v/v), 95% ethanol, and 5% trichloroacetic acid[10]. Homogenate samples were then hydrolyzed for 2 h with 0.6 N KOH and RNA was removed by precipitation with 1.2 N perchloric acid. DNA was determined by the diphenylamine reaction of Burton[11] and spectrophotometrically estimated as the difference in absorbance at 610 and 650 nm. Significant differences between control and experimental animals were assessed by the Student's t-test.

Results. Body weights (table) for all methadone-treated animals were significantly less than controls, with experimental animals weighing about 80% of saline-injected rats. Brain weight (table) were reduced in rats treated during gestation (12%) or lactation (30%), but were comparable to control values in animals exposed during gestation and lactation. Brain DNA-values were reduced in all methadone-treated animals (table). Drug exposure during gestation decreased DNA content by 50%, while a 34% reduction was noted in offspring treated only during lactation. Animals in the gestation-lactation group had reduced (but not significant) DNA-values.

Weights and DNA-composition of whole brains from 21-day-old rats subjected to different schedules of methadone

Methadone treatment	Body weight (g)	Brain weight (g)	DNA (mg/brain)
Control	51.25 ± 1.18	1.61 ± 0.56	2.22 ± 0.03
Gestation	37.00 ± 0.47*	1.41 ± 0.07*	1.10 ± 0.00*
Lactation	40.58 ± 0.97*	1.12 ± 0.03*	1.46 ± 0.00*
Gestation-lactation	40.11 ± 3.14*	1.56 ± 0.02	2.14 ± 0.05

Values for weights represent means ± SE for 10 animals per group. DNA-values represent means ± SE for quadruplicate samples from 4 assays per group. * Significantly different from controls at $p < 0.05$.

Discussion. Perinatal exposure to methadone produces decreases in the amount of DNA present in the brains of drug-exposed rat pups, with significant reductions observed in animals treated during gestation or lactation. Since estimations of the DNA content in brain tissues provide a useful measure of cell number[12], this retardation in brain growth in methadone-exposed animals appears to be accompanied by a reduction in neural cells. At this time it is difficult to determine which neural cells are involved in these cell losses, however neurogenesis is nearly completed in the rat brain by day 21[13], so that these decreases in cell number probably include neuronal deficits. Although it is not known whether perinatal exposure to methadone in humans has the same deleterious effects as in rats, it is interesting to note that infants delivered by methadone-treated mothers have a retardation in body growth[3,4], head circumference measurements below normal[4], and behavioral abnormalities during the first 2 years of life[3].

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2 G. Blinick, R. Wallach, E. Jerez and B. Ackerman, Am. J. Obstet. Gynec. *125*, 135 (1976).
3 R. Ting, A. Keller and L. Finnegan, Proc. 2nd natl Drug Abuse Conf. (1975).
4 G. Wilson, Addict. Dis. *2*, 333 (1975).
5 W. F. Geber and L. C. Schramm, Am. J. Obstet. Gynec. *123*, 705 (1975).
6 A. Jurand, J. Embryol. expl Morph. *30*, 449 (1973).
7 M. Crofford and A. Smith, Science *181*, 947 (1973).
8 I. S. Zagon and P. J. McLaughlin, Biol. Neonate *31*, 271 (1977).
9 I. S. Zagon and P. J. McLaughlin, Expl Neurol. *56*, 538 (1977).
10 W. Schneider, J. biol. Chem. *161*, 293 (1945).
11 K. Burton, Biochem. J. *62*, 315 (1956).
12 J. Dobbing and J. Sands, Brain Res. *17*, 115 (1970).
13 J. Altman, in: Developmental Neurobiology, p. 197. Ed. W. A. Himwich. C. C. Thomas, Springfield, Ill. 1970.

Effects of palytoxin on the electrical activity of dog and rabbit heart[1]

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Summary. Reversible effects of palytoxin, extracted from colonies of the soft coral *Palythoa caribaeorum*, are described. There is a decrease of both membrane resting potential and overshoot during activity. Rise time of the action potential is prolonged, while repolarization is shortened. The electrical events resemble those seen with metabolic poisons.

Palythoa caribaeorum, a soft coral (coelenterate) found in shallow waters on the shores of the Caribbean Islands, contains a highly toxic substance which has been extracted, purified, and partially characterized[3,4]. These carnivorous animals hold their prey by protruding cell organelles (nematocysts) and by releasing their powerful toxin to paralyze their prey. Toxicity of various fractions of the crude extract is usually measured by noting the survival time of mammals[5,6] or small fishes[7]. Thus, the i. v. LD_{50} of a highly purified toxin is 0.025 µg/kg for rabbit[6]. The partially purified material available to us killed a dog when injected i. v. at a dose of 5 µg/kg[8]. My own interest in palytoxin started when it had become evident that ventricular trabeculae from poisoned dog hearts could be used for in vitro experiments of a different kind. The apparent phenomenon of reversibility of palytoxin effects prompted me to look for possible modes of action.

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3 D. H. Attaway, Doctoral thesis, University of Oklahoma, 1968.
4 M. Y. Sheikh, Doctoral thesis, University of Hawaii, 1969.
5 R. E. Moore and P. J. Scheiner, Science *172*, 495 (1971).
6 J. S. Wiles, J. A. Vick and M. K. Christensen, Toxicon *12*, 427 (1974).
7 S. García Castiñeiras, Doctoral thesis. University of Puerto Rico, 1976.
8 J. Santos Martínez, personal communication (1977).

Methods. Palytoxin had been partially purified according to procedures of García Castiñeiras[7]. The 70% acetone precipitable fraction was used. Rabbit papillary muscles or dog ventricular trabeculae having a diameter of 0.8–1.0 mm were superfused in a 5 ml bath by Tyrode solution at room temperature (26–28 °C). The flow rate was increased to 10 ml/min when solutions were changed. Stimuli were applied at a rate of 0.5/sec through a concentric needle electrode (Disa, Copenhagen). Conventional microelectrode recording was used.

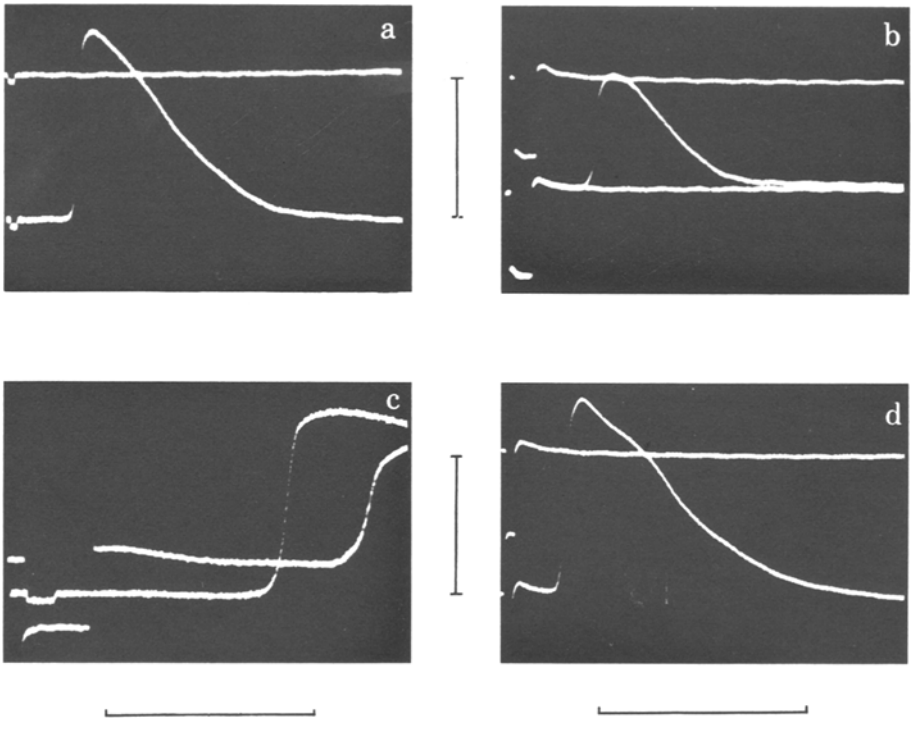
Results. The figure is taken from a sequence of records obtained with a rabbit papillary muscle. A concentration of the toxin-containing fraction was used (28 µg/l) that resulted in marked effects on the membrane potential and on the other hand did not damage the preparation in an irreversible way. Assuming a mol. wt of the toxin of 3300 (3), this concentration would correspond to less than 4×10^{-9} moles/l. For 15 min there was practically no change (not shown in figure), after which time a) threshold for excitation rose continuously, b) resting potential decreased considerably, c) 'overshoot' decreased less pronouncedly, d) rise time lengthened, while e) total duration of action potentials shortened. 30 min after starting the superfusion with palytoxin-containing solution, all-or-nothing excitation failed, at a resting potential of 45 mV. Washing with toxin-free solution resulted in recovery. The first conducted action potential appeared 7 min following the change to toxin-free solution. Values close to the initial ones were re-established at the end of a total washing time of 30 min. The only parameter, which consistently did not recover its pre-palytoxin value, was the threshold for excitation. This may be explained by damage inflicted to the fibres closest to the electrode by the very strong pulses applied during the time of conduction failure.

A 4fold lower concentration of the toxin had practically no effect when applied for 30 min. A 4fold higher concentration lead to changes that were irreversible over an observation period of 55 min in toxin-free Tyrode solution. 1 out of 6 preparations treated by the typical concentration of 28 µg/l was left in contact with the toxin for an additional 10 min after having become inexcitable. In this case, there was but partial recovery. Clearly, both exposure times and concentrations were highly critical if reversible effects were to be obtained.

Discussion. Palytoxin is known to cause contracture of both smooth and skeletal muscle[9]. Cardiac failure has tentatively been ascribed to a pronounced vasoconstriction of the coronary vessels[10]. Whether ischemic cardiac tissue or tissue poisoned by palytoxin has originally been reanimated for in vitro experiments, must remain an open question. Nevertheless, the present experiments provide some clue as to the possible mode of action of palytoxin on excitable cells. It is noteworthy that the effects take a comparatively long time to develop and to disappear; this may mean either that the substance has to cross the cell membrane and act at an intracellular site, or that the substance has a membrane effect which does not become apparent until intracellular concentrations of ions have had time to change. There seems to be no specific effect on Na^+ channels (as e.g. with tetrodotoxin), since the decrease of the rate of rise of the action potential (depending on Na influx) can be looked upon as a consequence of Na^+ channel inactivation by progressing membrane depolarization.

A specific increase of K^+ current leading to repolarization[11], or a specific decrease of the slow inward current carried by Ca^{++}[12], would account for the shortening of the action potentials, yet additional assumptions would be required to explain the decrease of resting potential. In an attempt to think of other agents leading to a similar combination of findings, one is reminded of substances interfering with metabolism, e.g. dinitrophenol[13], cyanide[14], or oxygen lack in a glucose-free medium[15].

9 J. del Castillo, personal communication (1977).
10 P. N. Kaul, Proc. 4th Food-Drug from the Sea Conf., p. 311. Marine Technology Society Press 1976.
11 G. Isenberg, Nature *253*, 273 (1975).
12 H. Reuter and U. Wollert, Naunyn Schmiedebergs Arch. *258*, 288 (1967).
13 W. C. De Mello, Am. J. Physiol. *196*, 377 (1959).
14 W. V. McFarlane, Circulation Res. *8*, 47 (1960).
15 T. F. McDonald and D. P. MacLeod, J. Physiol. *229*, 559 (1973).



Action potentials of a rabbit papillary muscle. *a* Control in Tyrode solution, *b* with palytoxin, 29 min, immediately preceding conduction block, *c* control record and 22 min palytoxin record superimposed, both taken at higher speed, *d* after a washing period of 30 min in palytoxin-free solution. Voltage calibration: from zero reference level to 70 mV, inside negative. Time calibrations: 100 msec for *a*, *b* and *d*; 25 msec for *c*.

In this connection it is of interest that O_2-uptake by Ehrlich ascites tumor cells is not affected by palytoxin at a concentration of 2 mg/l[16]. An inhibition of membrane ATPase as a mode of action of the toxin seems unlikely[7]. While the crude alcohol extract contains an inhibitor of Na-K-activated ATPase, the bulk of this substance, which has been identified as serotonin[17], is extracted into acetone in a following isolation step, while the precipitate (containing the toxin) has no inhibitory action on Na-K-activated ATPase[7].

Clearly, other methods than microelectrode recording of electrical events would now be required. The question of entrance of the toxin into the cells might be solved, especially since a passive distribution of the positively charged molecule[3,6] would have to result in accumulation within the cells. Measurements of ionic fluxes and ionic concentrations might eventually lead closer to an understanding of the mode of action of this and other toxins found in coelenterates.

16 A. M. Preston, personal communication (1977).
17 S. Garcia-Castiñeiras, J. I. White and E. Toro-Goyco, Molec. Pharmac. *13*, 181 (1977).

Direct toxic effect of isoproterenol on cultured cardiac muscle cells[1]

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Summary. Isoproterenol at relatively high doses (2.5 mg/ml) has a marked toxic effect on rat heart muscle cells cultivated in vitro. This effect is not prevented by propranolol and therefore is not mediated by beta adrenergic receptors.

Catecholamines administered in large doses can induce myocardial damage. A marked cardiotoxic effect is shown by isoproterenol, a synthetic catecholamine with a selective action on beta receptors[2]. Isoproterenol-induced myocardial necrosis resembles the ischemic necrosis induced by vascular occlusion and has been widely used as a model of 'infarct-like' lesion[3–6]. However, there is no direct evidence for an ischemic pathogenesis of the isoproterenol effect. Reports on coronary vascular changes and thrombosis after injection of isoproterenol[7] have not been confirmed[8]. The current interpretation is that myocardial damage by isoproterenol is due to its strong inotropic and chronotropic action which causes an increased oxygen demand by the heart muscle. This cannot be met by improved blood supply because the drug reduces systemic blood pressure by means of peripheral vasodilatation[8]. An alternative interpretation is that catecholamines exert a direct toxic effect on myocytes. The recent demonstration of myocardial necrosis induced by isoproterenol in the isolated perfused rat heart gives support to this view[9]. We have used cardiac muscle cells in culture for studying the direct cardiotoxic effect of isoproterenol.

Explants of newborn rat heart were grown in gelatin-coated plastic dishes containing Dulbecco medium with 20% horse serum under a continuous flow of 90% air–10% CO_2. Cells dissociated by trypsinization from newborn rat heart were also cultured under the same conditions. 1 week after plating, spontaneous rhythmic contractions could be observed in different areas of the cultures. Isoproterenol at a concentration of 0.01 mg/ml, or higher, increased the frequency of beating. However, at doses of 2.5 mg/ml, or higher, contractions soon became irregular and stopped completely and irreversibly after a few min. In the following h, cardiac cells underwent cell death, as shown by trypan blue exclusion test, and detached from the bottom of the dishes. Small pieces of newborn rat heart incubated for 2 h in culture medium in the presence of 2.5 mg/ml of isoproterenol did not show any sign of growth when transferred to normal medium in culture dishes.

The toxic effect of isoproterenol on myocardial cultures was not prevented by propranolol, a beta blocking agent. In fact, pieces of newborn rat heart preincubated with propranolol at 0.5, 1.5 or 2.5 mg/ml for 15 min before addition of isoproterenol showed no growth in culture. By contrast, propanolol alone did not interfere significantly with cardiac cell proliferation. These findings indicate that the cardiotoxicity of isoproterenol in this in vitro system is not mediated by beta adrenergic receptors. It is possible that oxidation products similar to adrenochrome are responsible for the direct toxic effect of isoproterenol, as suggested by Yates and Dhalla[9]. We have observed a rapid shift in the absorption spectrum of isoproterenol after incubation at 37 °C in the oxygenated culture medium, with a decrease of the 205 nm peak and an increase of the 225 nm peak within 10 min of incubation. These results on in vitro systems support the view that a direct cardiotoxic effect may contribute to the pathogenesis of myocardial necrosis induced by isoproterenol in vivo. Indeed, it has been reported that the isoproterenol effect in vivo is only partially prevented by propranolol[10]. The demonstration of extremely rapid permeability alterations of the sarcolemmal membrane in cardiac muscle cells after infusion of norepinephrine or isoproterenol[11] is also consistent with this interpretation.

1 This work has been supported in part by a grant from the Muscular Dystrophy Association, Inc. to Prof. M. Aloisi.
2 G. Rona, C. I. Chappel, T. Balazs and R. Gandry, Archs Path. *67*, 443 (1959).
3 G. Rona and D. Khan, Rev. Can. Biol. *22*, 241 (1963).
4 G. Zpinden and R. Bagdon, Rev. Can. Biol. *22*, 257 (1963).
5 V. Ferrans and R. Hibbs, Am. Heart J. *68*, 71 (1964).
6 Z. Csapo, J. Dusek and G. Rona, Archs Path. *93*, 356 (1972).
7 G. E. Cox and B. C. Wexler, Fed. Proc. *27*, 413 (1968).
8 D. Khan and G. Rona, Ann. N. Y. Acad. Sci. *156*, 285 (1969).
9 J. Yates and N. Dhalla, J. molec. cell. Cardiol. *7*, 807 (1975).
10 B. C. Wexler, Atherosclerosis *18*, 11 (1973).
11 M. Boutet and I. Hüttner, Lab. Invest. *34*, 482 (1976).