these apparently 'normal' surviving animals, we gave intraperitoneal injections of amphetamine and apomorphine and measured induced turning behavior.

Adult gerbils (60–80 g) were subjected to pentobarbital anesthesia and the left common carotid was dissected away from the vagus nerve and adjoining blood vessels. The artery was then either permanently ligated or temporarily clamped for 30 min using a suture.

In a pilot study 20 animals received permanent unilateral ligation and after one week the 15 survivors were tested for turning behavior to amphetamine. As compared to the effects of amphetamine prior to surgery there was a significant increase in turning behavior towards the side of the ligation. Subsequently, the effects of saline, amphetamine, and apomorphine were compared before and 1 or 8 weeks after a 30 min unilateral carotid clamp. Turns in both directions were measured separately. With regard to the side of the surgery ipsilateral turns were designated positive and contralateral turns were designated negative. Net turns refer to the arithmetic sum of these 2 values. Where total turns are analyzed both these numbers are signed positive and added. Large increases in turning behavior after amphetamine injection were again observed in these animals (table). The use of net turns in the data analysis implies that animals should turn consistently towards the stroked side in the case of amphetamine and away from the stroked side in the case of apomorphine. Although the overall effects are significant it should be pointed out that some animals do not increase turning behavior to amphetamine after the carotid clamp and some animals are induced to turn in the contralateral direction, thus subtracting from the significance of ipsilateral or net turning for the group. In this study 55% of all animals showed increased ipsilateral turning to amphetamine after carotid clamp. However an additional 4% of clamped animals responded to amphetamine by turning consistently in the contralateral direction. Therefore total turns summed irrespective of direction were also subjected to analysis as, a priori, the drug induced direction of turning may not be predicted without a knowledge of the site of any ischaemic damage. Apomorphine produced no significant effect at 1 week but was significant at 8 weeks when total turns are considered. In the case of apomorphine injections 26% demonstrated increased contralateral turns after 8 weeks but the group effect was significant only where computed as total turns irrespective of direction. The brains of 17 gerbils from the combined survivors were prepared for histological examination at the end of the experimental regimen. These gerbils were selected from the animals who were most sensitive to the effects of amphetamine as judged by turning behavior. Nissl stains of coronal sections were examined for evidence of swelling and infarction. As has been previously reported[4] no ischaemic lesions were evident in any of the survivors as revealed by the light microscope.

	Mean difference scores for postoperative minus preoperative turns per min			
	Saline	Amphetamine	Apomorphine	N
1 week postoperative net turns	−0.03	+1.16*	+0.4	16
1 week postoperative total turns	0.24	1.13*	0.78	16
8 weeks postoperative net turns	+0.09	+1.64*	−0.21	12
8 weeks postoperative total turns	0.24	1.25*	1.9*	12

Effect of (+)-amphetamine (5.0 mg/kg) and apomorphine (2 mg/kg) before and after a 30 min unilateral carotid clamp. Turning was measured for 1 min periods at 40 and 50 min after injection and averaged. Drugs were administered in 0.1 ml saline/20 g b.wt. Ipsilateral turns were designated as positive (+) and contralateral turns were designated as negative (−).

*Significant at $p < 0.05$ using a paired t-test. $N \geq 12$ for all groups.

It would appear then that gerbils that recover from this procedure are not normal but are markedly affected by amphetamine, causing turning behavior. Therefore, it may be assumed that amphetamine now has a differential action on both sides of the brain which was not observed prior to the carotid clamp. It is possible that some neurochemical or enzymatic disturbance is induced by the clamp on the ipsilateral side thus interfering with the ability of amphetamine to act on this half of the brain. Since the initiation of our own studies a report has appeared suggesting that in the Mongolian gerbil DA levels but not norepinephrine levels are markedly reduced in the ipsilateral hemisphere after carotid ligation[5]. As one of the major effects of amphetamine in brain is to increase available dopamine in the synaptic region then it is possible that the turning behavior which we have observed is due to the unavailability of dopamine for release on the lesioned side. These studies suggest that although a certain proportion of gerbils recover from unilateral ligation with no visible deficit, either behavioral or histological, an underlying pathology may be revealed by the administration of amphetamine which induces turning behavior. This test which apparently amplifies an inherent imbalance in brain function will provide a sensitive method by which to correlate the functional aspects of ischaemia with the underlying neurochemical alteration.

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Preferential neurotoxicity of pentobarbital on nerve and glial cells in culture[1]

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Summary. The effect of pentobarbital was studied in a mixed population of nerve and glial cells dissociated from brains of 7-day chick embryos and maintained in culture. Pentobarbital-Na was added in various concentrations ranging from 5×10^{-5} M to 1×10^{-3} M. The neuronal density was monitored by counting of neurons, neuronal identity was established by staining for Nissl Bodies and acetylcholinesterase. Over a culture period of 3 weeks, it was found that the barbiturate exerts a preferential dose-dependent cytotoxic effect on neurons.

Chronic administration of a barbiturate to newborn rats has been found to retard brain growth[2]. It is not clear whether this phenomen is caused by an effect on neurons, on glial cells or a combination of both cell types. In view

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of our previous findings that pentobarbital induces profound morphological and biochemical changes in various types of glial cells grown in culture[3,4], it was of interest to investigate the effect of pentobarbital on a mixed culture of dissociated embryonal nerve and glial cells. The results indicate that pentobarbital exerts a dose-dependent cytotoxic effect on neurons.

Primary cultures of a population of nerve and glial cells were prepared from the brains of 7-day-old chick embryos, and maintained in culture according to the method of Booher and Sensenbrenner[5]. Dissociation of the brains was achieved by forcing them gently through a nylon mesh[6] (48 μm pore size). Falcon plastic flasks (surface 25 cm^2) were coated by 1% collagen[7] upon which the dissociated cells were grown. The cells were seeded at a starting population of approximately 2×10^5 cells/cm^2. The cultures were observed and photographed under a Leitz inverse phase contrast microscope and camera assembly. Neurons were counted on various days of culturing from day 7 through 21 as follows: The Falcon flask was placed on a movable stage, graduated in mm and observed at each of 5 designated coordinates (corresponding to locations with vertical coordinates 100, 110, 120 mm and horizontal coordinates 20, 30, 40 mm) through a quadrant eye piece at a magnification of 200 ×. The actual area counted at each point corresponded to 44.2 mm^2. At least 3 flasks were counted at each dose and each day, resulting in a minimum evaluation of 15 squares/dose/day. Values represent the means of 3–5 flasks (SEM 10%). Pentobarbital-Na was added after 7 days of culture in concentrations varying from 5×10^{-5} M to 1×10^{-3} M to at least 3 flasks per dose. The medium was changed every 4th day and drug-free and barbiturate-treated cultures were handled identically throughout the experiment. The pH was monitored and ranged from 7.45 or 7.47.

Effect of pentobarbital-Na on total protein

	mg protein/flask ± SE	Percent reduction
Control medium	1.913 ± 0.140 (3)	
Pentobarbital medium 5×10^{-5} M	1.851 (2)	4.2
Pentobarbital medium 5×10^{-4} M	1.483 ± 0.139 (3)	22.5

For experimental details, see 'methods'. Protein was determined according to Lowry et al.[9] at day 21 of the experimental period.

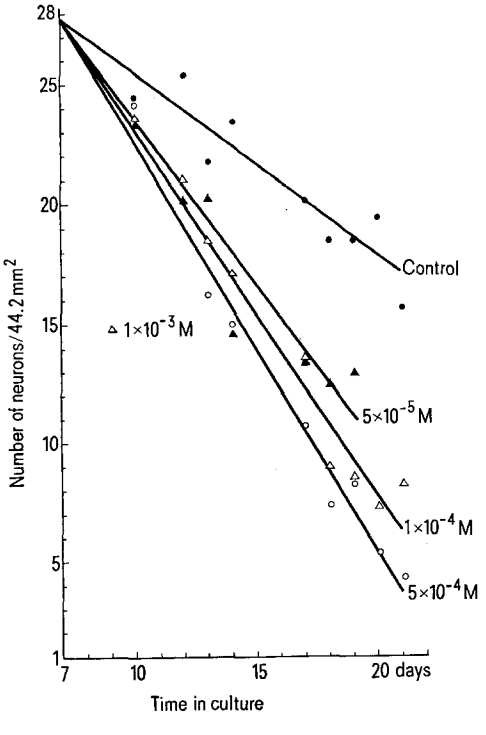

Fig. 1. Effect of pentobarbital-Na on number of neurons in cultures prepared from the brains of 7-day chick embryo. The rate of disappearance of neurons was estimated by determining the slope of the line fitted to the data points by least square fit.

Figure 1 shows for the first time in a quantitative fashion the well-known phenomenon of spontaneous neuronal degeneration as a function of time in culture[5]. The addition of pentobarbital-Na to the culture of nerve and glial cells dissociated from embryonic chick brains results in a dose-dependent reduction of the neurons which were identified by staining of Nissl bodies. Statistically significantly less neurons (p at least < 0.05) are present in all barbiturate-treated cultures beginning with day 10 of drug treatment (corresponding to day 17 of culture). In addition, the rate of disappearance of the neurons is significantly greater in all barbiturate-treated than in control cultures ($p < 0.01$).

Some morphological aspects of the cultures are shown in figure 2. Panel A is a representative photo of an untreated culture after 7 days of incubation. Large numbers of neurons can be seen which are attached to the not yet confluent layer or undifferentiated glial cells. Panel B shows an example of the same control culture at day 21 which corresponds to the last culture period studied. In both cases, well differentiated neurons are dispersed on a glial layer, but the number of neurons is reduced at day 21 when compared to day 7. This reflects the cell count depicted in figure 1. Panel C and D represents cultures incubated in drug-free medium for 7 days and then treated with 5×10^{-4} M pentobarbital for 7 and 14 days, respectively. As can be seen in Panel C, the number of neurons is reduced and their neuritic extensions are less elaborate or non-existent, when compared to the control cultures. In addition, the glial cells have changed their appearance to a more fusiform shape. This morphological change is even more apparent in those cultures treated with pentobarbital for 2 weeks (Panel D). At this time, very few neurons remain in the culture and the glial cells exhibit a parallel fusiform morphology. The morphological appearance of cultures treated with lower concentrations of the drug fall between these extremes. For

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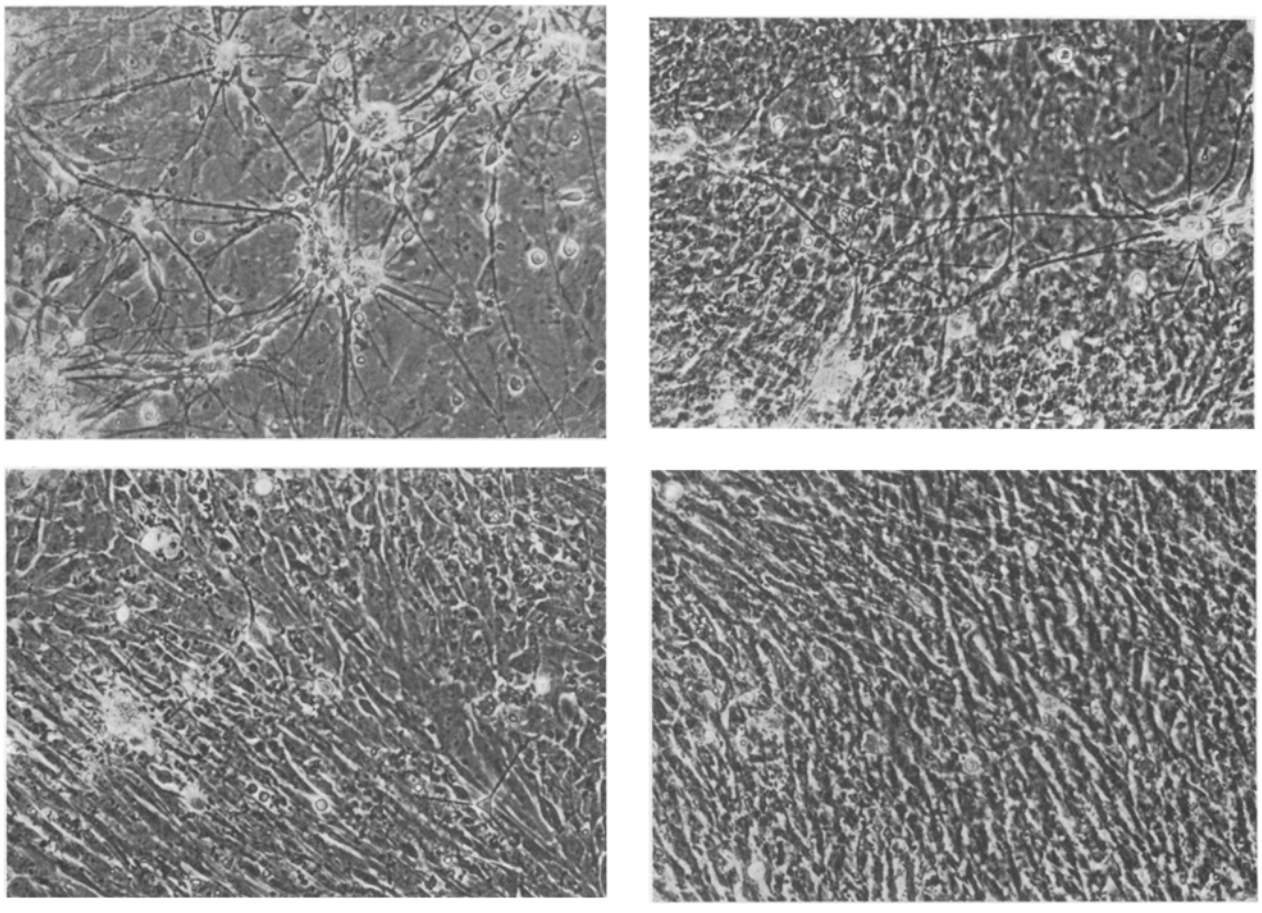

Fig. 2. Effect of pentobarbital-Na on the morphology of 7-day chick embryo dissociated brain cells in culture. All cultures were incubated for 7 days in drug-free medium. *A* Control day 7; *B* control day 21; *C* 5×10^{-4} M pentobarbital day 14 (7 days of treatment); *D* 5×10^{-4} M pentobarbital day 21 (14 days of treatment). $\times 200$.

example, a culture treated with 5×10^{-5} M pentobarbital for 2 weeks resembles one treated with 5×10^{-4} M for 5–7 days.

Throughout the experiment, some cultures were stained for the presence and distribution of acetylcholinesterase (AChE)[8]. This enzyme is known as a 'marker' enzyme in nerve cells in culture[10]. It was found that all neurons present in control as well as barbiturate-treated cultures were stained uniformly. There was no difference in the distribution or the intensity of the coloration between control and treated neurons. However, the cell bodies of all treated cultures appeared smaller and the neurites shorter, but this was not quantified.

At the end of the 3-week culture period, total protein was measured in some control cultures and in some cultures treated with pentobarbital at several doses (table). Although there is a 20% reduction of total protein in cultures treated with the high concentration, this does not reach statistical significance (Student's t-test, $p = 0.05$) probably due to the small number of determinations.

Our results demonstrate that, in a mixed culture of neuronal and glial cells, pentobarbital exhibits a specific neurotoxic effect. The rate of disappearance of the neurons during the culturing period was significantly accelerated in a dose-dependent fashion by the drug. The fact that AChE distribution and apparent activity were found unchanged implies normal functioning of the surviving neurons. Simultaneously with the neurotoxic effect, pentobarbital induced the same morphological changes in the supporting glial cell population as seen previously in cultures consisting of glial cells only[3]. In those cell populations, it was also established that pentobarbital reduces glial growth rate by between 15 and 30% for culture periods of 3–5 weeks. This phenomenon was reconfirmed with the total protein determination and the end of this study. It may be assumed that the 20% loss of total protein therefore primarily reflects glial growth retardation. The more than 80% loss of neurons is disproportionally greater and can be interpreted to indicate a preferential neurotoxic action of the barbiturate. The cell specific toxicity of pentobarbital in cultures of developing brain cells may explain the effect of barbiturates on developing brain in vivo[2]. Since barbiturates readily cross the placenta[11], and have been shown to have a teratogenic action[12], these findings have obvious implications when barbiturates are used in clinical practise.

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