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INITIAL INVESTIGATIONS INTO ADRENALINE ACCUMULATION AND ADRENERGIC RESPONSIVENESS IN CULTURED NEONATAL RAT HEART CELLS

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1 Accumulation of tritiated adrenaline (and/or its products) has been studied by a variety of techniques in viable, attached and free-floating myoblasts cultured from neonatal rat heart cells.

2 Accumulation increased with the number of cells used, the culture age of cells, incubation time and the concentration of adrenaline in the incubation mixture.

3 Accumulation-concentration data were not linearly correlated with dose-response data.

4 Accumulation did not correlate well with β-adrenoceptor responsiveness in a number of situations of varying responsiveness.

5 Accumulation was not blocked by a variety of uptake blocking agents or α- and β-adrenoceptor antagonists.

6 A much smaller accumulation process was observed in fibroblasts which respond to adrenaline with reversible morphological changes.

Introduction

The distribution and accumulation of catecholamines in tissue has been extensively investigated and both neuronal and extraneuronal uptake systems have been described (Iversen, 1973). The occurrence of such uptake systems, together with their associated binding sites, can complicate attempts to identify and extract catecholamine receptors (Iversen, 1975). Initial attempts to identify the β-adrenoceptor by binding studies were frustrated by the occurrence of non-specific binding sites (Cuatrecasas, Tell, Sica, Parikh & Chang, 1974) but recent experiments with more specific β-adrenoceptor labels have met with greater success (see Iversen, 1975 for references). There are, therefore, two main aims in investigating catecholamine distribution and binding in whole tissue and cellular fragments: the isolation of β-adrenoceptors and the identification of amine distribution and uptake systems.

Few attempts have been made to account for the total distribution and binding of catecholamines in a homogeneous tissue containing β-adrenoceptors and although objections can be raised to the use of cultured cells as physiologically suitable tissue for such studies, the binding of catecholamines to cultured chick heart cells has been investigated (Lefkowitz, O'Hara & Warshaw, 1973; 1974) as has the uptake and metabolism of catecholamines in isolated smooth muscle cells (Powis, 1973). In view of such studies it seemed worthwhile to study the distribution and binding of adrenaline in cells cultured from neonatal rat hearts with a view to correlating the information so obtained with pharmacological responses in the same tissue. This study, therefore, describes attempts to demonstrate adrenaline accumulation in myoblast and fibroblast cultures obtained from neonatal rat heart and to correlate such data with adrenaline responses under varying conditions (age of culture, exposure time to adrenaline, presence of other drugs). A preliminary report of this work has been published (Walker, 1974).

Methods

Cell culture

Cell cultures were prepared by a variation on a method due to Harary & Farley (1963). Ventricles from 5-day-old neonatal rats were diced (1 mm cubes) and subjected to trypsin digestion. Digestates, collected at 20 min intervals, were added to bovine serum at 4°C before a final harvest of cells by centrifugation (200 *g* for 3 minutes). Harvested cells were washed and suspended in a tissue culture medium

of the following composition: 20 ml F-10 (Colorado Serum Co.), 4 ml foetal bovine serum (Microbiological Assoc.), 1 ml antibiotic solution (containing 6.2 mg penicillin-G, 10 mg streptomycin) diluted to 100 ml with Hanks' basic salt solution (without KCl or $NaHCO_3$). The final cell suspension was placed in Falcon flasks and left for 2 h at 37°C in order to allow fibroblasts to become attached to the flask surface: the myoblast-rich suspension could then be decanted off for culture in separate flasks. Variations in initial incubation periods, and number of transfers allowed mainly (at least 95% pure) myoblast, or fibroblast, cultures to be obtained.

Measurement of pharmacological responses in cultures

Myoblasts. The beating activity of single (or networks) of myoblasts cells was monitored by a photoelectric device similar to that of Schanne (1970) with photoresistors measuring optical density variations in television images of cells or cellular networks. Output was displayed on a Grass Polygraph and processed to give an index of beating rate (Grass Tachograph) and the first differential of optical density variation (Tetronix Type O operational amplifier).

Drug responses of cultures were obtained by either continuously monitoring single cells during the addition of drug (cumulative or single dose) or by sampling the activity of 8–10 cells for each concentration of drug in a number of cultures.

Fibroblasts. Adrenaline exposure induced morphological changes in fibroblasts which consisted of shape changes and a general reduction in cell area. Changes varied with dose such that, with 10^{-4} mol/l adrenaline, fibroblasts had the appearance of a cytoplasmic sack wrapped tightly around the nucleus whilst the area previously occupied by the cell body was left littered with apparent cellular debris, some connected to the cell. These events took 15–20 min to develop fully and only reversed hours after adrenaline removal.

Similar changes could be induced in fibroblasts by dibutyryl cyclic adenosine 3',5'-monophosphate (AMP) at concentrations above 10^{-4} mol/litre. There was a tendency towards similar morphological changes with myoblasts but they were much less noticeable.

Accumulation of radio-labelled adrenaline, water and inulin

The accumulation of the above substances was studied according to the methods outlined in Table 1.

Attached cells. Portions of flasks with growing cells attached were cut into slides 45 × 15 mm and incubated in the following media: 40% N-16 medium, 60% Hanks' basic salt solution with 0.28 g/100 ml bovine serum albumin (Sigma). This medium was chosen as omission of serum ensured reproducible adrenaline figures whilst protein was required for cell survival.

Table 1 Method of studying isotope distribution in cultured heart cells

Primary culture (myoblast or fibroblast) from 4-5-day-old neonatal rats

I	II
Adhering cells	*Free-floating cells*
(15 × 45 mm slides cut from flask)	*(petroleum jelly-treated flasks)*
Myoblasts or fibroblasts	50,-200,000 Cells (80–90% myoblast)
(60 min in control incubation media at 25°C)	
Incubate slide at 25°C	Isotopically labelled incubation media ^3H and ^{14}C labels mixed or with appropriate controls — Incubate in 10 ml at 25°C
Either: (1) Transfer (2) Drain in air (3) Wash in control media at 4°C (15 s)	Filter and wash with control media (2 × 10 ml at 4°C)
(All counted in Aquasol)	

Isotope distribution studied in free-floating or attached myoblasts and on attached fibroblasts. Cells, either on slides or in free suspension, were incubated for a control period prior to addition of appropriate isotope(s) and subsequent varied procedures to account for, and reduce, counts due to physical contamination.

At the concentration used, albumin was assumed to bind 22% (±)-adrenaline at 10^{-9} mol/l (Powis, 1974).

After equilibrating for 1 h, slides were rapidly transferred to media containing [^{3}H]-(±)-adrenaline (New England Nuclear, N.E.N. 047), [^{14}C]-carboxylinulin (New England Nuclear, NET-164) or ^3H_2O (New England Nuclear, NET-001B) either as dual labels or as appropriate matched controls. Isotopes were generally used at equi-isotopic concentrations whilst, if necessary, the concentration of adrenaline was adjusted with non-labelled drug before incubation at 25°C.

At the end of the required incubation period, slides were either washed in a controlled manner in isotope and drug-free media, drained in air, or immediately immersed in scintillation fluid. Counting was performed in Aquasol (New England Nuclear) on a Model 3380 Packard Liquid Scintillation Spectrometer using internal standards and channels ratio methods; results were expressed as d/minute. Inulin and tritiated water determinations allowed free adrenaline carried over with the different techniques to be estimated but, where slides were air-dried, evaporation invalidated the tritiated water figure. The above methods allowed an estimate of the excess counts due to binding or cellular accumulation of adrenaline, or its derivatives, to be made and such estimates were performed on both myoblast and fibroblast cultures.

Free-floating cells. Free-floating viable myoblasts could be produced by culturing cells in vaseline-coated (~0.5 mm thick) Falcon flasks. Cells so grown would attach and grow if transferred to control flasks but with such techniques, only 20% of the original cells were viable (dye exclusion) after 5 days. Free-floating cells were harvested by gentle centrifugation (220 *g* for 2 min) and suspended in incubation media for accumulation studies.

Accumulation was measured by a filtration technique similar to those described for binding studies. Portions of medium (10 ml) containing 0.2–1.0 × 10^6 cells were incubated with the appropriate isotopes and then vacuum filtered through 47 mm diameter mixed cellulose esters filters (Millipore HAWP). The filter, plus contents, were washed in a standardized manner with 2 × 10 ml portions of incubation medium (total wash time = 15 s) before being placed in 10 ml of counting solution; counts were corrected to d/minute.

Expression of accumulation of adrenaline. From inulin and tritiated water distribution the amount of adrenaline present by contamination, on a slide or cells, could be found. Excess counts for adrenaline therefore represented adrenaline and/or its derivatives either bound to, or accumulated within, cells and, as no differentiation was made between these two sites, the excess was expressed as moles of adrenaline accumulated per unit number of cells (usually 10^6).

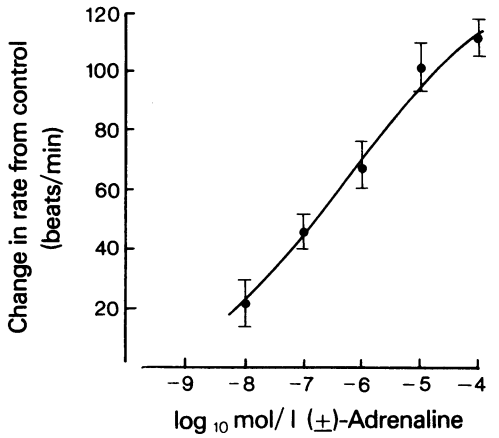

Figure 1 The chronotrophic response of cultured heart cells to (±)-adrenaline. Varying concentrations of (±)-adrenaline were added cumulatively to different cultures containing beating heart cells. Rate was recorded electronically (Grass tachograph) from single cells by monitoring optical density changes with contraction cycles. Each point is the mean rate from 6–8 cells. Vertical lines show s.e. mean.

Results

Adrenaline responsiveness in cultured neonatal rat heart cells

As reported elsewhere (Walker & Au, 1975) adrenaline responsiveness in cultured neonatal rat heart cells develops over a period of time to reach an effective maximum within 3.5 days (double exponential with $T_{\frac{1}{2}} = 0.95 \pm 0.13$ day for slowest process). A dose-response curve for adrenaline (using 4-day-old myoblasts) is shown in Figure 1, where each point represents the mean of determinations from five different cultures with different cells monitored for each dose in every culture. There was little evidence of a true response maximum being reached with the above technique. With cumulative dose-response curves obtained on single cells, indications of maxima were sometimes seen but the mean curve from such data showed no such levelling-off.

The rate response to adrenaline in incubation media faded with time of exposure (half response lost in 40–50 minutes). This possibly represented breakdown of adrenaline, as fresh drug added after 100 min exposure restored the response to the original. The receptor involved in this chronotropic response was investigated with propranolol and phentolamine (10^{-8} to 10^{-5} mol/l); the latter was without effect

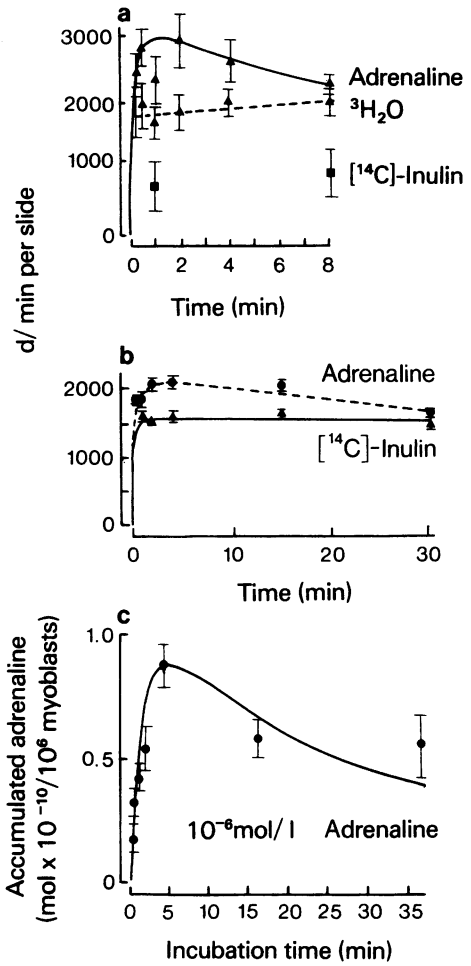

Figure 2 Accumulation of tritiated (±)-adrenaline by cultured heart cells with non-drained slides (a), with drained slides (b) and expressed as accumulated adrenaline (c). Viable cells on Falcon plastic slides were exposed to 10^{-6} mol/l [^{3}H]-(±)-adrenaline together with [^{14}C]-inulin or alone with ^3H_2O. After varying exposure periods, cells were either removed and counted immediately (a) or drained in a standardized manner in air (b). Using the latter technique the excess d/min due to accumulated adrenaline (and/or its derivatives) could be readily expressed as mol adrenaline per 10^6 myoblasts. All points are the mean of 6 determinations. Vertical lines show s.e. mean.

but propranolol antagonism was found. The development of antagonism with culture age was slower than that of adrenaline responsiveness (see Walker, 1977), despite a constant pA_2 value of 7.9 (calculated from Schild plots).

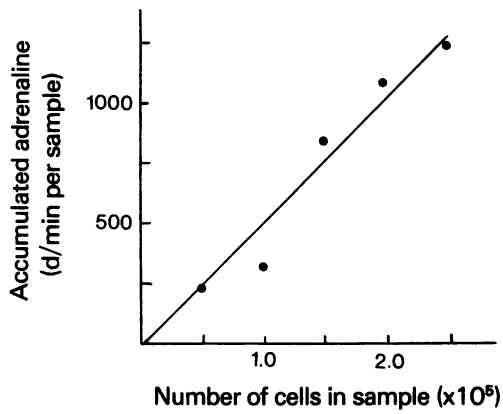

Figure 3 Accumulation of tritiated (±)-adrenaline on free-floating myoblasts as a function of the number of cells incubated. Various numbers of free-floating cells (see Methods) were incubated for 5 min with [^{3}H]-(±)-adrenaline at 10^{-6} mol/l in the incubation media described in Methods. After incubation, cells were filtered on a Millipore filter, washed, and the filter, together with contents, dissolved in Aquasol for estimation of tritium count by scintillation counting. Each point is the mean of 3 determinations.

Isotope accumulation in myoblasts

Non-drain distribution studies with isotopically labelled adrenaline, water and inulin on slide-attached cells showed (Figure 2a) that adrenaline accumulation occurred at 10^{-6} mol/litre. However, the large error inherent in the method, together with its poor reproducibility, led to inulin and adrenaline distribution studies in which slides were allowed to drain. In Figure 2b an example of [^{3}H]-(±)-adrenaline (10^{-6} mol/l) so accumulated with incubation time is shown while in Figure 2c the actual accumulated adrenaline figures are given. Similar excess counts for adrenaline were also obtained with the free-floating filtration technique and in this case the accumulation of adrenaline was a linear function of the number of cells in the incubation medium (Figure 3).

Attempts were also made to determine the rate of adrenaline accumulation and, with the drain technique, this (Figure 2c) was found to be fairly rapid reaching a maximum within 8 minutes. However, the results in Figure 2b and c were subject to the errors inherent in the draining procedure and whilst exclusion of the drain showed faster accumulation rates the larger errors inherent in this methodology prevented accurate quantitation. Accumulation rates for free-floating cells subject to filtration and the washing procedure described in the Methods section was essentially that of drained slides.

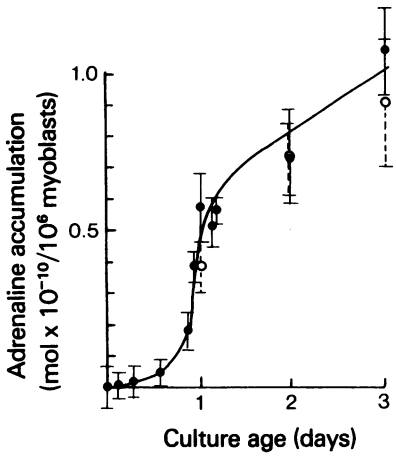

Figure 4 The growth in [^{3}H]-(±)-adrenaline accumulation capacity with culture age. Cells were cultured for varying times either as free-floating cells (●) or attached to the surface of flasks (○). After appropriate growth periods, the accumulation of [^{3}H]-(±)-adrenaline was determined, as in the previous figures, for 10^{-6} mol/l adrenaline and a 2 min incubation time. Points represent the mean of 5 determinations. Vertical lines show s.e. mean.

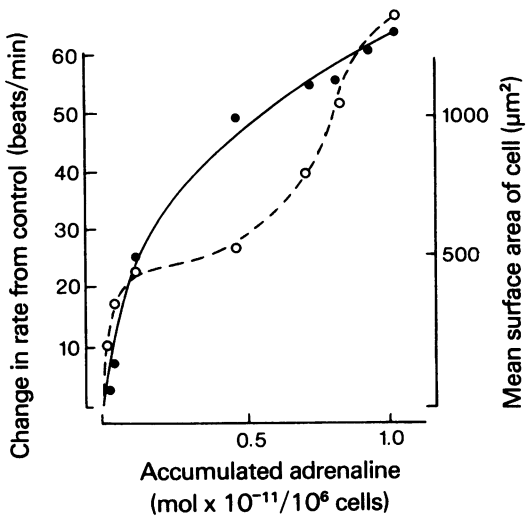

Figure 5 Correlations between the accumulation of [^{3}H]-(±)-adrenaline on attached cells and the rate response to adrenaline (●) or the mean surface area of such cells (○). Accumulation of [^{3}H]-(±)-adrenaline at 10^{-6} mol/l for various ages of culture were plotted against the mean beating rate increase induced by 10^{-6} mol/l adrenaline (left ordinate scale) or the mean surface area of cells (50 cells) (at different culture ages) as determined by planimetry from T.V. images (right ordinate scale).

Where adrenaline was applied instantaneously (within 1 s) to single cells under continuous observation the mean time to maximum response in 12 cells from different cultures was 42 ± 6.3 (s.e.mean) ($T_{\frac{1}{2}}$ approx. 10 s) which was much faster than the accumulation process. In a similar manner the rate of loss of adrenaline response on adrenaline wash-out could be measured and its $T_{\frac{1}{2}}$ was again approximately 10 seconds. Myoblasts exposed to 10^{-6} mol/l adrenaline for 2.0 min accumulated 0.8 (±0.10) × 10^{-10} mol adrenaline/10^6 cells (n = 5) and this fell within 15 s of transfer to adrenaline-free media to 0.37 ± 0.01 whilst the corresponding figure for 25 s was 0.14 ± 0.01. These temporal relationships were not investigated further.

As noted previously, adrenaline responsiveness grew in an exponential manner with culture age while similar growth curves could be constructed for adrenaline accumulation (Figure 4). Growth in accumulation capacity correlated non-linearly with the growth in adrenaline responsiveness (rate) and surface area increases with age (Figure 5). The accumulation-responsiveness relationship was such that with 10^{-6} mol/l adrenaline the first 0.1 × 10^{-10} mol/10^6 cells growth in accumulation capacity was accompanied by a 22.5 beat/min increment in response. After 3 days of culture the same increment in accumulation was associated with a 2.5 beat/min rate increase. The correlation between accumulation and the mean surface area of cells was biphasic and non-linear.

Response and accumulation were also investigated in terms of dose-response-accumulation relationships. The accumulation-concentration curve shown in Figure 6 was obtained with varying specific activity adrenaline (NEN stock suitably diluted) at an incubation time of 2 min and can be compared with Figure 1. Accumulation capacity saturated at adrenaline concentrations of 10^{-4} mol/l from which a K_m of 8 × 10^{-5} mol/l can be interpolated for the accumulation process which compares with the extrapolated K_D value of at least 10^{-4} mol/l from Figure 1. If accumulation and response are causally related, Figures 1 and 6 would suggest that log accumulation of adrenaline gives a linear rate response.

Further comparisons between response and accumulation were attempted in terms of the effect of the following drugs: propranolol, tranylcypromine, pargyline, phentolamine and 17-β-oestradiol (Table 2). With a variety of concentrations and incubation conditions, propranolol only inhibited accumulation at obviously toxic concentrations despite a pA_2 of 7.9 in the same cells, whilst phentolamine and pargyline had no effect. High concentrations of tranylcypromine produced some inhibiton as did 17-β-oestra-

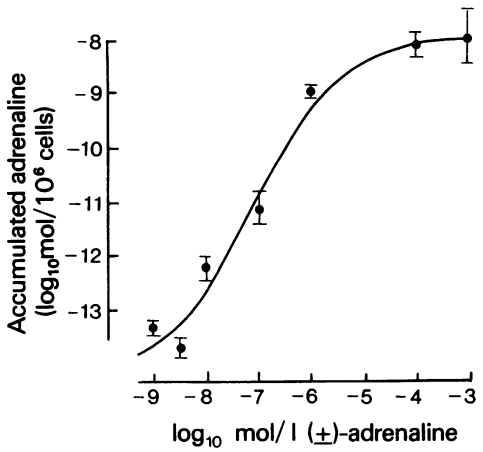

Figure 6 Accumulation capacity for [^{3}H]-(±)-adrenaline in 5-day-old cultured heart cells. The accumulation capacity of slide attached cells was determined for various concentrations of tritiated (±)-adrenaline (varying specific activity) in 5-day-old cells incubated for 2 minutes. The drained slide-inulin blank method (see Methods) of determining accumulation was used. Each point is mean of 6 to 10 determinations. Vertical lines show s.e. mean.

diol at the highest concentrations compatible with solubility (5×10^{-6} mol/litre). Of all the above drugs tested for inhibitory effects on accumulation, only propranolol was found to alter significantly the chronotropic responses to adrenaline.

In the experiments considered above, no consistent correlation between adrenaline accumulation and responsiveness was found. During the above studies, fibroblast slides, prepared according to Methods, were occasionally used as blanks to ensure that myoblast accumulation of adrenaline was not a technique artefact representing only non-specific cell accumulation. In these experiments, it was noted that fibroblasts not only microscopically responded to high concentrations of adrenaline with morphological changes but also accumulated adrenaline at concentrations producing morphological effects. The accumulation-concentration curve for fibroblasts is shown in Figure 7 from which it can be seen that this accumulation was easily saturated and of small total capacity compared with myoblast. The maximum accumulation of 5×10^{-11} mol/10^6 fibroblasts at 10^{-4} mol/l adrenaline is almost three orders of magnitude less than that of myoblasts (1×10^{-8} mol/10^6 cells). Accumulation in Figure 7 was measured at 2 min, the time generally used for myoblast measurements, but the process in fibroblasts was much slower for there was an increasing accumulation of radioactivity for at least 15 min (Figure 8).

Attempts to compare accumulation with physiological responses in fibroblasts were difficult owing to difficulties in quantifying such responses. As indicated previously, the response of fibroblasts to adrenaline consisted of a drawing-up process which took 15–20 min to develop and occurred over a dose range of 10^{-6} to 10^{-3} mol/litre. The most marked morphological changes occurred with higher doses

Table 2 Drug effect on adrenaline distribution in cardiac cell cultures

Drug	*Drug conc. (mol/l)*	*Adrenaline conc. (mol/l)*	*Pre-incub. time (min)*	*Incub. time (min)*	*Technique & culture type*	*% Inhib. of binding*	*°C*	*No. of expt.*
(±)-Propranolol	10^{-6}–10^{-5}	10^{-9}–10^{-6}	0–10	2–20	SFF*	0	25	5
(±)-Propranolol	10^{-4}	10^{-8}	0–10	2–10	SD**	0	37	2
(±)-Propranolol	10^{-3}	10^{-8}–10^{-6}	0–10	2	SD	100	25	2
(10^{-3} mol/l propranolol toxic to cells producing vacuolization and disruption of cells whilst 10^{-4} mol/l propranolol produces morphological changes in cells within 20 min)								
Tranylcypromine	10^{-4}	10^{-9}	10	2	SD	100	25	2
Tranylcypromine	10^{-4}	10^{-6}	10	2	SD	80	25	2
Tranylcypromine	10^{-5}	10^{-9}	10	2	SFF	0	25	2
Tranylcypromine	10^{-6}	10^{-9}	10	2	SFF	0	25	2
17-β-Oestradiol	5×10^{-6}	10^{-9}	10	2	Slide	20	25	2

Pargyline and phentolamine had no effect on adrenaline [10^{-8} mol/l] accumulation at concentrations up to 10^{-4} M.

* = Slide and free-floating; ** = Slide-drain.
Myoblasts were preincubated with drug before addition of tritiated (±)-adrenaline. Control slides contained no drug and % inhibition was found by difference. Each experiment consisted of 5 slides or 5 cell samples.

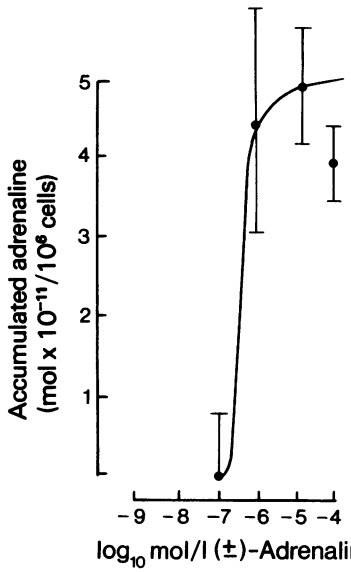

Figure 7 Accumulation capacity for [^{3}H]-(±)-adrenaline in slide-attached fibroblasts. The drained slide-inulin blank method was used to assess adrenaline accumulation in fibroblasts attached to flasks. Measurements were made after 2 min incubation with adrenaline. Points represents the mean of 5 to 6 determinations. Vertical lines show s.e. mean.

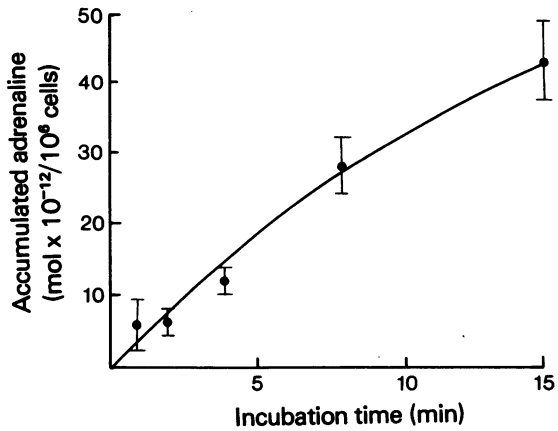

Figure 8 Accumulation of [^{3}H]-(±)-adrenaline with varying incubation times in fibroblasts. The drained slide-inulin blank method was used to measure adrenaline accumulation in fibroblasts incubated for various times with 10^{-4} mol/l [^{3}H]-(±)-adrenaline. Points represents the mean of 5 determinations. Vertical lines show s.e. mean.

such that 10^{-4} to 10^{-3} mol/l gave a complete response as far as could be gauged subjectively. Planimetric measurements were attempted in an endeavour to quantify the response but such efforts were not very successful owing to the variation in response occurring between cells. Subjective evaluation, however, did show that the response of fibroblasts to 10^{-5} mol/l adrenaline could be blocked by (±)-propranolol at 10^{-7} mol/l whereas the latter, even at 10^{-6} mol/l, did not affect adrenaline (10^{-6} mol/l) accumulation.

Discussion

This study represents an attempt to describe the accumulation or the uptake process for adrenaline in a homogeneous tissue whereas most uptake and binding studies (e.g., Bonisch, Uhlig & Trendelenburg, 1974; Harden, Wolfe & Molinoff, 1976) have been performed on heterogeneous tissues such as whole hearts or heart homogenates. Powis (1973) has investigated the accumulation of catecholamines by cultured smooth muscles and found a process similar to Uptake$_2$, in that its K_m was 2.6×10^{-4} mol/l as compared with the 2.5×10^{-4} mol/l for Uptake$_2$ given by Iversen (1965), and it was inhibited by normetanephrine and 17-β-oestradiol (1.3×10^{-5} mol/l = IC$_{50}$). Accumulation in these cultured smooth muscles was associated with the production of catechol-*O*-methyl transferase metabolites. The smooth muscle process accumulated 1.5×10^{-9} mol/10^6 cells at 10^{-4} mol/l which can be compared with the accumulation of 0.9×10^{-8} mol/10^6 cells for myoblasts and 5×10^{-11} mol/10^6 for fibroblasts reported in this study.

In the absence of complete information as to the effect of various drugs on the accumulation process in myoblasts it is difficult to identify part or all of the process as being Uptake$_2$. The blockade of Uptake$_2$ by various drugs is well documented (Iversen, 1973) and IC$_{50}$ values of 2.7 μmol/l from 17-β-oestradiol in rat heart tissue and 13 μmol/l in smooth muscle cells have been reported. The comparative lack of effect of 17-β-oestradiol in myoblasts was not fully evaluated (because of solubility) but phentolamine was also without effect although it has been reported to block Uptake$_2$ (Eisenfeld, Axelrod & Krakoff, 1966). Other inhibitors were not investigated.

By histochemical fluorescence the site of Uptake$_2$ has been localized on the cardiac muscle membrane of rat hearts (Clarke, Jones & Linley, 1969), but the existence of more than one extraneuronal uptake mechanism is a possibility in heart tissue for Bonisch *et al.* (1974) have described two extraneuronal uptake mechanisms; one associated with *O*-methylation and the other with storage alone; both uptakes were inhibited by corticosterone. Uptake$_2$ mechanisms are as-

sociated with catechol-*O*-methylation but no investigation was made in this study as to the chemical nature of the myoblast accumulated radioactivity whilst a total·intracellular localization of the accumulated radioactivity would seem unlikely in view of the rapidity with which accumulation occurred and was lost in amine-free solution.

The cell-type selective nature of the accumulation process seen in myoblasts can be compared with the very limited accumulation, with different kinetics, seen in fibroblasts which apparently also have a β-adrenoceptor mechanism. With regard to the fibroblast adrenaline-response, mimicked by dibutyryl cyclic AMP, a similar response to the latter compound in cultured fibroblasts has been described by Willingham & Pastan (1975).

A catecholamine binding has previously been described for fragmented cultured myocardial cells from chick embryos (Lefkowitz *et al.*, 1973, 1974). In their studies, myoblasts were not separated from fibroblasts and binding studies were performed on cell fragments. Binding (K_m = 2 and 10 μmol/l) of tritiated noradrenaline to such fragments was displaced by other catecholamines but not by propranolol and it did not show stereospecificity. In attempts to show specific β-adrenoceptor binding, Maguire, Wikland, Anderson & Gilman (1976) have applied iodinated hydroxybenzpindolol to rat glioma and human fibroblast cells. On glioma cells (C6TGIA) the K_D was 0.25 nmol/l with 4×10^3 binding sites/cell. Sheppard (1976) has attempted to identify β-adrenoceptor sites on normal and transformed 3T3 cells with alprenolol as a radiolabelled marker. The K_D for alprenolol was 10^{-8} mol/l and the binding was rapid (5 min to equilibrium at 20°C). The number of accumulation sites in the present study was, on the basis of an average total cell area of 1,300 μm^2, 6×10^9 sites/cell or $\sim 5 \times 10^6$ sites/μm^2 which, from physical considerations alone, implies either accumulation within the cell or a total cellular coating. This number of sites is considerably greater than 2.75×10^6 sites/cell given by Lefkowitz *et al.* (1974).

For a complete analysis of catecholamine distribution at, and around, a β-adrenoceptor membrane site it must be an advantage to describe such events in a homogeneous tissue with well-defined responses easily subject to physiological, pharmacological and biochemical analysis. Cultured neonatal myocardial cells would appear to be such a tissue but experiments are complicated by the accumulation process described in this study which does not correlate with β-receptor activation or Uptake$_2$.

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