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STUDIES OF FETAL MOUSE HEARTS IN ORGAN CULTURE: INFLUENCE OF PROLONGED EXPOSURE TO TRIIODOTHYRONINE ON CARDIAC RESPONSIVENESS TO ISOPROTERENOL, GLUCAGON, THEOPHYLLINE, ACETYLCHOLINE AND DIBUTYRYL CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE[1]

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ABSTRACT

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Previous experiments have demonstrated that long-term exposure of isolated fetal mouse hearts to high concentrations of triiodothyronine increases the atrial rate response to submaximal doses of norepinephrine. To explore possible mechanisms of the hyperresponsiveness and, specifically, to test whether it is unique for a particular type of drug or is a generalized phenomenon, dose-response curves were determined for a variety of agents that alter heart rate, including several that are thought to alter intracellular levels of cyclic 3′,5′-adenosine monophosphate. The responses of atrial rate to theophylline, glucagon and acetylcholine were similar in hearts exposed for 2 days to triiodothyronine and in hearts of matched litter mates maintained in control medium. In contrast, responsiveness to submaximal concentrations of isoproterenol was significantly enhanced in triiodothyronine-treated hearts. Dibutyryl cyclic 3′,5′-adenosine monophosphate did not cause tachycardia in any group. It is concluded that high levels of triiodothyronine do not cause hypersensitivity to all chronotropic agents. Rather, of the drugs tested, there is an increase in responsiveness only to agents that specifically stimulate the *beta* receptor. Accordingly, it is suggested that cardiac hyperresponsiveness to catecholamines during prolonged exposure to triiodothyronine *in vitro* may be the result of altered numbers or characteristics of the myocardial *beta* receptor and or changes in the intracellular processes specifically related to *beta* stimulation.

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Whether or not thyroid hormones cause supersensitivity to various cardiotonic agents has remained controversial for many years. Definitive answers have been difficult to obtain, in part because the induction of thyrotoxicosis *in vivo* causes secondary alterations in neural and humoral factors that may obscure the primary effects on the heart. A second problem has been that thyrotoxic hearts generally operate at different base-line levels than do control hearts, which makes comparisons of subsequent changes in function difficult, if not impossible, to interpret.

Recently, at the expense of introducing a less physiological system than exists *in vivo*, an effort to minimize these difficulties was made by turning to an organ culture system. Under appropriate conditions in organ culture isolated hearts of late-fetal mice can be maintained in a reasonably stable functional state for several days (Wildenthal, 1971). The unique advantages of organ culture in this regard are that when the isolated hearts are exposed to high concentrations of thyroid hormone, manifestations of "thyrotoxicosis," including tachycardia and arrhythmias, develop gradually over a period of several days just as *in vivo* (Wildenthal, 1972), yet the confusing features of secondary neural and humoral changes are obviated. Furthermore, there exists a time in the development of the abnormal state when early manifestations of dysfunction (*i.e.*, arrhythmias) have already appeared in many hearts, even though the average heart rate has not yet increased significantly above control levels (Wildenthal, 1972). Thus the hearts can be tested at a time when the complicating influence of differing base-line rates can be avoided. With organ culture it has been possible to demonstrate that exposure of isolated fetal hearts to high concentrations of triiodothyronine for 2 days causes a significant shift of the atrial rate response to norepinephrine (Wildenthal, 1972).

To test whether the observed change is specific for norepinephrine or is a generalized phenomenon for all chronotropic agents, a similar model was used in the present experiments to test atrial sensitivity to a variety of other drugs. Special attention was focused on acetylcholine, because of its physiological importance, and on several chronotropic agents that, like norepinephrine, have been suggested to act by increasing intracellular levels of cyclic 3′,5′-adenosine monophosphate (cyclic AMP).

Methods

Intact, spontaneously beating hearts from 19 and 20 day fetal mice were isolated and maintained in organ culture as described in detail previously (Wildenthal, 1971). Briefly, the hearts were removed aseptically from the fetuses and placed on stainless steel grids at the liquid-air interface of shallow culture chambers. They were incubated at 37.2 ± 0.5°C in an atmosphere of 95% O_2 + 5% CO_2. Control hearts were cultured in Medium 199 (Grand Island Biological Company, Grand Island, N.Y.) supplemented with 50 μg/ml of insulin and 0.5 μmol/ml of sodium octanoate, and matched litter mates were maintained under the same conditions in an identical medium to which 5×10^{-7} or 5×10^{-6} M *l*-triiodothyronine (Mann Research Laboratories, New York, N.Y.) had been added. The hormone was dissolved in 1 ml of 0.1 N NaOH before being added to the medium, and an equal volume of NaOH was added to the control medium. After incubation either for 2½ to 3 hours or for 2 days, the culture chambers were transferred to a controlled-temperature water bath in room air where, over the next 5 to 10 minutes, a dose-response curve to one of several drugs was determined. Each drug was tested on a minimum of eight control hearts and eight hearts cultured at each concentration of triiodothyronine. The drugs tested were: acetylcholine HCl, 10^{-7} to 10^{-2} M (Sigma Chemical Company, St. Louis, Mo.); tyramine HCl, 10^{-6} to 10^{-3} M (Sigma); theophylline, 10^{-6} to 10^{-2} M (Calbiochem, Los Angeles, Calif.); glucagon, 10^{-9} to 10^{-5} M (Sigma); dibutyryl cyclic 3′,5′-adenosine monophosphate (dibutyryl cyclic AMP), 10^{-6} to 10^{-2} M (Calbiochem); and *l*-isoproterenol HCl, 10^{-9} to 10^{-5} M (Winthrop Laboratories, New York, N.Y.). The lowest concentrations used were determined in preliminary studies to be less than those necessary to produce minimal responses in heart rate, and the highest concentrations were found to cause maximal responses to all conditions.

Determination of dose-response curves was accomplished by procedures that have been described in detail in previous publications (Wildenthal, 1972, 1973). Briefly, the original medium was replaced by fresh medium that had been prewarmed to 37°C and buffered to pH 7.4 in room air with $NaHCO_3$. After a 1-minute stabilization period, the base-line beating rate was counted through a dissecting microscope and recorded to the nearest multiple of 10 beats/min. Then the medium was replaced by an identical one supple-

mented with the lowest concentration of the drugs, and after another 1-minute stabilization period the new steady-state rate was counted. Subsequently, consecutively increasing concentrations of the drug were tested in an identical fashion.

In the process of counting the beating rate, atrial rhythm was also monitored and noted. In some experiments atrial arrhythmias (defined as two or more irregular contractions during a 30-second observation period) were present in the control state and/or after exposure to the drug being tested, but atrial fibrillation was never observed. Tachycardia in response to a chronotropic agent always developed gradually and progressively over a period of several seconds after application of the drug, which suggests that the faster rates caused by the drugs were in no instances due to the onset of a regular paroxysmal tachycardia from an aberrant focus. For all drugs, results were similar whether or not the hearts that exhibited arrhythmias were excluded from calculations of mean beating rates.

Results

As observed previously (Wildenthal, 1972), the base-line beating rates of all groups of hearts cultured for 2 days were similar in the presence and in the absence of thyroid hormone. The mean atrial rate in 45 hearts maintained in the control medium for 2 days was 171 ± 7.4 (S.E.M.) beats/min. In 41 hearts exposed to 5×10^{-7} M triiodothyronine for the same period the rate was 178 ± 10.2, and in an equal number maintained at a concentration of 5×10^{-6} M it was 167 ± 8.5. As before, arrhythmias which occurred in the control period were more common in hearts that had been exposed to the hormone: the incidence was 9% in control hearts, 24% in the presence of the lower dose of triiodothyronine ($.1 > P > .05$ by the chi-square analysis), and 29% with the higher concentration ($P < .05$).

Acetylcholine induced bradycardia in all hearts. The dose-response curve was similar in groups maintained in the presence or absence of triiodothyronine (fig. 1). Neither the responses to the threshold concentration of acetylcholine nor those to the concentration that produced a half-maximal effect were altered significantly by exposure to thyroid hormone. All hearts developed standstill at an acetylcholine concentration of 10^{-2} M.

Both theophylline (fig. 2) and glucagon (fig. 3) caused tachycardia in all hearts and, as with acetylcholine, the responses to the drugs were not altered by thyroid treatment. Throughout the dose-response curves, absolute changes in

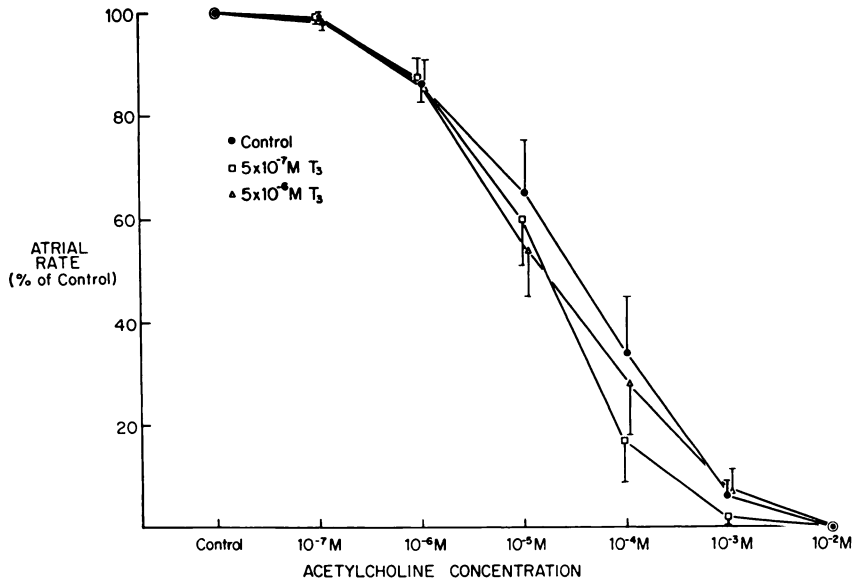

FIG. 1. Dose-response curves from isolated hearts maintained for 2 days in the presence or absence of triiodothyronine (T_3). The graphs relate changes in spontaneous atrial rates to the concentration of acetylcholine. Vertical bars represent ±1 S.E.M. There were no statistically significant differences at any point.

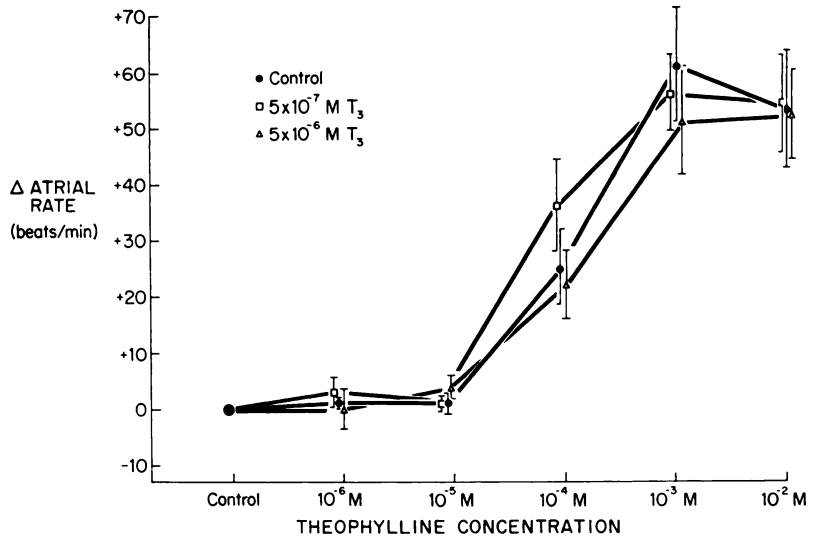

FIG. 2. Dose-response curves from isolated hearts maintained for 2 days in the presence or absence of triiodothyronine (T_3). The graphs relate changes in spontaneous atrial rates to the concentration of theophylline. Conditions and symbols as in figure 1. There were no significant differences among any of the groups.

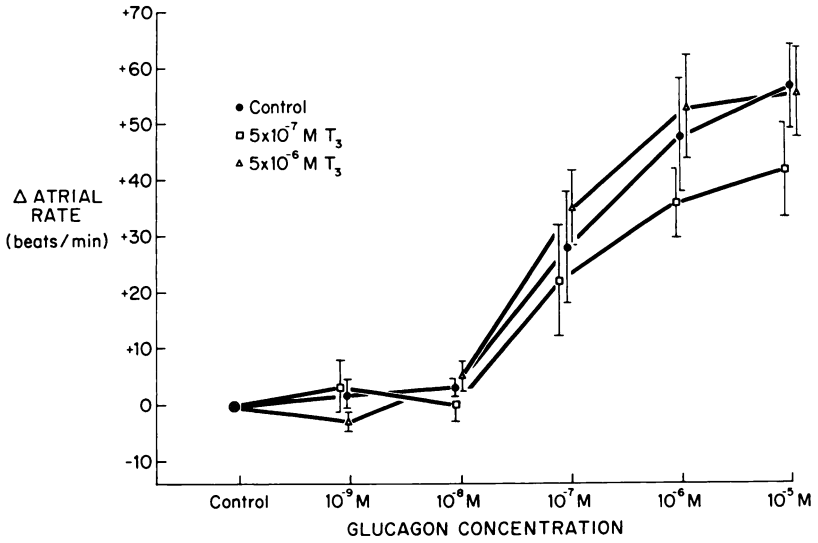

FIG. 3. Dose-response curves from isolated hearts maintained for 2 days in the presence or absence of triiodothyronine (T_3). The graphs relate changes in spontaneous atrial rates to the concentration of glucagon. Conditions and symbols as in figure 1. There were no significant differences among any of the groups.

heart rate were similar in control hearts and in hearts exposed for 2 days to 5×10^{-7} or 5×10^{-6} M triidothyronine.

In contrast, the dose-response curve to isoproterenol was significantly shifted in hearts exposed to very high concentrations of thyroid hormone (fig. 4). The absolute increase in atrial rate at maximal concentrations of isoproterenol was identical in all groups but, at submaximal doses, hearts previously exposed to 5×10^{-6} M triiodothyronine developed significantly higher rates. At 10^{-8} M isoproterenol, the atrial rate of

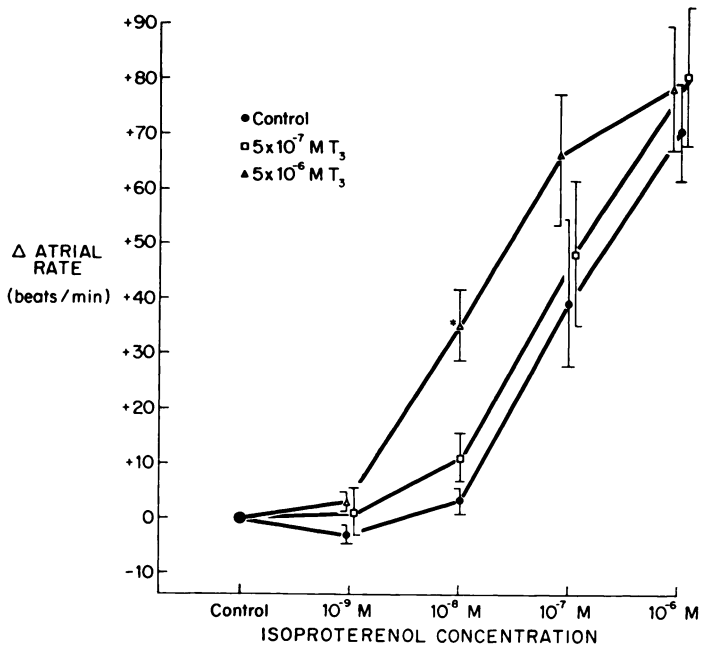

FIG. 4. Dose-response curves from isolated hearts maintained for 2 days in the presence or absence of triiodothyronine (T_3). The graphs relate changes in spontaneous atrial rates to the concentration of isoproterenol. Conditions and symbols as in figure 1. * Significantly different from control hearts ($P < 0.01$ by Student's t test for unpaired samples).

control hearts rose by 3 ± 3 beats/min ($P > .20$ by paired t test); in hearts exposed to 5×10^{-7} M triiodothyronine, atrial rate increased by 11 ± 4 ($P < .05$), and the same concentration of isoproterenol caused an increase of 35 ± 6 ($P < .01$) beats/min after a 2-day exposure to 5×10^{-6} M triiodothyronine. The latter change was significantly greater than the response of control hearts at the same concentration of isoproterenol ($P < .01$ by Students' t test for grouped data).

Absolute changes in rate might have a different meaning when a regularly firing pacemaker alters its frequency of firing than when an irregularly firing, aberrant focus appears. Accordingly, the results with isoproterenol were evaluated further by analyzing separately the data from hearts which maintained regular rhythm both during base-line conditions and throughout exposure to submaximal concentrations of isoproterenol. This subset of hearts revealed changes which were similar to those that occurred in the entire group (*i.e.*, statistically significant differences existed at 10^{-8} M isoproterenol), suggesting that the apparent supersensitivity to the drug was not simply a function of greater susceptibility to arrhythmias.

To test whether the changes induced by triiodothyronine might have been the result of nonspecific or toxic actions of the chemical used (Lee *et al.*, 1965), rather than due to the characteristic delayed action of the hormone, dose-response curves were tested in hearts maintained for 2½ to 3 hours in organ culture. The results are illustrated in figure 5. No differences were apparent in control hearts as compared to those exposed to triiodothyronine for only a few hours. This suggests that the subsequent change at 2 days was indeed a function of the usual, slowly developing effect of the hormone. Similar tests were made of the influence of acetylcholine, glucagon and theophylline after 2½ to 3 hours' incubation of the hearts. In all instances, the dose-response pattern was similar in control and treated hearts.

Finally, the influence of dibutyryl cyclic AMP, which causes tachycardia in isolated hearts under some circumstances, was tested in control and thyroid-treated hearts in culture. As was observed earlier (Wildenthal, 1973), no significant

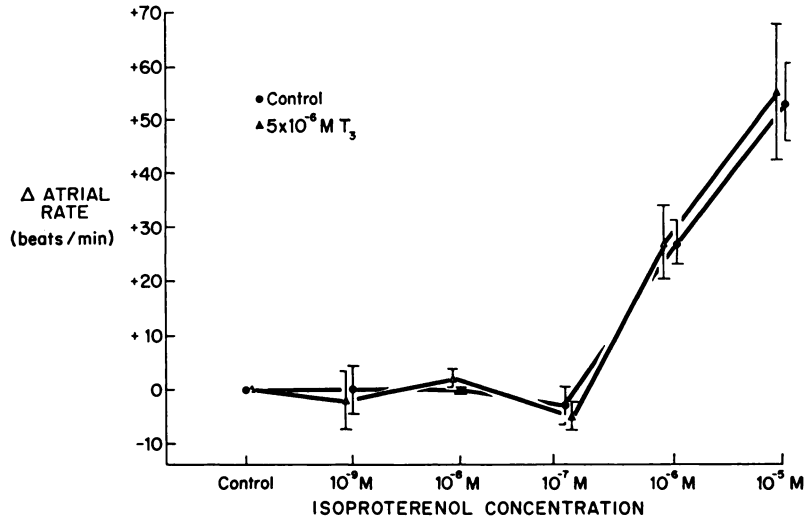

FIG. 5. Dose-response curves from isolated hearts maintained for 2½ to 3 hours in the presence or absence of triiodothyronine (T_3). The graphs relate changes in spontaneous atrial rates to the concentration of isoproterenol. Symbols as in figure 1. Each group contained six hearts. There were no significant differences at any point.

change in heart rate occurred after exposure of 10^{-4} to 10^{-2} M dibutyryl cyclic AMP in hearts maintained in culture for 2 to 3 hours. Similarly, hearts cultured for 2 days failed to increase their beating rate, either in the presence or absence of triiodothyronine.

Discussion

Previous evaluations of the influence of thyroid hormones on cardiac responsiveness to pharmacological agents have usually been made with hearts of animals that have been thyrotoxic *in vivo* for several days or weeks (McDonald *et al.*, 1935; Wise and Hoff, 1938; Brewster *et al.*, 1956; Harrison, 1964; Cravey and Gravenstein, 1965; Margolius and Gaffney, 1965; van der Schoot and Moran, 1965; Aoki *et al.*, 1967; Cairoli and Crout, 1967; Frazer and Hess, 1969; Anton and Gravenstein, 1970; Heimbach and Crout, 1972). This approach has two drawbacks that tend to limit its usefulness. First, use of intact animals makes it impossible to separate fully the actions of the hormone that are attributable to direct effects on the myocardial cells from actions that might be mediated indirectly by secondary alterations in neural activity or changes in circulating levels of other hormones or substrates. Second, since the hearts of thyrotoxic animals are already functioning at a significantly altered base-line level, even if they are removed from the animal and evaluated *in vitro*, subsequent changes from base line are difficult if not impossible to relate to the control state. This latter factor has been formulated by Wilder (1950) as the "Law of Initial Values."

Wilder argued that as a general rule the apparent functional response to a given agent "depends to a very large extent on the initial level of . . . function." Thus, if the initial level of function (*e.g.*, heart rate) is already high, further stimulation will tend to yield relatively less of an increase than would the same stimulation at a normal initial level. Conversely, the apparent effect of depressant agents will tend to be magnified if the initial level of function is abnormally high or minimized if it is abnormally low.

To avoid the difficulties inherent in the use of standard approaches, experiments were made on hearts made "thyrotoxic" *in vitro*. Such a study is not practicable with the techniques that are conveniently used for acute experiments *in vitro*, since the preparation usually deteriorates before the delayed effects of the hormone become manifest (Skelton *et al.*, 1973), but organ culture provides a satisfactory long-term system (Wildenthal, 1972). Such a system inevitably possesses unphysiological characteristics, of course. Nevertheless, such characteristics are shared

equally by control and experimental hearts, so that comparisons within the system should remain valid.

Experiments with this preparation indicate that atrial rate responsiveness to acetylcholine is not significantly increased after exposure of the hearts to high concentrations of triiodothyronine for 3 days. This conclusion is in agreement with that of Frazer and Hess (1969), who analyzed dose-response curves of isolated hearts of rats made thyrotoxic *in vivo*. On the other hand, when the effect of vagal nerve stimulation is tested in intact thyrotoxic animals, there is an apparent reduction in the degree of cardiac slowing compared to that in normals (Cairoli and Crout, 1967; Frazer and Hess, 1969). This diminished effect is not a consequence of increased acetylcholinesterase activity (Frazer and Hess, 1969). It has been suggested that it may be due to a reduction in the amount of acetylcholine released by vagal impulses, but this has not yet been demonstrated. It has also been held that vagal inhibition by atropine produces less of an increase in heart rate in hyperthyroid patients than in normal individuals, which might suggest that base-line vagal inhibition of heart rate is reduced in thyrotoxicosis (Heimbach and Crout, 1972). As Hall (1972) has pointed out, however, this latter finding could be explained at least in part by the operation of the Law of Initial Values.

In contrast to the results with acetylcholine, administration of graded doses of isoproterenol yielded significantly different responses in heart muscle cultured in the presence of high concentrations of triiodothyronine, compared to matched control heart muscle maintained in the absence of the hormone. The dose-response curve relating spontaneous atrial rate to the concentration of isoproterenol was shifted significantly to the left, with no change in the maximal rate that was ultimately achieved. This result is identical to that previously observed in the same experimental system for norepinephrine (Wildenthal, 1972). Early studies of pharmacological sensitivity in hyperthyroidism had also suggested that supersensitivity to catecholamines might occur *in vivo* (McDonald *et al.*, 1935; Wise and Hoff, 1938; Brewster *et al.*, 1956; Harrison, 1964), but several recent, well controlled studies have disputed this claim (Cravey and Gravenstein, 1965; Margolius and Gaffney, 1965; van der Schoot and Moran, 1965; Aoki *et al.*, 1967; Anton and Gravenstein, 1970). The extent to which conclusions drawn from such studies should be tempered by consideration of the complexities of secondary neurohumoral factors and the Law of Initial Values remains unclear, just as does the extent to which results from fetal hearts exposed to thyrotoxic concentrations of triiodothyronine can be extrapolated meaningfully to situations *in vivo*.

Be that as it may, the present data provide further evidence that at least one kind of functioning myocardial tissue gradually develops supersensitivity to some chronotropic agents during prolonged exposure to elevated levels of triiodothyronine. To determine whether this is true only for specific agents or is a general phenomenon for all interventions that raise atrial rate, substances whose mechanisms of action differ from that of norepinephrine and isoproterenol were also tested. In particular, since cyclic AMP has been suggested to be important in mediating the chronotropic effects of catecholamines (Sutherland *et al.*, 1968), attention was focused on agents that, like catecholamines, are thought to increase the intracellular levels of that substance. Glucagon, which activates adenylate cyclase through a receptor that is apparently separate from the *beta* receptor (Epstein *et al.*, 1971), was observed to cause similar rate increases in control and thyrotoxic hearts. Likewise, there was no demonstrable hypersensitivity to theophylline, an agent that among its other actions can inhibit phosphodiesterase (Sutherland *et al.*, 1968). Dibutyryl cyclic AMP itself had no chronotropic effect on control or thyroid-treated hearts in organ culture, despite the fact that it does enter cells and induce glycogenolysis (Wildenthal, 1973).

Thus, of the drugs tested, only agents that specifically stimulate the *beta* receptor appear to produce different responses in normal and thyrotoxic hearts. The shift in dose-response characteristics that occurs could be explained by increased numbers of *beta* receptors, increased affinity for the drug by the receptors or alterations in the secondary cellular responses induced by receptor stimulation. Differentiation among these various possibilities offers an interesting area for further investigation.

In addition, it is of interest that responsiveness to isoproterenol (but not the other chronotropic

drugs) was greater in hearts cultured in control medium for 2 days than in those maintained *in vitro* for only a few hours (figs. 4 and 5). A similar increase in responsiveness after long-term cultivation has been reported previously for norepinephrine (Wildenthal, 1972). In that instance, it was assumed that the altered reactivity of the hearts could be explained at least in part by a gradual degeneration of sympathetic nerve endings, loss of the ability to inactivate norepinephrine by neural uptake and resultant development of "denervation hypersensitivity." However, isoproterenol is not inactivated by neural uptake and denervation does not cause hypersensitivity to it (Trendelenburg, 1966; Dempsey and Cooper, 1968). Thus, the present study indicates that another mechanism is operative in mediating the increased effect of catecholamines in fetal hearts maintained for long periods *in vitro*. Unlike the effect of thyroid treatment, the effect of cultivation *per se* is both to increase the maximal responses to isoproterenal and norepinephrine and to change the concentrations of the drugs at which threshold and half-maximal responses occur. This difference suggests that the two interventions may alter catecholamine sensitivity by separate cellular mechanisms. It seems likely that studies of the mechanism of development of "cultivation hypersensitivity" may provide a valuable new approach to help elucidate the cellular processes that mediate the actions of catecholamines on the heart.

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