in Table 5. Withdrawn cells have an accelerated rate of respiration when compared to control cells. Most importantly, the depressive effect of a challenging dose of pentobarbital is identical in both types of cells. The addition of 1×10^{-3} M pentobarbital results in a reduction of 59% and 55% in control and barbiturate-withdrawn cells, respectively.

Table 3. The effect of short-term exposure of cultured glial cells to pentobarbital on pentobarbital sensitivity *in vitro*

Culture medium*	(μl O_2 consumed/mg protein/min) $\times 10^{-2}$ Control	5×10^{-4} M pentobarbital	% Depression
Normal medium	14·91 ± 1·80 (4)	8·59 ± 1·10† (4)	−43%
Medium containing 5×10^{-4} M pentobarbital	27·10 ± 1·40† (5)	23·47 ± 1·70‡ (5)	−13%

All values represent means ± standard error of the mean.
( ) Numbers of plastic (Falcon) tissue flasks (75 cm^2) used for a particular determination.
* 2 weeks culture period.
† $P < 0.01$ compared to control respiration of normal medium.
‡ $P < 0.05$ compared to control respiration of barbiturate medium.

Table 4. The effect of long-term exposure of cultured glial cells to pentobarbital on pentobarbital sensitivity *in vitro*

Culture medium*	(μl O_2 consumed/mg protein/min) $\times 10^{-2}$ Control	1×10^{-3} M pentobarbital	% Depression
Normal medium	13·87 ± 1·37 (9)	5·79 ± 0·84† (9)	−59%
Medium containing 5×10^{-4} M pentobarbital	15·01 ± 1·53 (9)	11·48 ± 3·03‡ (9)	−31%

All values represent means ± standard error of the mean.
( ) Numbers of plastic (Falcon) tissue flasks (75 cm^2) used for a particular determination.
* 4 weeks culture period.
† $P < 0{\cdot}01$ compared to control respiration in normal medium.
‡ $P < 0{\cdot}05$ compared to barbiturate respiration in normal medium.

Table 5. The effect of withdrawal of pentobarbital from cultured glial cells on pentobarbital sensitivity *in vitro*

Culture medium	(μl O_2 consumed/mg protein/min) $\times 10^{-2}$ Control	1×10^{-3} M pentobarbital	% Depression
Normal medium*	13·87 ± 1·37 (9)	5·79 ± 0·85§ (9)	−59%
Medium containing† 5×10^{-4} M pentobarbital	21·59 ± 2·59‡ (5)	9·74 ± 1·62¶ (5)	−55%

All values represent means ± standard error of the mean.
( ) Numbers of plastic (Falcon) tissue flasks (75 cm^2) used for a particular determination.
* 4 weeks culture in normal medium followed by 4 days in normal medium.
† 4 weeks culture in pentobarbital followed by 4 days in normal medium.
‡ $P < 0{\cdot}05$ compared to control respiration in normal medium.
§ $P < 0{\cdot}01$ compared to control respiration in normal medium.
¶ $P < 0{\cdot}01$ compared to control respiration in barbiturate medium.

DISCUSSION

Several facts can be unequivocally stated with regard to the morphological effects of pentobarbital on NN glial cells in culture. Pentobarbital induces a distinct morphological alteration in glial cell appearance and does so in a dose/time dependent fashion. The fact that neither alcohol and morphine nor bromodeoxyuridine induced similar changes can be interpreted to mean that the phenomenon observed is not common to all CNS depressants nor to known differentiation inducers. On the other hand, the possibility of pentobarbital interacting with a constituent of the medium and thus producing the changes observed has not been ruled out. Nevertheless, this effect observed implies some specificity as far as the barbiturate is concerned. Whether the barbiturate related morphological alterations are inducible in glial cells only, i.e. whether it is cell-specific, can hardly be ascertained at present, since dividing neurons are not yet available in culture. Pentobarbital was tested in a neuroblastoma line (M1) and found not to have any morphological effect. Assuming that neuroblastoma cells in general are representative of neuronal sensitivity [15, 16] cellular specificity of the barbiturate-induced morphological changes in glial cells could be implied. Further studies on different neuronal-cell type preparations are presently in progress.

Secondly, the data indicate that dependence on pentobarbital can be produced in normal hamster glial cells in culture. This conclusion is based on the two facts that (1) following withdrawal of the drug severe degenerative changes are observed quantitatively and qualitatively and (2) altered growth characteristics are observed in the surviving cells. The mechanism of this dependence at the cellular level is of great interest. It may reflect a selection of some resistant cells or an effect of gradual adaptation of all cells. The fact that glial cells do not exhibit dependence on bromodeoxyuridine, which is known to cause a selection of resistant cells, together with the fact that the surviving cells do not rapidly regain normal growth characteristics may indicate an adaptation of all cells. The long-term persistence of these changes is presently under investigation. Since barbiturate-adapted cells were found to be less sensitive to the depressive effect of pentobarbital on oxygen consumption and since barbiturate withdrawal was found to be blocked by ethyl alcohol, the barbiturate adapted cells exhibit similar pharmacological characteristics to what is observed *in vivo* [3].

The preliminary biochemical assays reported here were done to gain an understanding of the biochemical changes underlying the cellular adaptation observed. It was not surprising to find that pentobarbital, if added directly to normal respiring cells, depressed oxygen uptake in a dose-dependent fashion, since this has been reported to occur in numerous cerebral preparations [13]. Prolonged exposure of the glial cells to the drug, however, resulted in increased rates of respiration as well as of glucose uptake. The same effect was found to occur following long-term exposure to BrdU. Therefore, it is impossible at this point to decide whether the increased respiration is

a function of the drug-induced cellular change or independently an effect of the drugs per se, since differentiation in neuroblastoma cells by other means is known to coincide with increased oxygen consumption [18]. If there is a change in the sensitivity to a challenging dose of pentobarbital then one can assume that cellular tolerance has developed. Therefore, our results showing a reduced response to pentobarbital in those cells that had previously been exposed to the drug signify the development of such cellular tolerance. The possibility that it is due to increased oxidative metabolism of pentobarbital and thus reflecting metabolic tolerance cannot be ruled out at present. This aspect is under investigation. The fact that barbiturate-treated cells are cross-tolerant with ethyl alcohol may be taken as an indicator that additional adaptive processes have occurred that changed the cellular sensitivity independent of changes in metabolism.

It can be concluded therefore, that glial cells in culture provide a cellular system reflecting barbiturate-induced tolerance and dependence, since: (1) morphological dependence on pentobarbital was demonstrated in a qualitative and quantitative manner; (2) the biochemical response to a challenging dose of barbiturate was decreased and (3) blockade of withdrawal sequelae could be achieved by an agent (ethanol) effective *in vivo*. Further, pentobarbital concentrations used were comparable to total brain levels measured in animals having received an anesthetic dose. This is not to imply that the glial cell population *in vivo* is specifically responsible for barbiturate dependence, since it is known that non-cerebral cells in culture exhibit dependence on CNS depressants [18]. Nevertheless, normal glial cells in culture thus provide an excellent system to study the biochemical mechanism of barbiturate tolerance and dependence at the cellular level. How the findings presented here may relate to those obtained *in vivo* is presently under investigation.

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