Effect of Calcium Binding Substances on Rate and Duration of Narcosis. (23489)

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(Introduced by E. von Haam)
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Marked changes in the intracellular content of calcium have been shown to accompany narcosis induced by fat solvent anesthetics (1,2,3). Although a cellular loss of calcium with liquefaction of the cortical gel forms the basis of the currently popular colloidal chemical theory of anesthesia(3), to our knowledge no data exist on the effect of calcium binding substances on the induction and duration of narcosis. This report is the result of preliminary studies directed toward this end.

Materials and methods. One hundred and twenty wild-type male guppies (*Lebistes reticulatus*) weighing between 65 and 85 mg were kept in an aquarium in Ringer's solution at 25°C and fed Long Life Tropical Fish Food once daily. The experimental groups consisted of 20 fish each. After an experiment the groups were returned to the aquarium for a minimum rest period of 24 hours before further experiments were undertaken. Various experiments were carried out with a 50 mg% solution of sodium pentobarbital in Ringer's solution (NaCl 6.7 g, KCl 0.14 g, $CaCl_2$ 0.12 g) at 25°C (pH 6.5-6.9) modified with respect to the calcium level and the presence or absence of disodium ethylenediamine tetraacetate (EDTA) or sodium citrate. The following experimental groups were used: Group A (control) Ringer's solution (1 mM Ca); Groups B, H, and I Ringer's citrate (1 mM Ca, 1.3 mM citrate); Group C balanced Ringer's citrate (1 mM Ca, 0.6 mM citrate); Group D Ringer's EDTA (1 mM Ca, 2 mM EDTA); Group E balanced Ringer's EDTA (1 mM Ca, 1 mM EDTA); Groups F and G calcium-free Ringer's; and Group J Ringer's with excess calcium (10 mM Ca). Groups B, H, and I contained an excess of citrate ion theoretically capable of binding calcium ion in the fish, while Group C contained a balanced calcium citrate solution. Group D contained an excess of EDTA theoretically capable of chelating 1 millimol of calcium ion, while Group E contained a balanced solution of calcium EDTA.

Groups G and J were pretreated for 4 hours prior to narcosis. Group G fish were pretreated in calcium-free Ringer's solution and Group J in Ringer's with excess calcium. The solution in which Group G fish were pretreated was quantitatively analyzed for calcium ion after pretreatment by the method of Clark and Collip(4). Group H fish were placed in Ringer's citrate for 1 hour prior to the induction of narcosis. Group I fish were placed in Ringer's solution with pentobarbital for 7.5 minutes then transferred to Ringer's citrate with pentobarbital. The fish were placed in the various solutions and the time required for induction of narcosis was recorded. The endpoint for narcosis was taken when the fish lost purposeful motion and were unable to right themselves. Following narcosis the fish were removed from the pentobarbital solution and placed in Ringer's solution, where the period of recovery was observed and recorded.

Results. The data for induction and recovery of pentobarbital narcosis in the presence of various calcium binding substances are summarized in Table I. The true mean induction times in minutes with the standard error for the various groups are: (A) 14.9 ± .64, (B) 7.8 ± .45, (C) 15.2 ± .54, (D) 7.1 ± .43, (E) 10.7 ± .45, (F) 13.7 ± .73, (G) 28.6 ± .82, (H) 28.7 ± .88, (I) 16.1 ± .93, and (J) 13.9 ± .86. A comparison of the mean induction times for Groups B and D with A (control) shows that excess sodium citrate (B) shortened the induction time of narcosis by 47.7%, while excess disodium EDTA (D) shortened it by 52.3%. Essentially no change was observed in Group C, a balanced solution of calcium citrate, in con-

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TABLE I. Summary of Data for Induction and Duration of Narcosis in the Presence of Calcium Binding Agents and in Calcium-Free Ringer's Solution.

Group	Sol.: Ringer's containing 50 mg % pentobarbital and modified as follows: Ca (mM)	Citrate (mM)	EDTA (mM)	Pretreat. time (hr)	Induction time (min.)	% difference compared to control (A)	Recovery time (hr)	% increase compared to control (A)
A	1				14.9 ± .64†		1.1 ± .08†	
B	1	1.3			7.8 ± .45	−47.7	1.4 ± .13	27.3
C	1	0.6			15.2 ± .54	+ 2.0	1.2 ± .12	9.1
D	1		2		7.1 ± .43	−52.3	3.4 ± .22	209.1
E	1		1		10.7 ± .45	−28.2	2.2 ± .22	100.0
F					13.7 ± .73	− 8.1	1.6 ± .22	45.5
G				4	28.6 ± .82	+91.9	1.2 ± .13	9.1
H	1	1.3		1	28.7 ± .88	+92.6	1.2 ± .09	9.1
I*	1	1.3			16.1 ± .93	+ 8.1	1.4 ± .16	27.3
J	10			4	13.9 ± .86	− 6.7	1.1 ± .10	0

* Pretreated in 50 mg % pentobarbital Ringer's solution for 7.5 min.
† S.E.

trast to the 28.2% shortening of induction time by a balanced solution of calcium EDTA (E). There was only a minimal decrease in induction time with calcium-free Ringer's solution (F), while pretreatment in calcium-free Ringer's for 4 hours prior to narcosis (G) lengthened the induction time by 91.9%. Similar results were observed by pretreatment with citrate for 1 hour (H) where the induction time showed a 92.6% increase. Quantitative analysis of the solution after the 4 hours treatment period of Group G fish showed that an average of .55 mg of calcium was released by each fish. Pretreatment with pentobarbital for one-half the mean normal induction time, *i.e.* 7.5 minutes (I), or with Ringer's solution containing a 10-fold increase in calcium for 4 hours (J) only slightly altered the induction time.

The true mean recovery times in hours with the standard error for the various groups are as follows: (A) 1.1 ± .08, (B) 1.4 ± .13, (C) 1.2 ± .12, (D) 3.4 ± .22, (E) 2.2 ± .22, (F) 1.6 ± .22, (G) 1.2 ± .13, (H) 1.2 ± .09, (I) 1.4 ± .16, and (J) 1.1 ± .10. Marked prolongation of the mean recovery time was observed with Ringer's EDTA (D) which showed an increase of 209.1%, with balanced EDTA (E) which showed an increase of 100%, and with calcium-free Ringer's solution which showed a 45.5% increase.

Discussion. The presence of calcium binding agents(5,6) during pentobarbital narcosis shortened the period of induction by approximately 50%. While these results are generally in accordance with the concept that changes in calcium metabolism are of importance in narcosis(3), there are several findings which are of considerable interest.

Procedures capable of lowering the calcium level of the fish prior to narcosis by either pretreatment in Ringer's containing 1.3 millimols of citrate or in calcium-free Ringer's solution resulted in a prolongation of approximately 90% in the induction time. On the other hand, pretreatment of the fish for one-half the mean induction time of the control group in Ringer's solution with pentobarbital, prior to being transferred to Ringer's citrate with pentobarbital, resulted in essentially no change in induction time. Thus it appears that the synergistic effect of hypocalcemia on pentobarbital narcosis depends on the simultaneous introduction of both hypocalcemia and pentobarbital. The results observed with calcium-free Ringer's and Ringer's citrate both with and without pretreatment suggest that the rate of induction of hypocalcemia by diffusion or chemical binding may be of prime importance in altering the induction time of narcosis.

The failure of balanced calcium citrate solution to shorten the induction time and prolong recovery time to any extent in contrast to the shorter induction time and longer recovery time with balanced EDTA corroborates the observation of Bersin, *et al.*(7) that EDTA exerts a synergistic effect on barbiturate nar-

cosis. It is interesting to note that the excess EDTA solution, which was capable of chelating calcium, had a more pronounced effect on shortening induction time and prolonging recovery time than the balanced EDTA solution. The binding of calcium ions during the induction of narcosis by either citrate or EDTA resulted in a prolongation of recovery time. Thus, it appears that ionized calcium is of importance in the rate of recovery from narcosis. The anomalous results with reference to the recovery times observed when narcosis was induced in calcium-free Ringer's solution and following a 4 hour pretreatment period in this solution remain unexplained at the present time.

Pretreatment of the fish in calcium-free Ringer's solution resulted in a loss of calcium ions as shown by quantitative analysis. This is in accord with observations of Krogh(8) who showed that various ions may be removed by diffusion, primarily across the gill capillaries, when fish are placed in ion-poor solutions. The inability of Ringer's solution containing excess calcium to modify the induction of narcosis even after the 4 hour pretreatment period may indicate either that excess intracellular calcium has no effect on narcosis or that calcium diffuses across cellular membranes and becomes incorporated into the cell with great difficulty.

Summary. 1. The induction time of pentobarbital narcosis was shortened by 50% when carried out in the presence of calcium binding substances. 2. Removal of calcium by binding with citrate ion or pretreatment of the fish in calcium-free Ringer's solution prior to induction of pentobarbital narcosis resulted in a prolongation of the induction time (90%). 3. Balanced calcium EDTA solution shortened the induction time (30%) and lengthened the recovery time (100%) in spite of its theoretical inability to chelate calcium, while excess EDTA caused a greater shortening in induction time (50%) and lengthening in the recovery time (200%). 4. Excess calcium exerted no effect on induction or recovery of pentobarbital narcosis.

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Nutritional Requirements of *Micrococcus freudenreichii.** (23490)

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As a preliminary step in the investigation of certain enzyme systems in *Micrococcus freudenreichii,* it became desirable to grow the organism in a chemically defined medium which was capable of producing a high yield of cells. When a search of the literature failed to reveal the necessary information concerning the nutritional requirements of this organism, an investigation of these requirements was undertaken.

Materials and methods. Cultures and inocula. The culture employed in the studies was *Micrococcus freudenreichii* ATCC 8459. It was maintained on a stab in stock medium containing peptonized milk, 0.1%; tryptone, 0.1%; filtered tomato juice, 20%; and agar,

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