Long-term Reversible Arrest of Cell Growth by Amobarbital

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A mammalian cell culture system subjected to prolonged arrest of growth by sodium amobarbital returned to the pre-existing growth rate very soon after the barbiturate was removed. The increased glycolysis which occurred in the presence of the anesthetic ceased equally rapidly. (Key words: Amobarbital; Cell metabolism; Inhibition; Barbiturate; Cell growth.)

IN PREVIOUS STUDIES with monolayer cultures of mouse heteroploid cells,[1,2] we demonstrated a direct relationship between the logarithmic concentrations of various volatile and barbiturate anesthetics and the slowing of multiplication, the inhibition of oxidative metabolism and the stimulation of nonoxidative metabolism of these cells. These effects were immediate and, at least so far as the inhibition of oxygen uptake was concerned, immediately reversible. However, it was not clear to what extent the cells could recover from prolonged exposure to anesthetics. In the present study we have demonstrated the ability of the cells to recover after continuous partial or total inhibition of cell growth by sodium amobarbital for 24 days. The cells appear to return to the control rate of growth very quickly after the drug is removed.

Methods

The methods of culture have been described.[1] In the present experiments the culture medium contained sodium amobarbital* to a concentration of 0, 2, 3, 3.5, or 4 mM/l. Replicate monolayers were incubated for periods ranging from two to 28 days, the medium being renewed at intervals of two, three, or four days. Following incubation the cells were either harvested with trypsin or washed three times with Hanks' solution and then incubated in amobarbital-free medium for a further period of one to five days before harvesting. The gas phase consisted of 5 per cent CO_2 with air. The harvested cells were counted electronically and the protein content measured by the method of Lowry.[3] The glucose and lactate contents of the supernatant medium were determined by standard enzymatic methods. Duplicate measurements were made of pooled media from four replicates of each condition. The results are expressed as millimoles of glucose uptake or lactate output per million harvested cells over the period of incubation. The results cannot be expressed as per-day rates because the cell numbers were not stationary during the periods considered.

Results

Figures 1 and 2 refer to experiment 1, in which we measured the daily growth rates of replicate cultures incubated in 2-, 3-, and 4-mM amobarbital media for periods up to 12 days, the media being renewed every three or four days. The figures compare these growth rates with those of replicates maintained in amobarbital-free medium at the beginning of the experiment, or placed in amobarbital-free medium after four or eight days of exposure to amobarbital. It is clear that amobarbital inhibited the rate of cell multiplication and that the intensity of the inhibition increased with increasing concentration of the drug. In the presence of 4 mM amobarbital the number of cells attached to a plate remained virtually stationary or declined. After being switched to amobarbital-free medium the growth rates in all cases quickly returned to virtually the same rate of growth as the controls. The suddenness of the transition was manifest in the fact that the rate of multiplication of the "switched" cells on the first day of recovery was about the same as that of the controls, with the possible exception of the

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* Amytal Sodium, Lilly.

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FIG. 1 (left) and FIG. 2 (right). Growth curves of mouse heteroploid cells before, during and after exposure to 2, 3, and 4 mM sodium amobarbital. Medium: Eagle's minimum essential medium with 10 per cent fetal bovine serum and 9 mM TES buffer (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid). Atmosphere: carbon dioxide 40 torr, air to 650 torr. ●———● = no amobarbital; ●— — — —● with amobarbital.

4-mM concentration, with which recovery from the barbiturate effect was most marked on the third day.

Figure 3 presents results of experiment 2, in which cells grew in the presence of 3.5 mM amobarbital for periods of seven, 10, 17, and 24 days, and were then cultivated in barbiturate-free medium for a week more. In this experiment the medium was renewed at intervals of either three or four days. In 3.5 mM amobarbital the cells were unable to multiply and the number of harvested cells gradually decreased to about a third of the number in the original inoculum. Nevertheless, throughout the experiment the cells retained their ability to multiply in ordinary culture medium, ostensibly at the same rate as cells that had never been exposed to amobarbital.

The glucose uptake and lactate output of the cells at the different stages of experiment 2 are shown in table 1. These data are presented again in table 2, with arrows to indicate the timing of changes of medium. In a period of three days (days 1–3) the controls metabolized glucose at the rate of about 1.25 μM per million harvested cells. In the presence of 3.5 mM amobarbital the three-day (days 7–10) glucose uptake increased to 19.5 μM per million harvested cells, while the corresponding rate for cells "switched" back to amobarbital-free medium was about 3.9 μM. A week later (days 14–17) the three-day glucose metabolism of the cells in amobarbital was at the rate of about 34.5 μM per million harvested cells, whereas their recovering counterparts used only 1.8 μM. After still another week the three-day glucose consumption of cells exposed to barbiturate was at the rate of 38 μM per million harvested cells (days 21–24), while the rate for the corresponding recovery replicates was 0.9 μM.

Table 1 also presents observations from experiments 3 and 4. Some observations from these experiments are summarized for purposes of comparison in table 3. In experiment 3 the cells were cultured for two days in

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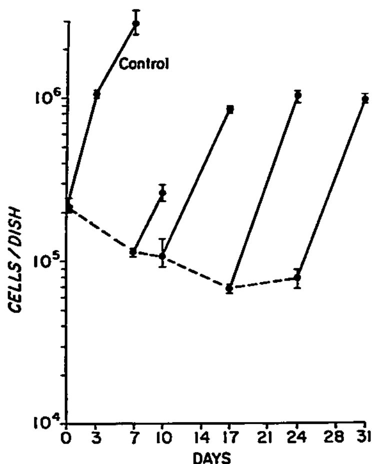

FIG. 3. Growth yields of mouse heteroploid cell monolayer cultures in the presence of 3.5 mM sodium amobarbital for 24 days. Medium and atmosphere as in figure 1 and 2. ●———● = no amobarbital; ●— — — —● with amobarbital.

media containing 0, 2, and 3.5 mM amobarbital. Following this the monolayers were incubated in fresh, amobarbital-free medium for two more days. In the presence of the barbiturate the multiplication of the cells was depressed. As regards metabolism, under control conditions the lactate that appeared was stoichiometrically equivalent to about half the glucose consumed. In 2 mM amobarbital the rate of glycolysis was more than four times, and in 3.5 mM nearly six times, that of the controls, and all of this glucose could be accounted for as lactate. However, after the removal from barbiturate (indicated in table 1 by a superscript) the cells returned to the original rate of growth and metabolism. Thus, the increased glycolysis which occurred in the presence of barbiturate apparently ceased as soon as the barbiturate was removed.

The results of experiment 4 illustrates similar effects in an eight-day experiment, in which the growth of replicates was measured daily for four days before, during and after exposure to 3 mM amobarbital. In the presence of 3 mM amobarbital the cell numbers increased at about 11 per cent of the control rate. These cells contained somewhat less protein than the controls, but metabolized seven times more glucose, with the appearance of stoichiometrically equivalent amounts of lactate.

Discussion

Although the magnitude of the effects of amobarbital on the rate of glycolysis was somewhat variable in the different experiments, the variability was not excessive considering the vagaries of cell culture. Qualitatively the effects were consistent. Most striking among the results was the rapidity of the reversal to the original growth rate after the removal of amobarbital, even after growth had been completely suspended for 24 days (experiment 2).

The protein concentration per cell was not increased in the cells maintained in barbiturate medium, suggesting a uniform retardation of the cell cycle. It was clear that the reversible inhibition of growth could be maintained at a steady level for many days without jeopardizing recovery. One may conclude that any alterations to the molecular mechanisms by the abnormal environment were temporary and that the normal mechanisms quickly regenerated when the environment returned to normal.

The increase in glycolysis due to amobarbital might have been expected to offset the shortfall of energy resulting from depression of oxidative metabolism,[1] and to minimize the depression of cell multiplication. Instead, cell multiplication always was depressed much more than expected from the amounts of glucose undergoing glycolysis and lactate produced, and increasingly so as the concentration of amobarbital increased. In experiment 2, both in the presence of and after removal of 3.5 mM amobarbital, the cultures produced lactate in amounts more than stoichiometrically equivalent to the glucose consumed, suggesting that substrates other than glucose also were being drawn upon. If all the measured changes in the glucose and lactate contents of the medium are ascribed to the surviving cells,

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TABLE 1. Growth and Metabolism of Mouse Heteroploid Cells before, during and after Cultivation in Amobarbital Medium

Experiment	Growth Day	Cells × 10⁻⁶/Dish				Protein/Cell × 10⁶ (μg)				Period of Uptake or Output (Days)	Glucose Uptake/Cell × 10⁶ (μM)				Lactate Output/Cell × 10⁶ (μM)			
		0 mM Amobarb.	2 mM Amobarb.	3 mM Amobarb.	3.5 mM Amobarb.	0 mM Amobarb.	2 mM Amobarb.	3 mM Amobarb.	3.5 mM Amobarb.		0 mM Amobarb.	2 mM Amobarb.	3 mM Amobarb.	3.5 mM Amobarb.	0 mM Amobarb.	2 mM Amobarb.	3 mM Amobarb.	3.5 mM Amobarb.
2	0	0.213																
	3	1.056				308				3	1.2				0.5			
	7	2.804			0.112	441			460	4	5.1			44.5	2.0			94.5
	10	0.256ˣ			0.108	420ˣ			420	3	3.0ˣ			19.5	7.5ˣ			65
	17	0.840ˣ			0.067	500ˣ			243	4	1.8ˣ			34.5	3.6ˣ			74.5
	24	1.023ˣ			0.078	496ˣ			260	4	0.9ˣ			38.0	3.9ˣ			83.5
	31	0.960ˣ								3	8.0*				20.0*			
3	0	0.44																
	2	1.49	0.63		0.41					2	4.2	18		23.5	4.35	37		20.5
	4	3.10				258				2	7.5				6.6			
		1.50ʸ				281ʸ				2	6.0ʸ				6.8ʸ			
		1.12ˣ				308ˣ				2	5.2ˣ				6.2ˣ			
4	0	0.088																
		0.273		0.134		414		158		2	3.3		10.5		0.0		41	
		0.806		0.154		424		324		3	1.8		37.5		10.0		75	
		1.266		0.160		500		177		1	2.0		18.5				35.5	
		2.813		0.300		368		333		2	4.8		33.5		6.0		53	
		0.595ˢ				520ˢ				1	2.3ˢ				2.5ˢ			
		1.303ˢ				513ˢ				2	3.2ˢ				5.0ˢ			
		2.510ˢ				368ˢ				3	4.4ˢ				4.5ˢ			

* = in 10 mM glucose medium.
ˣ = switched from 3.5 mM amobarbital on day of previous count.
ʸ = switched from 2 mM amobarbital on day 2.
ˢ = switched from 3 mM amobarbital on day 5.

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TABLE 2. Growth and Metabolism of Mouse Heteroploid Cells before, during and after Cultivation in Amobarbital Medium, Experiment 2

Growth Day	Cells × 10⁻⁶/Dish		Protein/Cell × 10⁶ (μg)		Glucose Uptake/Cell × 10⁶ (μM)		Lactate Output/Cell × 10⁶ (μM)		Period of Uptake or Output
	0 mM Amobarb.	3.5 mM Amobarb.	0 mM Amobarb.	3.5 mM Amobarb.	0 mM Amobarb.	3.5 mM Amobarb.	0 mM Amobarb.	3.5 mM Amobarb.	Days
0	0.213								
3	1.056		368		1.2		0.5		3
7	2.894	0.112	441	460	5.1	44.5	2.9	94.5	4
10	0.256	0.108	420	420	3.9	19.5	7.5	65	3
17	0.840	0.067	590	243	1.8	34.5	3.6	74.5	4
24	1.023	0.078	496	269	0.9	38.0	3.9	83.5	4
31	0.966				8.0*		20.0*		3

* = in 10 mM glucose medium.
↲ = change to amobarbital-free medium.

it appears that in the presence of 3.5 mM amobarbital little or no energy useful for growth was derived from the metabolized glucose. The last-mentioned cells, when switched to amobarbital-free medium, resumed a rate of growth similar to that of the controls, but the rate of lactate production remained as high as in the presence of amobarbital. The reason for this is not apparent. Possibly the presence of the anesthetic may have favored the survival of the more-highly-glycolytic members of the seed population. At the highest concentrations amobarbital may have interfered with enzyme systems in synthesizing pathways, consistent with the somewhat depressed complement of protein per cell. Interaction between anesthetic and protein is currently a subject of much interest.[4] A less likely explanation is that in high concentration amobarbital uncoupled glycolytic phosphorylation, in addition to the energy shortfall due to inhibition or uncoupling of oxidative phosphorylation. This idea was tested and apparently disproved by incubating erythrocytes, which are unable to oxidize glucose, with amobarbital. No effect on glycolysis was detected.

TABLE 3. Effects of Amobarbital Sodium on Two-day Metabolism of Glucose and Lactate (μM/cell × 10⁶)

	Control	In Amobarbital	Recovery
2 mM/l amobarbital			
Glucose uptake	4.2	18	6.0
Lactate output	4.4	37	6.8
3 mM/l amobarbital			
Glucose uptake	4.8	33.5	3.2
Lactate output	6.0	53	5.0
3.5 mM/l amobarbital			
Glucose uptake	5.1	44.5	3.9
Lactate output	2.9	94.5	7.5

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In this connection, a recent report by Shaw and Pace,[5] who experimented with the effects of anoxia and hyperoxia on the growth of cultures of Low-line fibroblasts, is of interest. Their cultures stopped growing when deprived of oxygen, but survived deprivation for eight days sufficiently to approach the control rate of cell multiplication soon after restoration to atmospheric air. As with the higher concentrations of amobarbital in the present experiments, the energy released by anaerobic metabolism was insufficient for growth but was sufficient to maintain viability of most of the cells as long as the increased demand for metabolic substrates (including glucose) was met. Hempling[6] has found that in suspensions of Ehrlich ascites tumor cells 2.1 mM amobarbital reduces respiration to 16.7 per cent of the control values, yet the ATP content of the cells is well maintained for at least 40 minutes provided glucose is present. Whether the multiplication rate of ascites cells is affected by amobarbital is not known, but Hempling did establish that amobarbital blocks 25 per cent of the potassium influx, which suggests that some of the available energy is no longer utilizable.

As to a bearing of the present results on metabolic effects of barbiturates *in vivo*, the possibility may seem remote, since a 2-mM concentration of amobarbital is ten to 20 times greater than that encountered in the sera of overdosed comatose patients.[7] However, it is well established that *in-vitro* cell systems vary in their susceptibility to growth inhibitors and a corresponding variability *in vivo* probably exists. Pomerat and co-workers[8] found that 0.6 mM amobarbital inhibited outgrowth of chick spinal-cord explants, but that a 1.2 mM concentration was required to inhibit growth of heart explants. Trowell[9] reported that 0.3 mM amobarbital killed more than half the lymphocytes in rat lymph-node cultures. That a reversible long-term *in-vivo* inhibition of growth may be obtainable with certain anesthetics is suggested by the reversible inhibition of myelopoiesis in man and the rat by nitrous oxide.[10] Regardless of the relevance of our results to *in-vivo* conditions, the value of barbiturates (and other anesthetics) as metabolic inhibitors in stored mammalian tissue deserves investigation.

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