Table 1. Intestinal calcium transport response to 5,6-*trans*-25-OHD_3.

Compound	Amount (μg)	Animal		^{45}Ca serosal/ ^{45}Ca mucosal (mean ± S.E.)
		Condition	No.	
None	0	Normal	5	1.8 ± 0.2
5,6-*trans*-25-OHD_3	25	Sham-operated	5	4.4 ± 0.3
5,6-*trans*-25-OHD_3	25	Anephric	6	3.3 ± 0.3
25-OHD_3	25	Anephric	5	1.9 ± 0.3
25-OHD_3	25	Sham-operated	5	4.5 ± 0.8

Table 2. Calcium mobilization from bone in response to 5,6-*trans*-25-OHD_3.

Compound	Amount (μg)	Animal		Milligrams of Ca/ 100 ml of serum (mean ± S.E.)
		Condition	No.	
None	0	Anephric	6	4.3 ± 0.1
5,6-*trans*-25-OHD_3	25	Anephric	6	4.9 ± 0.1
25-OHD_3	0.25	Anephric	6	4.5 ± 0.1
1,25-$(OH)_2D_3$	0.25	Anephric	6	6.1 ± 0.1

vehicle. Two other groups (either sham-operated or bilaterally nephrectomized) received either 25 μg of 5,6-*trans*-25-OHD_3 or 25 μg of 25-OHD_3 intrajugularly in 0.05 ml of 95 percent ethanol. Sixteen hours later the rats were decapitated. The small intestines were removed for the measurement of intestinal calcium transport by the everted gut sac technique described by Martin and DeLuca (*14*).

For the measurement of calcium mobilization from bone, male weanling Holtzman rats were fed for 2 weeks with a diet adequate in calcium and phosphorus and deficient in vitamin D (*15*), and then a low calcium (0.02 percent) vitamin D–deficient diet for another 10 days (*13*). In some groups the rats were bilaterally nephrectomized and immediately after surgery were injected with either 25 μg of 5,6-*trans*-25-OHD_3, 0.25 μg of 25-OHD_3, or 0.25 μg of 1,25-$(OH)_2D_3$ dissolved in 0.05 ml of 95 percent ethanol. Controls received 0.05 ml of 95 percent ethanol vehicle. Twenty-four hours after the administration of the dose, the animals were killed by decapitation, and the blood serum was collected.

Serum calcium was determined with an atomic absorption spectrophotometer (Perkin-Elmer model 214). For this determination, serum samples (0.10 ml) were diluted with 1.9 ml of 0.1 percent $LaCl_3$.

The results in Table 1 demonstrate that 5,6-*trans*-25-OHD_3 is effective in stimulating calcium transport in the duodenum of vitamin D–deficient rats. More important, however, is the observation that the 5,6-*trans*-25-OHD_3, like 1,25-$(OH)_2D_3$ (*7*), is more active than 25-OHD_3 in stimulating intestinal calcium transport in bilaterally nephrectomized rats.

The major difference in the biological activity between 5,6-*trans*-25-OHD_3 and 1,25-$(OH)_2D_3$ is shown in Table 2. Like 25-OHD_3, the 5,6-*trans* analog has little effect in the mobilization of calcium from bone in anephric rats, as is demonstrated by only a small rise in the serum calcium, whereas 1,25-$(OH)_2D_3$ elicited a marked response. Furthermore, this *trans* analog showed little if any potential to induce bone resorption in fetal rat bone tissue culture (*16*), whereas 1,25-$(OH)_2D_3$ was extremely effective (*17*).

These unusual properties make the 5,6-*trans*-25-OHD_3 a promising drug for the treatment of calcium abnormalities associated with chronic renal failure. Furthermore, the biological activity of this analog provides additional evidence that a hydroxyl function must be present on C-1 of vitamin D compounds for the stimulation of intestinal calcium transport.

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References and Notes

1. A. Windaus and R. Langer, *Justus Liebigs Ann. Chem.* **508**, 105 (1934).
2. A. Windaus, H. Lettre, F. Schenck, *ibid.* **520**, 98 (1935).
3. T. Suda, R. B. Hallick, H. F. DeLuca, H. K. Schnoes, *Biochemistry* **9**, 1651 (1970); J. R. Roborgh and T. J. DeMan, *Biochem. Pharmacol.* **3**, 277 (1960); H. E. Harrison, H. C. Harrison, F. Lifshitz, *Parathyroid Hormone and Thyrocalcitonin (Calcitonin)* (Excerpta Medica Foundation, Amsterdam, 1968), pp. 455–460.
4. A. Verloop, A. L. Koeveet, E. Havinga, *Rec. Trav. Chim.* **74**, 1125 (1955).
5. H. H. Inhoffen, G. Quinkert, H. J. Hess, H. Hirschfeld, *Chem. Ber.* **90**, 2544 (1957).
6. D. R. Fraser and E. Kodicek, *Nature* **228**, 764 (1970).
7. R. W. Gray, I. T. Boyle, H. F. DeLuca, *Science* **172**, 1232 (1971).
8. I. Boyle, L. Miravet, R. W. Gray, M. F. Holick, H. F. DeLuca, *Endocrinology* **90**, 605 (1972); J. Omdahl and H. F. DeLuca, *Science* **174**, 949 (1971).
9. M. F. Holick, M. Garabedian, H. F. DeLuca, *Science* **176**, 1146 (1972).
10. P. F. Neville and H. F. DeLuca, *Biochemistry* **5**, 2201 (1966).
11. A. Verloop, A. L. Koevoet, R. Van Moorsellaar, E. Havinga, *Rec. Trav. Chim.* **78**, 1004 (1959).
12. *The United States Pharmacopeia* (Mack, Easton, Pa., 15th rev. ed., 1955), p. 889.
13. T. Suda, H. F. DeLuca, Y. Tanaka, *J. Nutr.* **100**, 1049 (1970).
14. D. L. Martin and H. F. DeLuca, *Amer. J. Physiol.* **216**, 1351 (1969).
15. G. Guroff, H. F. DeLuca, H. Steenbock, *ibid.* **204**, 833 (1963).
16. C. L. Trummel, L. G. Raisz, M. F. Holick, H. F. DeLuca, unpublished observations.
17. ———, *Science* **175**, 768 (1972).
18. Supported by PHS grant AM-15512 and the Harry Steenbock research fund.

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Development of Sensitivity to Tetrodotoxin in Beating Chick Embryo Hearts, Single Cells, and Aggregates

Abstract. *The spontaneous activity of intact embryonic heart becomes progressively more sensitive to tetrodotoxin block with increasing age of the embryo. The activity of isolated single heart cells in culture was relatively insensitive, independent of embryo age. Aggregates formed from single cells responded to tetrodotoxin in the same manner as intact hearts; aggregated cells from older hearts were sensitive.*

Tetrodotoxin (TTX) specifically blocks inward sodium current in many excitable tissues (*1*), including heart (*2*), and abolishes spontaneous activity in cells whose action potential is dependent on a transient increase in sodium conductance (*3*). Such a mechanism underlies action potential generation in the embryonic chick heart (*4*); the fast inward sodium current in adult heart tissue is blocked by TTX (*5*, *6*). We have examined the effect of TTX on the spontaneous beating of chick embryo heart preparations, namely, whole hearts, single isolated cells, and aggregates of dissociated cells. We have

come to the following conclusions: (i) Chick embryo hearts at 4 days of age are insensitive to TTX at 10^{-5} g/ml ($3 \times 10^{-5} M$). Sensitivity to TTX increases dramatically between days 4 and 7 and thereafter remains constant. (ii) Only a small fraction of isolated cells are sensitive to TTX, and this fraction does not increase with age. (iii) Aggregates formed from single cells are quite similar to intact heart in both their age-related and dose-related sensitivity to TTX.

Hearts were dissected from chick embryos aged 2 to 12 days. Whole hearts or atria (12-day only) were incubated in tissue culture medium $818B_1$ (*7*) at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2. Hearts were allowed to equilibrate for 30 to 60 minutes before the experiment was begun, and beating activity was observed (magnification ×10 to ×30).

Cultures were prepared after the dissociation of hearts into their component cells by the multiple-cycle trypsinization method (*7*). Plates were seeded with 2×10^5 cells per plate and incubated at 37.5°C in a water-saturated atmosphere of 5 percent CO_2, 10 percent O_2, and 85 percent N_2. Various media were used, but the majority of cultures were in 818A or 818B (*7*, *8*) containing either 1.3 or 4.5 m*M* potassium. After 24 hours of incubation, plates were washed and placed on the constant-temperature stage (37°C) of an inverted microscope, in an atmosphere identical to that in the culture incubator. Beating in single cells was determined as described (*7*, *8*).

Heart cell aggregates were prepared by the technique of Moscona (*9*), following the dissociation of the hearts into their component cells (*6*, *7*). Erlenmeyer flasks (25 ml) containing 5×10^5 cells in 3 ml of medium were gassed with 5 percent CO_2, 10 percent O_2, and 85 percent N_2, stoppered, and placed in a gyratory shaker bath (37°C) at 70 rev/min. The medium was 818A or 818B containing either 1.3 or 4.5 m*M* potassium. After 18 hours of gyration, the contents of each flask were transferred to a Falcon tissue culture dish and allowed to attach to the bottom of the dish during a 2-hour period in the culture incubator. The plates were washed and placed on a microscope stage as described above. The volumes of the aggregates were between 5×10^4 and 5×10^6 μm^3; an aggregate of 10^6 μm^3 contained approximately 200 cells.

Table 1 shows the percentage of hearts that continued to beat after successive 15-minute exposures (*10*) to TTX (*11*), 10^{-8} to 10^{-5} g/ml. Sensitivity to TTX increased with increasing embryo age. All hearts from 2- to 4-day embryos (5 2-day, 22 3-day, and 20 4-day) continued beating in the presence of TTX, 1.1×10^{-5} g/ml. In fact, six hearts from 4-day embryos incubated overnight in the presence of TTX, 1.1×10^{-5} g/ml, were observed to still beat regularly the next morning. Hearts from 5-day embryos were slightly sensitive to TTX, spontaneous activity was blocked in 9 out of 20 of these hearts in the presence of TTX, 1.1×10^{-6} g/ml. By day 7, 43 percent of the hearts stopped beating at TTX, 10^{-8} g/ml. No further increase in sensitivity was apparent in hearts from 12-day embryos. Although beating activity was scored 15 minutes after the addition of each dose of TTX, TTX was effective, in all three kinds of heart preparations under study, within the first 3 minutes or not at all. The effect of TTX was reversible; all affected hearts resumed beating after the drug was removed. The average beating rate of 27 untreated hearts from 6-, 7-, and 12-day embryos was 118 beats per minute. Fifteen minutes after the added TTX was washed out (an accumulated dose of 1.1×10^{-6} g/ml) the average beating rate was 109 beats per minute.

Table 1. The percentage of spontaneously beating chick hearts still beating after tetrodotoxin treatment. Data on 12-day hearts are from isolated atria, all other data are from whole hearts. Activity was scored 15 minutes after the addition of TTX (20 μl was added to 2 ml of medium). The initial dose of TTX was 10^{-8} g/ml, and successive doses were cumulative; that is, when the final dose of TTX was delivered, the medium contained 1.111×10^{-5} g/ml. The numbers in parentheses are the number of hearts used.

TTX (g/ml)	Percentage of hearts beating in embryos at day				
	2 to 4 (47)	5 (20)	6 (20)	7 (23)	12 (22)
10^{-8}	100	100	80	57	55
10^{-7}	100	75	50	4	9
10^{-6}	100	55	35	0	0
10^{-5}	100	40	15	0	0

Table 2 compares the TTX sensitivity of isolated heart cells and cell aggregates from embryos aged 4, 7, and 12 days. The major inhibition by TTX of single cell beating activity was apparent after successive doses of 10^{-8} and 10^{-7} g/ml, by which concentration approximately 30 percent of the beating cells were suppressed. Further doses to the final TTX concentration of 1.1×10^{-5} g/ml had little effect. Although the TTX sensitivity of heart cells from embryos aged 7 and 12 days was slightly greater than that of cells from embryos aged 4 days, the majority of single cells in culture did not share with their parent hearts the increase in TTX sensitivity with age. Aggregates, on the other hand, responded to TTX in a manner similar to that of whole heart. Some 78 percent of the cell aggregates from 4-day embryos remained beating in TTX at 1.1×10^{-5} g/ml, whereas only 18 percent of the aggregates from 7- and 12-day embryos were beating in TTX at 1.1×10^{-7} g/ml. Experiments on cell aggregates from the hearts of 5- and 6-day embryos indicate that TTX sensitivity increases during this period in a fashion similar to that of intact hearts. Beating activity was restored in 98 percent of the single cells and in 97 percent of

Table 2. The normalized percentage of single heart cells and aggregates still beating after treatment with tetrodotoxin. Heart cells and aggregates were cultured from 4-, 7-, and 12-day chick embryos. The data include cultures obtained from atria, ventricles, and whole hearts. The actual percentage of beating cells and aggregates were normalized and are expressed as percent of control. Control percentages were determined immediately before the experiment and averaged 38 percent for isolated cells and 99 percent for aggregates. The percentage of beating cells and aggregates were determined 15 minutes after the addition of TTX (20 μl added to 2 ml of medium). The initial dose of TTX was 10^{-8} g/ml, and successive doses were cumulative; that is, when the final dose of TTX was delivered, the medium contained 1.111×10^{-5} g/ml. Each determination on single cells represents a count of 100 cells in two to three random fields. Each determination on aggregates involved a count of all the aggregates on a plate. The average number of aggregates per plate was 109. All values are mean ± S.E.; *n* is 7 to 10 for single cells and *n* is 4 to 6 for aggregates.

TTX concentration (g/ml)	Age of embryos (days)					
	Single cells			Aggregates		
	4	7	12	4	7	12
10^{-8}	78 ± 2	83 ± 3	85 ± 5	93 ± 4	62 ± 8	47 ± 5
10^{-7}	77 ± 4	68 ± 3	75 ± 5	73 ± 7	18 ± 6	18 ± 5
10^{-6}	75 ± 6	66 ± 6	66 ± 7	78 ± 3	4 ± 1	7 ± 2
10^{-5}	78 ± 4	63 ± 4	68 ± 7	78 ± 7	6 ± 3	5 ± 1

the aggregates after TTX, 10^{-5} g/ml, was washed out.

We conclude that there is a dramatic increase in the TTX sensitivity of spontaneously beating whole hearts from chick embryos between days 4 and 7 (*12*). Similarly, the effectiveness of TTX in blocking electrically stimulated action potentials in chick heart ventricle has also been shown to be related to the age of the embryo (*13*). Tetrodotoxin is widely accepted as a specific inhibitor of the sodium channel in nerve and muscle (*6*, *14*), and yet is without effect on channels that can carry both sodium and calcium (*6*, *15*). The observed change with age in the sensitivity to TTX of hearts and aggregates is open to three interpretations: (i) The channels responsible for the early inward current of the action potential are not specific for sodium in young hearts, but the sodium specificity of these channels increases between 4 and 7 days of development. (ii) New sodium-specific channels appear between days 4 and 7. (iii) Sodium-specific channels are always present in hearts, but the access of TTX to these channels changes with development. It has been reported that cardiac tissue from 3- and 4-day embryos can be stimulated to generate action potentials in sodium-free medium (*13*), whereas cardiac tissue from later embryos (6-day and 19-day) are not excitable in 30 m*M* sodium (*4*). This evidence tends to favor an interpretation of the observations based on an increasing sodium specificity of the membrane channels rather than a change in accessibility to TTX.

An unexplained finding is the response of single cells as compared with aggregates. In most experiments single cells and aggregates were cultured from the same population of cells; hearts were trypsinized and dissociated into single cells, and then the cells were either plated or aggregated. The diminished sensitivity of isolated cells to TTX does not result merely from lack of cell-to-cell contact. Confluent monolayer sheets were also resistant to TTX, as has been noted (*16*). It may be that treatment with trypsin, or other culture procedures, alters the structure of the sodium channel at the membrane surface, and that this alteration results in the observed insensitivity to TTX. Monolayer cultured cells (singlets or confluent sheets) may be unable to restore the specificity of the sodium channels although reaggregated cells are capable of doing so. It is interesting that embryonic retinal cells in confluent monolayers lack the ability to respond to hydrocortisone induction of glutamine synthetase, whereas aggregates show an enzyme activity similar to that of intact tissue (*17*). Similarly, brain cell reaggregates have several enzymes with specific activities in the same range as in vivo embryonic brain, but much higher than in monolayers (*18*).

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References and Notes

1. T. Narahashi, J. W. Moore, W. R. Scott, *J. Gen. Physiol.* **47**, 965 (1964); Y. Nakamura, S. Nakajima, H. Grundfest, *ibid.* **48**, 985 (1965); B. Hille, *Nature* **210**, 1220 (1966).
2. S. Yamagishi and T. Sano, *Proc. Jap. Acad.* **42**, 1194 (1966); J. Dudel, K. Peper, R. Rudel, W. Trautwein, *Nature* **213**, 296 (1967); E. Coraboeuf and G. Vassort, *J. Electrocardiol.* **1**, 19 (1968).
3. F. Strumwasser, in *Physiological and Biochemical Aspects of Nervous Integration*, F. D. Carlson, Ed. (Prentice-Hall, Englewood Cliffs, N.J., 1968), p. 329; P. A. Mathieu and F. A. Roberge, *Can. J. Physiol. Pharmacol.* **49**, 787 (1971).
4. B. K. Yeh and B. F. Hoffman, *J. Gen. Physiol.* **52**, 666 (1968); G. M. Oliveira Castro and A. Paes de Carvalho, *Acta Physiol. Latinoamer.* **20**, 242 (1970).
5. O. Rougier, G. Vassort, D. Garnier, Y. M. Gargoüil, E. Coraboeuf, *Pfluegers Arch. Gesamte Physiol. Menschen Tiere* **308**, 91 (1969).
6. M. Tarr, *J. Gen. Physiol.* **58**, 523 (1971).
7. R. L. DeHaan, *Develop. Biol.* **16**, 216 (1967).
8. ———, *ibid.* **23**, 226 (1970).
9. A. A. Moscona, *Exp. Cell Res.* **22**, 455 (1961); D. Fischman and A. Moscona, in *Cardiac Hypertrophy*, N. Alpert, Ed. (Academic Press, New York, 1971), p. 125.
10. The cumulative dose technique allowed each heart to serve as its own control and permitted the use of fewer embryos at each age. Control experiments on hearts from 5- to 7-day embryos indicated that the effectiveness of any particular dose of TTX did not increase during a 1-hour observation period.
11. Tetrodotoxin (Sankyo) was obtained from Calbiochem and from G. Camougis.
12. In an article which appeared after the completion of our wo→ K. Shigenobu and N. Sperelakis [*J. Mol. Cell. Cardiol.* **3**, 271 (1971)] reported that the spontaneous activity of embryonic chick hearts 5 days old and younger was insensitive to TTX at a dose of 2×10^{-5} g/ml, while hearts from embryos aged 8 days and older were sensitive to TTX at a dose of 1 to 4×10^{-6} g/ml.
13. Y. Ishima, *Proc. Jap. Acad.* 44, 170 (1968).
14. J. W. Moore, M. P. Blaustein, N. C. Anderson, T. Narahashi, *J. Gen. Physiol.* **50**, 1401 (196→ B. Hille, *ibid.* **51**, 199 (1968).
15. D. Geduldig and D. Junge, *J. Physiol. London* **199**, 347 (1969); S. G. Chamberlain and G. A. Kerkut, *Comp. Biochem. Physiol.* **28**, 787 (1969); D. Geduldig and R. Bruener, *J. Physiol. London* **211**, 217 (1970).
16. N. Sperelakis and D. Lehmkuhl, *Amer. J. Physiol.* **209**, 693 (1965).
17. J. E. Morris and A. A. Moscona, *Science* **167**, 1736 (1970).
→ N. W. Seeds, *Proc. Nat. Acad. Sci. U.S.* **68**, 1858 (1971).
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Possible Mechanism for the Antiarrhythmic Effect of Helium in Anesthetized Dogs

Abstract. *Breathing a mixture of 75 percent helium and 25 percent oxygen instead of 75 percent nitrogen and 25 percent oxygen reduced the occurrence of dangerous cardiac arrhythmias after ligation of the circumflex coronary artery in open-chest dogs anesthetized with pentobarbital. In dogs not subjected to circumflex ligation, the sensitivity of blood pressure, heart rate, and extrasystoles to epinephrine injected intravenously was not altered by the substitution of helium for nitrogen; however, helium did reduce the baseline heart rate and the concentration of endogenous plasma catecholamines. The antiarrhythmic effect of helium may thus be mediated by changes in sympathetic activity.*

It was recently reported that helium protects anesthetized dogs from ventricular fibrillation after ligation of coronary arteries (*1*). We repeated this work with particular attention to the control of body temperature and of other variables known to influence ventricular arrhythmias. Of 14 dogs anesthetized with pentobarbital, six control dogs breathed a mixture of 75 percent nitrogen and 25 percent oxygen (N_2-O_2), and eight dogs breathed 75 percent helium and 25 percent oxygen (He-O_2), beginning 10 minutes prior to ligation of the circumflex coronary artery. After ligation, all animals were observed for 2 hours [or until ventricular fibrillation (VF) occurred] for changes in the electrocardiogram and arterial blood pressure. During this time, arterial blood gases and *p*H were maintained by administration of bicarbonate or by changes in mechanical ventilation (*2*).

Dogs breathing He-O_2 had fewer premature ventricular contractions (Table 1) and a lower incidence of ventricular fibrillation than did the dogs breathing N_2-O_2, three of which died from this arrhythmia 15 minutes after ligation. At the time of ligation, the He_2-O_2 and N_2-O_2 groups were similar