Compartmentalization of Adriamycin and Daunomycin in Cultured Chick Cardiac Myocytes

Effects on Synthesis of Contractile and Cytoplasmic Proteins

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SUMMARY. The affinity of two anthracycline antineoplastics ([14C]adriamycin and [14C]daunomycin) for cultured embryonic chick heart cells was determined by measuring their uptake, compartmentalization into subcellular fractions, and effects on the synthesis of cytoplasmic and contractile proteins. Both drugs, at micromolar concentrations, were readily uptaken by myocytes and found to be concentrated in a light-buoyant-density fraction containing no lysosomes. Nuclear [14C]drug content accounted for 20-25% of the drug incorporated. Binding of adriamycin was saturable within 90 minutes of drug exposure, and the uptake of [14C]adriamycin was inhibited 50% by verapamil and adenosine triphosphate. Uptake of [14C]daunomycin was not influenced by these compounds. Cytosolic and contractile protein synthesis measured by [35S]methionine incorporation into proteins was blocked 70% overall in each fraction after 6 hours of incubation with 2 μM adriamycin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography and quantitative densitometry, revealed that actin synthesis was the least affected of the major proteins. Cardiac myocytes incubated for short periods of time with 2 μM adriamycin revealed subtle cytoplasmic changes in their organelles with the appearance of clear zones of cytoplasm containing short unorganized microfilaments. The deleterious effects of anthracyclines in heart cells are manifested early by rapid drug incorporation into myocytes and inhibition of cytoplasmic protein synthesis. (Circ Res 53: 352–362, 1983)

ADRIAMYCIN (ADR) and daunomycin (DM), both anthracycline antineoplastics (anthracyclines), are known to cause irreversible congestive cardiomyopathy (Lefrak et al., 1973). The ability of heart cells to incorporate ADR (Bossa et al., 1977) makes possible the systematic study of biochemical interaction and ultrastructural arrangements of myofibrillar components. Both anthracyclines induce structural changes in myocytes in vivo (Jaenke, 1974, 1976) and biochemical alterations of cardiac contractile proteins in vitro (Lewis et al., 1982). The nature of deficient myofibrillar functions induced by anthracyclines is poorly understood (Bristow et al., 1981; Balazs and Herman, 1976). Alterations in Z-disc structure and disarray of thin filaments are critical ultrastructural events observed in the development of ADR heart muscle disease (Jaenke, 1974; 1976; Billingham et al., 1978).

Evidence exists concerning the interaction of ADR with cardiac cell membranes (Duarte-Karim et al., 1976; Caroni et al., 1981), and its binding affinity for selected cytoskeletal and contractile proteins (Lewis et al., 1982; Someya et al., 1978; Na and Timasheff, 1977) and nucleic acids (Paul et al., 1981; Trouet and DePrez-Decampagneer, 1979). However, the binding sites of ADR, and its uptake pathways, subcellular compartmentalization, and ultimate metabolic fate have not been completely elucidated for heart cells (Skovsgaard, 1978; Dano, 1976). Our previous studies on the binding of ADR to selected purified contractile proteins demonstrated the importance of a specific site of the ADR molecule to alter the properties of actin in vitro. The free amino group of ADR was responsible for the binding of ADR to actin and for producing altered ultrastructure of actin polymers without hindering actin interaction with myosin. To examine whether this relationship exists in vivo, we initiated studies of uptake binding and internalization of ADR on primary cultured chick embryo heart cells, and, in addition, we explored the effects of ADR on cytoplasmic contractile proteins. Our study used [14C]ADR and [14C]DM to monitor the incorporation of anthracyclines into primary cultured, chick embryo heart cells. We examined the effects of these compounds on the synthesis of cytoplasmic proteins soluble in nonionic detergents and on protein components of the cytoskeleton and found that anthracycline exerted a selective inhibitory effect on the synthesis of these proteins.

Methods

All reagents were analytical grade I. [14C]ADR and [14C]DM were supplied by the Stanford Research Institute. Specific activity: 13.4 mCi/mmol for ADR; 30.9 mCi/
mmol for DM. Adriamycin and daunomycin are products of Farmitalia Carlo Erba, Milan, Italy. Purity was established by thin layer chromatography. Verapamil (V), 2.5 mg/ml in saline, was obtained from Knoll Pharmaceuticals. ATP and trypsin were purchased from Sigma. All media were prepared fresh from Gibco. Fertilized chick eggs (7-10-day-old embryos) were supplied by Shamrock Farms. Buffer A consisted of 20 mM phosphate-buffered saline (PBS). Buffer B consisted of PBS + 10⁻³ M ADR. Buffer C consisted of PBS + 0.1% Triton X-100. Medium A: Hanks’ F-10 + 10% fetal calf serum + 10% horse serum. Sodium dodecyl sulfate (SDS) was purchased from Bio-Rad. Adriamycin and DM were stored dry in aluminum foil-covered vials at room temperature.

Chick Heart Cell Culture

Chick heart cells were obtained by a modification of the methods described previously (Harary and Farley, 1963; Batra et al., 1976; Seraydarian and Luz, 1979). Cell monolayers were prepared from 7-day-old chick embryo hearts by multi-cycle trypsinization. Dishes of 60 mm containing 4 ml of medium A were seeded with 2 x 10⁶ cells/dish and incubated at 37°C in a water-saturated atmosphere incubator containing 5% CO₂, 20% O₂, 75% N₂ for 2 days prior to treatment. In parallel culture dishes, linear growth rates were established for the duration of the experimental procedure using incorporation of 0.1 μCi/ml of [U-¹⁴C]leucine (Amersham) according to methods previously described (Clark and Zak, 1981).

Cell Viability Determinations

Cell viability was determined on cells derived from identical pooled explants and grown in parallel Petri dishes. Equimolar amounts of nonradioabeled ADR were added to the time-matched dishes, and cells were harvested as described above. Trypan blue dye (Fisher Scientific) was added to the suspension, and exclusion was monitored on a hemocytometer by light microscopy, permitting both total cell counts and percentage of cells capable of excluding the trypan blue dye. Experimental determinations were completed on culture dishes exhibiting greater than 95% viability and 10⁶ cells/ml. Fibroblast growth was reduced using glutamine-free medium. All experiments in which cells were exposed to ADR were performed under steady state conditions as described elsewhere (Clark and Zak, 1981).

Binding Studies

Heart cells in culture were examined microscopically to detect spontaneous beating. Medium was decanted from all dishes; cells were washed gently with small volumes of buffer A; the wash was discarded, and buffer A (2 ml) was added to the culture dish. Cells in triplicate dishes were incubated with 0.1 μCi [³H]ADR or [¹⁴C]DM (2 x 10⁻⁶ M at specified time intervals. At each experimental time, the incubation buffer was decanted, cells washed in 1 ml of ice-cold buffer B to remove free [¹⁴C]Janthracycline, harvested with a rubber policeman, and placed in 10 ml of Scintiverse, a liquid scintillation cocktail. After 90 minutes binding studies were determined at 30-minute intervals of up to 4 hours. No difference in radioactivity was found in determinations retrieved after 30 minutes thus, binding calculations and experimental points are expressed for up to 30 minutes. Radioactivity was monitored with a Beckman Tricarb scintillation spectrometer.

Determinations of [³H]ADR and [¹⁴C]DM by the Nuclear Compartment

Cells in triplicate dishes were treated as above with some modification. Cells were incubated at 4°C for 20 minutes with addition of 10% SDS and 1.0 mM MgCl₂ to bring their final concentrations to 0.1% SDS, and 10 mM MgCl₂ in buffer B in a volume of 1 ml. This hypotonic medium with detergent and magnesium was used to permeabilize the cell and disrupt the cell membrane, yet leave the nuclei and nuclear membranes intact (Aaronson and Wool, 1981). Cell suspensions were placed in microfuge tubes and centrifuged at room temperature for 10 minutes in a Beckman Eppendorf microcentrifuge. The supernatant extracts were decanted and 100 μl of supernatant solutions were counted for radioactivity. Sedimented material was washed with 1–2 ml of buffer B and pelleted as before. The supernatant was decanted and the pelleted material was washed twice in 1 ml of ice-cold buffer B, decanted, and the wash material counted for radioactivity. The pellet then was resuspended in 0.5 ml of NCS tissue-solubilizing reagent (Amersham) and the final pellet was placed in 10 ml of Scintiverse for radioactive counting as described previously.

Cytoplasmic Compartmentalization Studies

Cells were treated as described above with some modifications. After the cells were incubated with [¹⁴C]DM for specified time intervals, they were harvested in 0.7 ml of buffer B. The suspension was placed in a glass-hand homogenizer and homogenized for 30 seconds at 4°C at 60 strokes/min. The homogenate was placed on a discontinuous sucrose gradient built with 0.5-ml fractions ranging from 5 to 30% or 5 to 50% sucrose in 20 mM N-(morpholino)ethanesulfonic acid (MES) buffer, pH 6.5. Gradients were placed in a Beckman SW55Ti rotor and centrifuged at 125,000 g for 1 hour at 4°C. The gradients were fractionated by suction from above from 0.3-m1 fractions using a peristaltic pump (Buchler Instruments) and a Gilson micro-fraction collector. Aliquots of 100 μl were counted for radioactivity. Pellets from this procedure were treated as described above for the nuclear compartment, and the final pellet was placed in 0.5 ml of NCS and 100 μl of the solubilized pellet solution similarly counted.

Enzyme Marker Determinations

Specific enzymatic markers for the various subcellular fractions were used to establish the identity of the organelles. For the lysosomal fraction marker, acid phosphatase at pH 5 using 50 mM sodium β-glycerophosphate in 0.1 mM sodium acetate, is measured. For cytochrome oxidase levels (marker for mitochondria), reduced 35 μM cytochrome c in 30 mM sodium phosphate, pH 7.4, is measured. For 5'-nucleotidase level (plasma membrane marker), 1 mM sodium 5'AMP is the substrate in 50 mM Tris, 20 mM MgCl₂, pH 8.5. For NADH cytochrome c reductase levels (endoplasmic reticulum marker), 45 μM oxidized cytochrome c in 30 mM sodium phosphate, pH 7.4, is measured (Tulens et al., 1974).

Electron Microscopic Studies

Morphology of Myocytes

Parallel to the [¹⁴C]JADR binding determinations, heart cells in Petri dishes were incubated in buffer B for various
time periods and prepared for electron microscopic ex-
amination. Cells were washed of excess ADR with buffer
A as above, and fixed in PBS containing 1.5% glutar-
dehyde. Fixed cells were harvested from the dishes, gently
pelleted at 500 g for 5 minutes, and processed for electron
microscopy. Examination of the ultrathin sections were
made with a JEOL 100-B electron microscope at 80 V.

Morphology of Sucrose Gradient Fractions

Electron microscopy was performed on specimens ob-
tained from the various sucrose gradient fractions, and
their pellets were used to ascertain the composition of the
subcellular organelles. Sucrose gradient fractions were
aspirated from the top of each tube, diluted 10 times in
buffer A, and sedimented by ultracentrifugation at
100,000 g for 1 hour. The pellets that were obtained were
fixed in 2% glutaraldehyde for 2 hours at room tempera-
ture, washed, osmicated, dehydrated, embedded in Epon,
and poststained with lead citrate. Electron microscopy was
performed with a JEOL 100-C electron microscope at
80kV.

Protein Incorporation using [35S]Methionine

Treatment of cells was essentially similar to that de-
scribed above, with some modifications (Martin et al.,
1977). Cells were incubated in medium A in the presence
of 10⁻⁵ M ADR and [35S]methionine (specific activity, 1
mCi/ml, Amersham) for the specified time periods. After
incubation, the medium was decanted and cells were
washed in 1.0 ml of PBS. The wash was discarded, cells
were harvested with a rubber policeman in 1.0 ml of
buffer C (containing Triton X-100), and homogenized at
4°C for 30 seconds in a glass-glass hand homogenizer
fuge tube and centrifuged in a Brinkman microcentrifuge
for 5 minutes. The supernatant with most of the cyto-
somal-soluble proteins was decanted and 100 µl were
counted for radioactivity as before. The pellet was resus-
pended in 1.0 ml of buffer C and repelleted. This material
which had been treated with buffer C is considered the
cytoskeletal matrix (Yu et al., 1975). This pellet then was
placed in 150 µl of 0.1% SDS, repelleted, and the super-
natant used for slab gel electrophoresis. The insoluble
material remaining was dissolved in 300 µl of NCS at
37°C for 24 hours and 20 µl of this mixture were counted
for radioactivity.

Slab Gel Electrophoresis and Autoradiography

The Triton X-100 supernatant (20 µl) was placed on 5–
15% SDS polyacrylamide slab gel electrophoresis, run at
7 mA for 16 hours fixed, and stained with Coomassie blue
(Laemmli, 1970). Gels were dried on filter paper (What-
man) with a drying apparatus (Bio-Rad). Autoradiography
was performed on the dried gel, using a Kodak safety NS-
5 x-ray film, and exposed for 96 hours.

Densitometric Scanning

Relative intensity of the radioactive imprint on the x-
ray film was determined with a Beckman densitometer
with integrating computing attachment, using a slit width of
0.1 mm. Absorbance was monitored at a speed of 1
cm/min.

Anthacycline and ATP Purity and Stability
Determinations

Drug purity of both the nonradioactive ADR and DM
and the stability of their radiolabeled counterparts were
assessed by thin layer chromatography using silica gel-
covered plastic sheets (Eastman Chemicals). The solvent
system consisted of 7:1.2 proportions of n-propanol:ethyl
acetate:water, respectively. Chromatography proceeded
for 8 hours at room temperature in darkness, and visual-
ization of spots was performed under a long-wave flu-
orescent light. A single spot migrating with a relative front
value = 0.45 was observed for ADR; a relative front value
= 0.55 was determined for DM. When [14C]ADR and [14C]
DM were used, spots were excised with scissors, placed in
10 ml of Scintiverse, and counted. Areas not containing
fluorescent spots also were routinely checked for free
counts.

Results

Chick Heart Cell Culture

Normal growth of primary cultures of chick heart
cells was observed following explantation and seed-
ing. Cells appeared confluent within 48–72 hours.
Cells were detected beating spontaneously at room
temperature by light microscopy. Fibroblasts, endo-
thelial or other noncardiac myocyte cells accounted
for <10% of the total cell population. Electron mi-
oscopic examination revealed large, polygonal,
primitive myocytes with peripheral nuclei and char-
acteristic contractile elements arranged as sarcom-
eres (Fig. 1A). The total number of sarcomeres ob-
served in each section examined varied from 12 to
15.

Anthraccline Binding Determinations

Cells incubated with [14C]ADR (Fig. 2A) were
found to bind 280 femtomoles (fmol) per cell after
1 minute. About 1% of the drug was bound or
incorporated by the cells from the total amount
applied. Uptake proceeded asymptotically there-
after. The uptake was 480 fmol/cell after 30 minutes
of incubation and remained unchanged for up to 4
hours. The uptake rate decreased from 280 fmol/
cell per min to 18 fmol/cell per min after 30 minutes.
These values were derived from hemocytometer
counts of the cell number present in each culture
dish.

Similar results were determined for [14C]DM bind-
ing, although initial binding levels were lower than
with [14C]ADR at comparable early points of incu-
bation, increasing to over 600 fmol after 30 minutes
of incubation. The rate of binding declined from 40
to 20 fmol/cell/min after 30 minutes of incubation
(Fig. 2B).

When a Ca++ channel blocker (CCB), V, was
present in the incubation medium at concentrations
ranging from 5 x 10⁻⁷ to 5 x 10⁻⁵ M, binding of
[14C]ADR decreased from 280 to 160 fmol at the
earliest point of incubation tested (1 minute); at 30
minutes, inhibition was approximately 50% of that
from control cells (Fig. 3). To examine the effect of
ADR on cardiac cell membrane receptor sites, we
chose ATP as a ligand. ATP is a compound that is
not transported into the cell cytoplasm, yet is bound
by the heart cell purinergic receptors (Burnstock,
1980). The results obtained indicated that ATP ex-
Figure 1. Electron micrographs of chick embryo heart cells in culture. Panel A: untreated myocyte; panel B: myocytes exposed to ADR (2 μM) for 60 minutes revealing osmophilic vacuoles (v) and zones of cytoplasmic clearing (CZ) filled with microfilaments. Inset: detail from square in panel B shown at higher magnification. The microfilaments (arrowheads) have a width of 12-14 nm. N: nucleus; S: sarcomere; M: mitochondria.
habited blocking effects similar to those produced by verapamil on the binding of [\(^{14}\)C]ADR (Fig. 3).

Determinations of Drug Binding by the 125,000 g Pellet

[\(^{14}\)C]ADR was bound to the pellet. Radioactivity in the pellet fraction accounted for 20–25% of the total radioactivity recovered in the cell homogenates (Fig. 4A) The pellet fraction contained primarily nuclear material. The remainder of radioactive tracer was found in the soluble fraction. Levels of quenching were uniformly standardized for all samples using internal controls to permit comparison of insoluble vs. soluble compartments. Levels of cyto-

![Figure 2](image_url)

**Figure 2.** Incorporation of [\(^{14}\)C]ADR (panel A) or [\(^{14}\)C]DM (panel B) by cultures of chick embryo heart cells. Cells grown in Petri dishes (1 x 10\(^5\) cells/dish) were incubated with the anthracyclines. Amount of radioactivity incorporated was determined after washing and harvesting of cells. [\(^{14}\)C]ADR incorporated is expressed per cell; [\(^{14}\)C]DM is expressed per cell/min. Values between bars represent an average of three separate determinations performed in triplicate.

![Figure 3](image_url)

**Figure 3.** Effect of V or ATP on the uptake of [\(^{14}\)C]ADR by cultured heart cells. Cells were preincubated with either V or ATP (5 x 10\(^{-5}\) M) for 30 minutes before addition of [\(^{14}\)C]ADR to the medium. Cells were treated as described in the legend for Figure 1. Values between bars represent an average of three separate determinations performed in triplicate.

![Figure 4](image_url)

**Figure 4.** Panel A: binding of [\(^{14}\)C]ADR and [\(^{14}\)C]DM by a soluble cytoplasmic and insoluble nuclear fraction of cultured heart cells. Cells were incubated as indicated in legend for Figure 1. Cells were harvested in 1 ml of a medium containing 0.1% SDS and 10 mM MgCl\(_2\). Supernatant was centrifuged and radioactivity was determined in aliquots of the clear supernatant. The pellet of nuclear material was solubilized in NCS, and aliquots were used for measurements of radioactivity. Values between bars represent an average of three separate determinations, each performed in triplicate.
Adriamycin Uptake by Cardiac Cells in Culture

FIGURE 5. Sucrose gradient centrifugation of cardiac cells (1 × 10^6 cells/ml) incubated with [14C]ADR (panel A) and [14C]DM (panel B). Cells were washed, harvested, and homogenized in 0.7 ml of PBS containing 2 × 10^{-6} M unlabeled ADR. The suspensions were layered on top of gradients and centrifuged at 125,000 g for 1 hour at 4°C. Fractions were collected and aliquots counted for radioactivity. The plasmic radioactivity were reduced by 50% if cells were concomitantly treated with micromolar concentrations of V after 60 minutes of exposure. Values of [14C]ADR radioactivity in the V-treated nuclear pellet were proportionately reduced. The differences observed appeared at 60 minutes of incubation (Fig. 4B). Similar results were obtained when [14C]DM was used as a tracer instead of [14C]ADR (not shown). Rate of [14C]DM uptake in the soluble fraction increased with time up to 60 minutes, and remained constant after 90 minutes of incubation. As with binding determinations previously described, the amount of [14C]DM incorporated by the heart cell was higher than that calculated for [14C]ADR.

Cytoplasmic Compartmentalization of [14C]ADR and [14C]DM by Cultured Heart Cells

Determination of [14C]ADR incorporation by cytosolic organelles was performed by subcellular fractionation on discontinuous sucrose gradients. The amount of radioactivity determined accounted for approximately 1% of the total [14C]ADR applied to the cells in the incubation medium. The highest concentration of [14C]ADR was found in the subcellular fraction equilibrated within the 10–15% sucrose layer. When examined by electron microscopy, this fraction contained numerous vesicles and small membrane fragments. Cytoplasmic markers chosen to estimate relative position of the major subcellular organelles in sucrose gradients were: cytochrome oxidase for mitochondria, NADH-cytochrome-c reductase for endoplasmic reticulum vesicles, 5'-nucleotidase for plasma membrane, and acid phosphatase for lysosomes (Noel et al., 1978). Results from cytoplasmic marker studies indicated that the light-buoyant density fractions that contained [14C]ADR did not contain significant levels of contamination by plasma membranes, mitochondria, lysosomes, or endoplasmic reticulum.

There was radioactivity in the pellets at the bottom of the sucrose gradient layers (>30% sucrose). Radioactivity levels in fractions retrieved between 15 and 30% sucrose density, were essentially down to baseline levels. Only the light-buoyant density (10–15% sucrose) and heavy-buoyant density (>30% sucrose) fractions demonstrated substantial radioactivity (Fig. 5, A and B). The fractions used for analysis in our studies were retrieved from both of the latter fractions. The amount of [14C]ADR found in the 10–15% sucrose interface did not increase with longer periods of [14C]ADR incubation, up to 8 hours. Similar patterns were observed when [14C]ADR was replaced by [14C]DM as the tracer. The amount of [14C]ADR found in the subcellular fraction (10–15% sucrose) diminished 3-fold in the presence of either 10^{-7} M ATP or 5 × 10^{-6} M V (Fig. 6).
When ATP was added to this system with [14C]DM, there was no difference from that of [14C]DM controls in radioactivity incorporated (Fig. 7).

**Electron Microscopic Studies**

Control myocytes in culture examined by electron microscopy demonstrated the characteristics of embryonic heart muscle cells (Fig. 1A): nuclei with euchromatin, abundant mitochondria, and numerous primitive sarcomeres. Upon exposure to ADR for 60 minutes, cells demonstrated dispersed heterochromatin in their nuclei. Ultrastructural changes noted in cytoplasmic organelles included swelling of the mitochondria with occasional myelin figures present and an increase in osmophilic vacuoles, presumed to be lipid-containing vacuoles. There were zones of decreased staining density in the cytoplasm which we labeled “clear zones” containing short, 12-14 nm microfilaments (Fig. 1B, Table 1).

**Protein Incorporation Using [35S]Methionine**

Cellular incorporation of [35S]methionine into Triton-soluble cytoplasmic proteins or SDS-soluble myofibrillar proteins was determined. Adriamycin exposure for 6 and 24 hours respectively, revealed overall decreases in the amounts of [35S]methionine incorporated into proteins (Fig. 8). At all incubation times, the total cytoplasmic counts of radioactivity were similar for both ADR-treated cells and untreated controls. This suggested that ADR did not interfere with the active transport of [35S]methionine from the medium into the cell, and indicated adequate preservation of membrane functional integrity (Table 2).

**The densitometric scan of myocardial cell proteins was performed on polypeptides resolved on SDS-polyacrylamide gels (Fig. 8A and B) according to their solubility in ionic (SDS) and nonionic (Triton X-100) detergents.** Total protein radioactivity in these fractions was reduced by 70%. The amount of [35S]methionine incorporated into the pellets was less than 1%. Quantitative autoradiographic scans using computerized integration of peak area were performed on x-ray film (Fig. 8, C and D). Figure 8E shows further, that the total amount of protein extracted from ADR-treated cardiac cells did not vary from that of untreated cells. There were marked differences in the polypeptide composition of both fractions. The highest peak found in all gels, however, corresponded to a protein with a molecular weight of 43,000 co-migrating with authentic cardiac muscle actin (Fig. 8E). This protein apparently

<table>
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<tr>
<th>Qualitative Evaluation of Morphological Changes Produced in the Cytoplasm of Cultured Myocardial Cells Exposed to ADR</th>
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<tbody>
<tr>
<td>Subcellular organelles</td>
</tr>
<tr>
<td>Mitochondria</td>
</tr>
<tr>
<td>Sarcomeres</td>
</tr>
<tr>
<td>Vacuoles, osmophilic</td>
</tr>
<tr>
<td>Clear zones</td>
</tr>
</tbody>
</table>

Cells were treated as indicated in the legend of Figure 1. The values above (+) were derived from the examination of over 100 electron photomicrographs obtained from control and ADR-treated cells. Plus (+) indicates presence with little change, ranging to (+++), indicating increased number and/or increased altered morphology(ies).
FIGURE 8. Effect of ADR on the incorporation of [35S]methionine into myocyte cytosolic and cytoskeletal proteins. Cells were incubated with 5 μCi/ml of [35S]methionine in the presence or absence of 2 × 10⁻⁶ M ADR. Cells were harvested after 6 and 24 hours and were homogenized in Triton X-100, followed by SDS solubilization of the Triton X-100-insoluble material. Aliquots of these proteins were electrophoresed on 5–15% polyacrylamide gels containing SDS, stained with Coomassie blue, dried, and autoradiographic imprints of the cytoskeletal and cytosolic gels made on x-ray films exposed for 96 hours. Denstometric tracings of autoradiographs of matched time points were superimposed to illustrate decreased [35S]methionine incorporation. Panels A and B: autoradiographic imprints of separate runs on SDS-polyacrylamide gel electrophoresis of Triton X-100-soluble proteins (panel A) and SDS-extracted proteins (panel B). Code for panel A: lane I: controls after 6 hours of incubation; lane II: ADR-treated cells after 6 hours of incubation; lane III: controls after 24 hours; lane IV: ADR-treated cells after 24 hours of incubation. Code for panel B: lane I: ADR-treated cells for 6 hours; lane II: untreated control; lane III: ADR-treated cells for 24 hours; lane IV: untreated control. Panels C and D: time-matched superimposed densitometric scans of cytosolic and cytoskeletal proteins, respectively. Upper lines of shaded area indicate untreated controls. Lower lines indicate ADR-treated cells. Gray shaded areas between upper and lower lines illustrate decrease of protein synthesis. Arrowheads indicate proteins co-migrating on gels with purified cytoskeletal markers. Panel E: Coomassie blue-stained gel of polypeptides found in the cytoplasmic fraction. Code of lanes is identical to that of panel A.

Discussion

To examine the nature of anthracycline-induced cardiomyopathy at the cellular and subcellular levels, we used embryonic chick heart cells grown as a primary culture in vitro. This system offered the advantages of isolating the myocyte from its integral relationships in tissue, and of studying the effects of anthracycline without the influence of systemic input from either the organ or the organism. Furthermore, using these heart cells made it possible to observe and quantify uptake and compartmentalization of anthracycline and the effect of anthracycline on the incorporation of radioactive amino acid precursor in proteins. Because these cells are embryonic, they differ from adult cells in their sarcomeric
and was found to saturate at 30 minutes. The anthracyclines were concentrated in the cytosol in a light-buoyant density fraction consisting of vesicles and resealed plasma membrane vacuoles. The finding that ADR appears to be localized in a light-buoyant density fraction of cultured heart cells exposed to the drug supports the concept that ADR can be cytotoxic to cultured cells at the cell membrane level (Tritton and Yee, 1982). Our previous studies (Lewis et al., 1982) and those of others (Trouwet and DePrez-Decampaneere, 1979; Noel et al., 1978; Lampidis et al., 1980) point to other intracellular sites as alternate and/or additional sites for ADR effects. Of note is the fact that both [14C]ADR and [14C]DM demonstrated confinement of radioactivity in light-buoyant density regions (Noel et al., 1978). Electron microscopic examination of these sedimented materials revealed the presence of membrane fragments and numerous resealed vesicles not resembling sarcoplasmic reticulum. Previous reports using embryonic fibroblasts (Noel et al., 1978) demonstrated [14C]ADR concentration at higher-buoyant density fractions which co-migrated with lysosomes. These incubations, however, were performed using longer exposure times to anthracyclines than those in this study. Our findings support previous studies demonstrating the absence of anthracycline accumulation in other subcellular organelles such as sarcoplasmic reticulum, mitochondria, and heavy plasma membranes.

Approximately 20% of the radioactivity incorporated in the cells was detected in the pellet from the sucrose gradients. These observations are compatible with the known high affinity of anthracyclines for DNA in tissue culture (Lampidis et al., 1980). Electron microscopic examination of this material revealed the presence of numerous intact nuclei. No

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**Table 2**

Total Uptake of [35S]Methionine by Heart Cells in Culture in the Presence or Absence of ADR

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>Experimental conditions</th>
<th>[35S]methionine (cpm ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>115,000 ± 12,500</td>
</tr>
<tr>
<td></td>
<td>ADR-treated</td>
<td>93,000 ± 11,700</td>
</tr>
<tr>
<td>6</td>
<td>Untreated</td>
<td>182,000 ± 28,500</td>
</tr>
<tr>
<td>24</td>
<td>ADR-treated</td>
<td>135,000 ± 22,200</td>
</tr>
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After incubation with [35S]methionine in presence or absence of ADR, cells were washed, harvested, and aliquots of cell extracts were placed in scintillation fluid and monitored for uptake of the [35S]methionine.

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**Table 3**

Effect of ADR on the [35S]Methionine Incorporation into Protein by Cardiac Myocytes Based on Densitometric Tracings of Autoradiographic Gels

<table>
<thead>
<tr>
<th>Identified cytoskeletal proteins</th>
<th>Area (cm²) (% total)</th>
<th>Area (cm²) (% total)</th>
<th>Area (cm²) (% total)</th>
<th>Area (cm²) (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>0.051</td>
<td>0.0051</td>
<td>0.136</td>
<td>0.012</td>
</tr>
<tr>
<td>(3.8)</td>
<td>(1.1)</td>
<td>(5.9)</td>
<td>(1.4)</td>
<td></td>
</tr>
<tr>
<td>α-Actinin</td>
<td>0.072</td>
<td>0.0098</td>
<td>0.112</td>
<td>0.039</td>
</tr>
<tr>
<td>(5.4)</td>
<td>(2.1)</td>
<td>(4.8)</td>
<td>(4.4)</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>0.201</td>
<td>0.0493</td>
<td>0.432</td>
<td>0.125</td>
</tr>
<tr>
<td>(15.1)</td>
<td>(10.8)</td>
<td>(18.6)</td>
<td>(15.7)</td>
<td></td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>0.115</td>
<td>0.0261</td>
<td>0.179</td>
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</tr>
<tr>
<td>(8.9)</td>
<td>(5.7)</td>
<td>(7.7)</td>
<td>(7.9)</td>
<td></td>
</tr>
</tbody>
</table>

**Structural proteins in the cytosol**

| Tubulin                         | 0.40                 | 0.23                  | 0.60                  | 0.27                 |
| (6.9)                           | (6.9)                | (5.7)                 | (5.3)                 |
| Actin                           | 0.96                 | 0.82                  | 2.44                  | 1.44                 |
| (16.0)                          | (24.5)               | (22.9)                | (29.2)                |

Percentage total is defined as the percentage area that a given densitometric peak describes as compared to the integrated total area of all peaks detected on that gel scan.
radioactivity was found in the uppermost portion of the gradient, suggesting that anthracyclines were particulate bound.

We found that the CCB, V, inhibited the uptake of \(^{14}\)C]ADR from the medium. Decrease of \(^{14}\)C]ADR incorporation in the presence of pharmacological concentrations of V was noted similarly in the light-buoyant density fraction, implying that incorporation of ADR is related to membrane phenomena whereby molecules such as V, or ATP, known to bind to myocyte membranes through receptors (McDonald et al., 1980), may sterically hinder anthracycline incorporation. The inhibition of ADR uptake by V may be related to recent findings where V restored drug responsiveness of anthracycline-resistant tumor cells (Slater et al., 1982). The effects described are considered unique for Ehrlich ascites tumor cells which are resistant to DM, and may be specific for their system. Heart cells are mesenchymal in origin and cannot be equated to this ectodermal cell line used (Slater et al., 1982). Our findings suggest that the effect of V on heart cells exposed to ADR differs from the effects on Ehrlich ascites carcinoma cells. It is feasible that ADR may enter the cell by multiple pathways, one of which is related to the calcium channel and is blocked by V. The remaining portals of entry for ADR would still be available for its internalization.

Although adenosine triphosphate, a nucleotide known to bind to cardiac myocyte membranes through purinergic receptors (Seraydarian and Luz, 1978; Burnstock, 1980), was found to reduce \(^{14}\)C]ADR uptake, it did not affect \(^{14}\)C]DM uptake. This would preclude possible binding of anthracycline to the nucleotide's phosphate group (Gabbay et al., 1976) and would suggest a less complex uptake pathway of \(^{14}\)C]DM into myocytes.

ATP had effects similar to V on ADR-treated heart cells. Incorporation of \(^{14}\)C]ADR into the light-buoyant density fraction was diminished by 50%. On the other hand, this ATP effect on DM-treated cells was absent, implying a potentially greater specificity of the purinergic receptor for ADR than for its congener, DM (Burnstock, 1980). ADR and DM, although sharing lipophilic properties that would permit passive diffusion through the plasmalemma, nonetheless may use alternate, possibly separate routes to enter the cell, as has been reported previously with other systems (Dano, 1976; Skovsgaard, 1977; Noel et al., 1978). \(^{14}\)C]ADR levels of incorporation into cells at 2 minutes, the shortest exposure time, were almost 50% of maximum values. \(^{14}\)C]DM showed minimal incorporation at 2 minutes, rose rapidly over 10 minutes, and did not reach maximum levels after 30 minutes of exposure. A possible explanation for the different levels of incorporation detected between these two compounds may reside in different association or dissociation kinetics. Alternatively, multiple classes of binding sites may exist for each drug.

Based on our previous work demonstrating binding affinity of ADR for protein components of the myofibril (Lewis et al., 1982), it appeared important to dissect the contractile elements of heart cells affected by ADR exposure. We demonstrated recently that ADR interacts strongly with monomeric cardiac actin molecules, forming 12-14 nm filaments. These filaments appeared to be a form of altered, short actin polymers demonstrating different ultrastructural characteristics from F-actin.

Our biochemical findings on protein levels of cultured heart cells treated with ADR and utilizing \(^{35}\)S)methionine as a tracer revealed decreased overall protein incorporation of the labeled precursor by these cells after 6 hours. Depression of incorporation of the \(^{35}\)S)methionine label into cytoplasmic proteins and contractile proteins was comparable, as judged by integrated areas of densitometric gel scans of autoradiographs. Analysis of the two protein fraction profiles revealed that dense bands co-migrating with actin appeared in both the Triton-insoluble pellet (myofibrillar) and in the cytoplasmic Triton-soluble protein preparations. Densitometric scans of autoradiography obtained after 6 hours of exposure to ADR showed that the amount of actin incorporating the radiolabel in heart cells did not decrease in proportion to the rest of protein populations. This unusual finding could be related to the postulated multiple number of actin genes present in chicken (Korn, 1982; Rubenstein and Spudich, 1977) and the well documented interaction of ADR with DNA (Trout and DePrez-DeCampaneere, 1979).

Ultrastructural morphology of cardiac myocytes revealed subtle changes after short exposure to these compounds. Electron micrographs showed that the integrity of major myocyte organelles remained essentially intact. Of note was observation of large cytoplasmic zones exhibiting numerous short stubby microfilaments ranging from 12 to 14 nm in thickness. It remains to be determined whether these filaments are related to our previously described ADR actin-polymerized filaments in vitro.

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