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Authors

Siemens, AE
Kitzes, MC
Berns, MW

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Hydrazine Effects on Vertebrate Cells *in Vitro*¹

ANN E. SIEMENS, MARGARITA C. KITZES, AND MICHAEL W. BERNES

Developmental and Cell Biology, University of California, Irvine, Irvine, California 92717

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Hydrazine Effects on Vertebrate Cells *in Vitro*. SIEMENS, A. E. KITZES, M. C., AND BERNES, M. W. (1980). *Toxicol. Appl. Pharmacol.* 55, 378-392. This study was designed to elucidate the cellular effects of hydrazine on four established tissue culture vertebrate cell lines (rat kangaroo kidney, *Xenopus* toad kidney, human diploid fibroblast, and Chinese hamster cells) and primary cultures of neonatal rat myocardial cells. Cells were exposed to hydrazine in various concentrations (0.001 to 10 mM) for varying time periods. The resulting growth and morphological data revealed a possible site of hydrazine action. In all cell lines tested, population growth was depressed by low concentrations of hydrazine (0.01 to 0.1 mM). Cell growth was initially depressed, but it eventually returned to normal log phase growth even when fresh hydrazine was added to the culture medium. At higher concentrations (0.5 to 2.0 mM), hydrazine was lethal. Most cell types first showed population growth depression at 0.01 mM hydrazine, but the lethal concentration varied with the cell type. Cultures treated with hydrazine yielded a significantly higher number of giant, multinucleated cells. Autoradiography studies employing [³H]thymidine confirmed that the large, multinucleated cells resulted from cell fusion. The increase in cell fusion in hydrazine treated cell cultures implicated the cell surface as a possible target site. Scanning electron microscopy confirmed concentration related surface differences between control and hydrazine-treated cells. Further membrane studies examining the effects of hydrazine on the contractile and intercellular spontaneous electrical activity of myocardial cells in culture indicated that hydrazine also altered these membrane-related activities in a concentration and time-dependent manner.

Hydrazine (NH₂NH₂) is a hygroscopic, highly polar reducing agent (Raphaelian, 1966). This reactive compound, described as the ammonio analog of hydrogen peroxide, can be converted to a variety of widely used alkyl derivatives. Both hydrazine and its derivatives are used extensively in the production of photographic developers, agricultural chemicals, and pharmaceutical products. The use of hydrazine both as an oxygen scavenger in industry and as a major component in high-energy

rocket fuel cells constitutes its major commercial uses. As a result of these applications, hydrazine and its derivatives are becoming more prevalent in the environment, and their use has been criticized as a source of biological hazard.

Previous studies have detailed the hazardous effects of hydrazine and related derivatives. Hydrazine is known to effect pyrimidine-related mutations in DNA (Brown *et al.*, 1966; Brown, 1967; Gupta and Grover, 1970; Kak and Kaul, 1975), and it is easily derivatized into a number of detrimental agents which act as both toxins and carcinogens. The most studied of these agents include the toxins, hydralazine, which interferes with smooth muscle con-

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traction (McLean *et al.*, 1978), phenylhydrazine, a hemolytic agent including anemia and Heinz body formation (Jain *et al.*, 1978), and monomethyl hydrazine, a metabolic inhibitor (Dost *et al.*, 1976). A carcinogenic relative of the latter derivative, dimethylhydrazine, has produced both colon and blood vessel tumors in several laboratory animals (Toth and Wilson, 1971; Toth *et al.*, 1976; Mak and Chong, 1978; Barkla and Tutton, 1978). More specifically, some of these effects are produced in isolated cellular components only under specific conditions of treatment (Brown, 1967; Kak and Kaul, 1975). The importance of these studies may be underestimated or misinterpreted without a basic understanding of the impact such compounds have on a wide variety of cell types when tested under controlled conditions.

The present study investigates the basic cellular responses of diverse vertebrate cell types *in vitro* to the compound hydrazine. The results implicate the cell membrane as one of the major targets of hydrazine action.

METHODS

Maintenance of Cell Cultures

The PTK₂ cell line, which was derived from normal adult male kidney of *Potorous tridactylus*, was obtained from the American Type Culture Collection (CCL 56). Cells were grown in minimum essential medium (Eagle) with Earle's salts supplemented with 0.085% sodium bicarbonate, 10% heat-inactivated fetal calf serum, and 0.011% pyruvic acid. Cultures were maintained at 37°C in a 5% CO₂, 95% air atmosphere in Falcon T-75 plastic flasks. Cells were enzymatically detached from the flasks as follows: the supernatant overlying the cell monolayer was aspirated from the T-75 flask and replaced with 4 ml of an enzymatic solution (0.25% pancreatin, 0.1% EDTA in a balanced salt solution, pH 7.0). Cells detached from the substrate during incubation (6 min, 37°C) and light Pasteur pipetting. The enzymatic solution was diluted out with 5 ml of medium, and the cells were pelleted from the suspension (5 min, 200g). The resulting supernatant was discarded, and the cells were diluted in fresh medium for plating into either T-75 flasks (4

× 10⁵ cells/ml in 10 ml of medium/flask) for growth curves or into Rose multipurpose chambers (50 × 10³ cells/ml, Berns *et al.*, 1972) for microscopic evaluation.

The A6 cell line (American Type Culture Collection, CCL 102), derived from primary culture of normal male toad (*Xenopus laevis*) kidney, was maintained in Eagle's minimum essential medium (Hank's salts) fortified with 10% heat-inactivated fetal calf serum, penicillin (100,000 IU/liter) and streptomycin sulfate (0.0714 g/liter) in a 5% CO₂, 95% air atmosphere at room temperature. For weekly subculturing and experimental setups, the cells were enzymatically removed from the flasks as previously described (4 min of incubation at room temperature) and plated into T-75 flasks for growth curves (4 × 10⁵ cells/flask in 10 ml of medium) or into Rose chambers (75 × 10³ cells/ml) for microscopic evaluation.

Human diploid embryonic lung cells (W138) were purchased from the American Type Culture Collection (CCL 75). These cells were maintained in Eagle's basal medium (Earle's salts) with 10% unactivated fetal calf serum in a 5% CO₂, 95% air atmosphere. For weekly subculturing and growth curve experiments, the cells were enzymatically detached from the flask as previously described with the following modification: the cells were incubated 5 min at 37°C in an enzymatic solution consisting of 0.25% trypsin in a balanced salt solution, pH 7.0.

Chinese hamster cells (CH), also referred to as the MC-3 line, were generously provided by Dr. Joe Gray (Lawrence Livermore Laboratory, Livermore, Calif.). They were maintained in minimum essential medium (Earle's salts) supplemented with 15% heat-inactivated fetal calf serum, 4.6% NCTC-135 (GIBCO, Grand Island, N.Y.) and 60 mg/liter gentamycin sulfate (Schering Corp., Kenilworth, N.J.) in a 10% CO₂, 90% air atmosphere at 37°C in T-25 flasks (Falcon, Pittsburg, Pa.). After enzymatic detachment from flasks (incubated 5 min, 37°C), these cells were plated in Falcon T-25 flasks at 2.5 × 10⁴ cells/flask in 5 ml medium for growth experiments.

Hydrazine Stock Solutions

Hydrazine (MW 32.05, anhydrous, 97%) was supplied through the courtesy of Dr. Ronald Shank (University of California, Irvine). Stock solutions of 100 and 10 mM hydrazine were prepared by dilution into 0.01 N HCl. The stock solutions were diluted from 50- to 1000-fold in tissue culture medium just prior to application on the cultures. Control cultures received a corresponding amount of 0.01 N HCl without hydrazine in the medium. In the quantities used, the addition of 0.01 N HCl with or without hydrazine did not significantly alter the pH of the medium.

Growth Response Curves

A. Normal growth curves. To study the growth effects of hydrazine on a particular cell line, the specified quantity of cells were plated out in Falcon T-75 flasks in tissue culture medium. Sufficient flasks were prepared such that two flasks were counted to determine each data point for a given hydrazine concentration. At the specified times (indicated by arrows, Fig. 1), two flasks from the entire population were harvested as follows: the cells in a flask were examined by inverted phase microscopy, washed with fresh medium to remove debris, enzymatically removed from the flask as previously described, and counted and sized by a Coulter counter-channelizer (Model ZBi, Coulter Electronics). At this time, the medium in all the remaining flasks was replaced with fresh medium and supplemented with the appropriate concentration of fresh hydrazine stock solution. Control cultures received fresh medium and a corresponding aliquot of 0.01 N HCl without hydrazine. At each subsequent point of harvest, two flasks of cells representing each hydrazine concentration were similarly harvested, and the medium in the remaining flasks was replenished again with fresh medium containing the appropriate concentration of fresh hydrazine. The medium was changed at different intervals in the various experimental schemes. The particular timing for each experiment is depicted in the figures.

B. Time growth curves. To determine how the length of hydrazine exposure might correlate to hydrazine-induced effects on cell cultures, both PTK₂ cells and A6 cells were treated with a growth suppressive concentration of hydrazine (1.0 mM) for varying lengths of time. In this experiment, the cells were set up and harvested in the previously noted manner incorporating the following modifications: at the indicated initial exposure time (Fig. 2, arrows at Day 2), an aliquot of hydrazine stock solution was introduced into each flask of established cell cultures containing freshly replenished medium. The flasks of cultures were then divided into sets which would receive exposures to hydrazine for 24 or 96 hr, or throughout the duration of the experiment (continuous). In the A6 cell line, the 24-hr flasks received just one exposure to hydrazine (at the time point indicated); the 96-hr exposure received fresh hydrazine after medium changes at the initial time point (arrow, Fig. 2), Day 3, and Day 5; the continuous exposure group received fresh hydrazine after medium changes at the initial time point (arrow, Fig. 2), and Days 3, 5, and 8. The PTK₂ cells received the same schedule of treatment except the exposure time was at Day 6 rather than at Day 5, as indicated for the A6 cells. Beyond the designated time of exposure, each flask in a set was replenished with fresh medium and an equivalent aliquot of 0.01 N HCl without hydrazine. The control

flasks received fresh medium containing 0.01 N HCl at all time points indicated in Fig. 2.

C. Selection growth curves. Flasks of A6 cells were plated out as previously described. Forty-eight hours after establishing cell cultures, the medium in the flasks was discarded, replenished with fresh medium, and supplemented with an aliquot of stock hydrazine solution to yield a final concentration of 1.0 mM. This procedure was repeated at 48-hr intervals up to the 10th day when the cultures approached confluency. At this point, these hydrazine "selected" cells were harvested and plated out in flasks for growth analysis as previously described. The above procedure was then repeated with the following modifications: both flasks of "selected" A6 cells and parallel flasks of non-selected, "naive" A6 cells were treated as described with 1.0 mM hydrazine at the points indicated in Fig. 3 throughout the experiment. Parallel flasks of "selected" cells receiving a corresponding aliquot of 0.01 N HCl without hydrazine served as a control.

PTK₂ Large Cell Analysis

PTK₂ cells were established in Rose chamber cultures as previously described except that experimental chambers were plated out in medium containing 1.0 mM hydrazine. The chambers received fresh medium (control set) and medium containing 1.0 mM hydrazine (experimental set) at 48 and 96 hr post-plating. Each of three chambers constituting a set was then photographed at 20 random sites (40X, obj. Kodak Pan X film, ASA 32) with a Zeiss photomicroscope. From the photographs, the frequency of multinucleation occurring in both the control and experimental population was tabulated. Statistical differences between the two populations were determined by the two tailed *t* test with at least 95% confidence.

PTK₂ Cell Fusion Assay

Two sets of duplicate T-25 flasks were set up with 10⁵ PTK₂ cells in 5 ml of medium. One set of duplicate flasks received 1.0 mM hydrazine 24 hr after plating, and the second set served as a control. Fresh medium was substituted 36 hr later with one flask from each set receiving [³H]thymidine (2 μ Ci/ml, ICN Pharmaceuticals, Irvine, Calif.). The cells in each set of flasks were pulsed for 8 hr, rinsed, enzymatically removed, and cosuspended into a common culture containing both [³H]thymidine-labeled and regular cells. Each mixed culture set was plated into two Rose chambers (40 $\times$ 10³ cells/ml medium) with experimental chambers again receiving 1.0 mM hydrazine. After 12 hr, glass coverslips with the adherent cells were fixed in medium containing 2% diglutaraaldehyde (EM grade, Polysciences, Warrington, Pa.) for 1 hr at

room temperature and overnight at 4°C. The coverslips were then washed in phosphate-buffered saline and mounted on glass microscope slides. Autoradiography was performed in total darkness as follows: the slides were dipped into a 33% aqueous solution of Ilford nuclear research emulsion type L-4 (batch PL-729, Ciba-Geigy Co., Ilford, Ltd., Basildon Essex, Essex, England), dried for 1 hr at room temperature and stored in a dessicant-containing, light-tight box (4°C). One week later, the slides were developed using Kodak D-19 developer (5 min), 1% aqueous acetic acid stop (15 sec), Kodak fixer (5 min), and water rinse (20 min); all solutions were used at 18°C. The autoradiographs were stained in a filtered, saturated aqueous methylene blue solution (15 min) and destained with water. Permanent mounts were made of the slides after a series of ethanol dehydrations.

Electron Microscopy

Xenopus cells (75×10^3 cells/ml) were plated into Rose chambers in medium containing either 0.01 N HCl with hydrazine (final experimental culture hydrazine concentrations from 0.01 to 1.0 mM) or corresponding amounts of 0.01 N HCl alone (control cultures). Fresh medium containing hydrazine was added at 48 hr. After 3 days, the cells were fixed in medium with 2% glutaraldehyde (30 min at room temperature, and overnight at 4°C), and the adherent cells on the glass coverslips were processed for SEM according to the procedures described by Cohen *et al.* (1968). The coverslips were rinsed 5 min in phosphate-buffered saline and run through a series of 5-min dehydrations in aqueous ethanol (50–100%). This was followed by a series of 10-min ethanol–freon dehydrations (30–100% Freon 113), critical point drying (Omar SPE-900EX) utilizing Freon 113 and gold evaporation (Technis Hummer II, 3 min at 10 V). Coverslip specimens were mounted with silver paint on aluminum studs and analyzed on a Hitachi HS500 scanning electron microscope (15–20 kV, tilt angle of 30–55°). Random samples of cells on the coverslips were examined with regard to the quantity of cell surface projections (light, moderate, or heavy surface detail) that each cell displayed. Tabulations of the number of cells displaying each type of surface detail were prepared from each specimen by an investigator who was unaware of which specimen corresponded to each hydrazine concentration. Evaluation of the observed data was verified with Student's *t* test (confidence level at least 95%).

Heart Culture/Electrophysiology

Neonatal rat (1–2 days old) ventricular cells were cultured in Rose chambers according to the methods

previously described (Kitzes and Berns, 1979). Glass micropipet microelectrodes filled with 2.7 M K-citrate were utilized for intracellular recording. Electrodes were selected with resistances between 20 and 50 megohms. Individual contracting myocardial cells were selected for impalement by observation through a Nikon inverted-phase microscope. The microelectrode was carefully lowered into the selected cell using a de Fonbrune pneumatic micromanipulator, and the electrical properties of the cell were recorded and analyzed according to the procedures described earlier (Kitzes and Berns, 1979). Recordings were made both before exposure to hydrazine and during the indicated times after the medium bathing the cells was replaced by medium supplemented with 0.01, 0.1, or 1.0 mM hydrazine.

RESULTS

Figure 1 demonstrates the basic dose-related growth response of four cell types to hydrazine concentrations of 0.01 to 10 mM. Although the different cell types expressed varying degrees of sensitivity toward hydrazine, all cell types showed several common responses. Hydrazine was cytotoxic to all populations tested at dosages ranging from 0.5 to 4 mM, depending on the cell type. At lower concentrations, hydrazine produced a dose-dependent suppression (but not complete inhibition) of population growth with 0.01 to 1.0 mM being the threshold range of response for most cell types tested. For each cell type, there appeared an optimum dose (OD) of hydrazine which initially suppressed population growth from control levels and yet allowed the treated population to recover to log phase growth. This dosage appeared to be 1.0 mM for PTK₂, 1.0 mM for A6, 0.1 mM for WI38, and 0.05 mM for CH cells. The return of OD-treated populations to log phase growth implies that either the action of hydrazine on a cell may be short-lived or that nonhydrazine sensitive cells in the population are being selected for.

In addition to being dose dependent (Fig. 1), the growth suppression seen in both A6 and PTK₂ cell types appeared to be related to the length of time of hydrazine exposure.

FIG. 1. Growth response curves of four cell lines (PTK₂, WI-38, A6, and CH) to solutions containing the indicated hydrazine concentrations. Cells were exposed to fresh hydrazine at every point along the graph throughout the duration of the experiment. Arrows indicate the point of initial treatment.

The effects caused by various exposure lengths of 1.0 mM hydrazine on PTK₂ and A6 cells are illustrated in Fig. 2. These cultures were replenished with medium containing fresh hydrazine at the initial exposure point (arrow) and at every subsequent data point up to 24 or 96 hr. The cultures designated "continuous" received fresh hydrazine-containing medium at each data

point throughout the experiment. Beyond the designated time of treatment, the medium was replaced at each data point with fresh medium containing no hydrazine. The net population increase observed in cultures receiving fresh hydrazine containing medium at every point beyond induction is noteworthy. It suggests either a selection of genetically resistant cells or a physio-

logical adaptation of cells to hydrazine. It is not likely that the observed effect is due to hydrazine breakdown in the medium; if this were occurring, at least an initial depression in growth rate should be observed after exposure to each fresh hydrazine application.

To test for the selection of hydrazine resistant cells, OD-treated A6 cells which achieved log phase growth (Day 10) were replated and treated anew with fresh hydra-

zine (Fig. 3). The "selected" cells responded to hydrazine treatment with severe population growth suppression similar to the untreated "naive" cells. The difference between the growth rates of "selected" and "naive" cells was insignificant when compared to the control growth rate. There was no appreciable difference between growth rates of untreated "naive" and untreated "selected" controls (data not shown).

FIG. 2. Growth curves of A6 cells and PTK₂ cells treated with 1.0 mM hydrazine for varying exposure periods. Established cultures received medium containing fresh hydrazine at each point from the initial point of treatment (indicated by arrow) up to the designated time—24 or 96 hours, or throughout the duration of the experiment (continuous). Beyond the indicated treatment, the cultures received fresh medium without hydrazine at each subsequent point.

These results indicate that no genetic selection was operating in the observed recovery from hydrazine-induced growth suppression. Further attempts to detect hydrazine-induced genetic mutation in several cell lines via ouabain resistance and growth in sloppy agar were completely unsuccessful.

Besides unsuccessful mutation assays, several other experiments were performed to detect hydrazine-induced cellular anomalies which would account for the behavior of the experimental populations. Examination of earlier Coulter counter data of control and hydrazine treated populations revealed an apparent cell size increase in the experimental cultures (Fig. 4). In a typical dose response growth curve of 72-hr-treated populations, the experimental cultures had a measurably larger mean cell volume than the control cultures (plots of cell populations are taken from Day 5 samples of control and 1.0 mM-treated A6 and PTK₂ cells shown in Fig. 1). Further

FIG. 3. Effects of 1.0 mM hydrazine on the growth rates of two subpopulations of A6 cells: "selected" cells, which had been grown to confluency in the presence of 1.0 mM hydrazine, and "naive" or unselected A6 cells. Both populations were treated with hydrazine throughout the experiment from initial time of exposure (indicated by arrow).

TABLE 1

INCIDENCE OF MULTINUCLEATION OCCURRING IN BOTH CONTROL PTK₂ CULTURES AND EXPERIMENTAL CULTURES TREATED FOR 2 DAYS WITH 1.0 mM HYDRAZINE

	Uni-nucleated cells (%)	Multi-nucleated cells (%)	Total No. cells
Control cultures	92	8	170
Experimental cultures (hydrazine treated)	78	22 ^a	159

^a Hydrazine-treated cultures had significantly more multinucleate cells than did controls (at least 95% confidence).

microscopic analysis of PTK₂ cells revealed significantly more multinucleate cells in 1.0 mM hydrazine treated populations than in control populations (Table 1, Fig. 5). This threefold increase in multinucleation resulted in each of triplicate experiments performed.

The increase in cellular multinucleation suggests that hydrazine may act to produce either abnormal mitosis resulting in multiple nuclei or cell surface alterations promoting cell fusion. Experiments were undertaken to test for both possibilities in 1.0 mM-treated cell populations. Because PTK₂ cells remain perfectly flat throughout mitosis (Rattner and Bernis, 1974), it was possible to carefully observe mitosis by light microscopy. Studies employing still and time lapse photography revealed no difference in mitotic abnormalities between control and treated populations. However, evidence for increased cell fusion in hydrazine-treated populations implicated the cell surface as a possible target site for hydrazine action (Table 2). In a mixed population of PTK₂ cells having either regular or ³H-tagged nuclei, the presence of multinucleated cells containing both types of nuclei (tagged and untagged) suggested that cell fusion was occurring. There was a fivefold increase of these types of cells in the hydrazine-treated populations as compared to the con-

FIG. 4. Effect of 1.0 mM hydrazine on the relative distribution of individual cell volumes from PTK₂ and A6 suspended cell cultures (from Fig. 1, Day 5) as determined by Coulter counter-channelizer plots. Abscissa represents 100 graded relative cell volume channels. Ordinate illustrates the number of cells in a population fitting a certain channel size; maximum cell number per channel = 1000.

trol populations (Fig. 6 and Table 2). Furthermore, the quantity of these cells comprising the entire multinucleate population of a culture was significantly higher (1.5-fold) in the hydrazine-exposed cultures than in control cultures.

To further investigate membrane effects, cell surface morphologies of hydrazine-

treated and control A6 cells were studied (Fig. 7). Table 3 presents data showing the effect of hydrazine on the cell surface. It is clearly evident that there is a significant, inverse relationship between the hydrazine concentration a cell received and the amount of SEM detectable cell surface projections it displayed. Furthermore,

FIG. 5. Phase contrast micrographs of PTK₂ control cultures (A) and 72 hr, 1.0 mM hydrazine-treated cultures (B). Magnification, 2200 $\times$.

TABLE 2

INCIDENCE OF CELL FUSION OCCURRING WITHIN THE MULTINUCLEATED POPULATIONS IN BOTH CONTROL PTK₂ CULTURES AND EXPERIMENTAL CULTURES TREATED FOR 3 DAYS WITH 1.0 mM HYDRAZINE

	Uni-nucleated cells (%)	Multi-nucleated cells (%)	Similarly labeled nuclei ^a (%)	Dis-similarly labeled nuclei ^b (%)
Control cultures	95	5	4	1
Experimental cultures	85	15	10	5 ^c

^a Refers to multinucleated cells containing either all normal or all [³H]thymidine-tagged nuclei.

^b Refers to multinucleated cells containing both normal and [³H]thymidine-tagged nuclei.

^c Hydrazine-treated cultures had significantly more dissimilarly labeled multinucleated cells than controls did (confidence level at least 95%).

this inverse relationship is concentration dependent, paralleling the results of the growth response curves.

Since the cell fusion data and the SEM data implicated the cell membrane as a primary site of hydrazine action, additional studies were undertaken to examine the effect of hydrazine (0.01 to 1.0 mM) upon membrane-associated electrical and contractile activities of neonatal rat myocardial ventricular cells in culture.

A typical intracellular recording of spontaneous action potentials recorded in a rhythmically contracting heart cell is shown in Fig. 8a. The resting membrane potential is approximately -60 mV, and the action potentials occur at a rate of approximately 1 per second. In Figs. 8b and c are shown typical recordings 10 and 20 min following exposure of the culture to 0.01 mM hydrazine. Three effects are apparent: (1) a depolarization of the resting membrane potential, (2) a reduction of action potential amplitude, and (3) a disruption of discharge rhythmicity. These electrical changes were accompanied by a disruption of the rhythmic contractile behavior of the cell. However, electrical activity returned to almost

normal status 30 min after cells initially received medium containing 0.01 mM hydrazine (Fig. 8d).

Exposure to 0.1 mM hydrazine (Fig. 9b) resulted in (1) a more severe reduction in membrane potential that is still evident 15 min after exposure, (2) a complete absence of action potential discharge, and (3) arrhythmic baseline activity. During this time, the cell was not contracting. At this hydrazine concentration, the cells resumed normal electrical and contractile activity after 45 min in the hydrazine-supplemented medium (Fig. 9c). Cells exposed to 1.0 mM hydrazine (Fig. 10) did not recover after 45 min to 1 hr in the experimental medium. At this time (Fig. 10), only very aberrant electrical activity accompanied by occasional small and atypical contractile activity was observed. However, these cells resumed normal electrical and contractile activity 15 min after the hydrazine-containing medium was replaced with normal medium (data not shown).

DISCUSSION

The purpose of this study was to examine the basic cellular effects of hydrazine. Previous studies have linked hydrazine and its derivatives with carcinogenic and mutagenic effects. Carcinogenic effects of the hydrazine analog 1,2-dimethylhydrazine have been reported *in vivo* in studies of rodent intestines (Barlka and Tutton, 1977, 1978; Jacobs, 1977; Mak and Chong, 1978; Richards, 1977; Sunter *et al.* 1978; Toth *et al.* 1976) and blood vessels (Toth and Wilson, 1971). Hydrazine has been shown to mutate DNA from a variety of sources (Raphalian, 1966; Dave, 1977; Brown, 1967; Brown *et al.*, 1966; Gupta and Grover, 1970; Kak and Kaul, 1975; Kimball, 1977; Kimball and Hirsch, 1975, 1976; Lemontt, 1977). These effects appear as the result of the chemical treatment of selected target tissues under specific conditions. In this study, however, a diverse variety of tissue culture cell types were employed to ascertain the basic cellular effects of hydrazine.

FIG. 6. PTK₂ cell cultures, stained after autoradiography with methylene blue, from control (A) and 32 hr, 1.0 mM hydrazine-treated (B) cultures. Cells containing both labeled and unlabeled nuclei were formed by cell fusion. Magnification, 1900 $\times$.

FIG. 7. Scanning electron micrographs of A6 cells which typify (A) heavy, (B) moderate, and (C) light cell surface projections. Bar, 5 μ m.

The noted hydrazine-induced growth effects shown in this study could be classified into two basic types of responses. First of all, the cells demonstrated a concentration-dependent response to hydrazine treatment (Figs. 1, 8, 9, 10 and Table 3). In these studies, different hydrazine concentrations, usually spanning three or four orders of magnitude, caused effects ranging from imperceptible cellular changes to lethality. Within this concentration range, the cells could survive, proliferate, and function.

TABLE 3

EFFECTS OF VARIOUS HYDRAZINE DOSAGES ON SEM DETECTABLE XENOPUS CELL SURFACE PROJECTION

Hydrazine concentration (mM)	Cells with light surface projection (%)	Cells with moderate surface projection (%)	Cells with heavy surface projection (%)	Total No. cells sampled
0	24	37	39	196
0.01	35.5	31.5	33	186
0.1	60	31.5	8.5	188
1.0	79	17	4	203

Note. Statistical analysis verified a significant difference in the quantity of light or heavy cell surface projection present between populations of control cultures and populations receiving either 0.1 or 1.0 mM hydrazine (confidence level at least 95%).

Second, in the growth response curves, the growth rate remained depressed while cells received intermittent rechallenges to the higher hydrazine concentrations; however, the growth rates resumed normal log phase levels when hydrazine-containing medium bathing the cells was replaced with normal medium (Figs. 2, 3, Control—see Methods). At the lower concentrations of hydrazine treatment, the cells exhibited an

FIG. 8. Effects of exposure to 0.01 mM hydrazine on the spontaneous intracellular electrical activity of rhythmically contracting myocardial cells in culture: (a) Control activity (no exposure to hydrazine); electrical activity after exposure to 0.01 mM hydrazine for (b) 10, (c) 20, and (d) 30 min.

FIG. 9. Effects of exposure to 0.1 mM hydrazine (a) control; (b) 15 min after culture received 0.1 mM hydrazine solution; (c) 45 min after culture received 0.1 mM solution.

initial depression in growth, but, upon re-exposure to fresh hydrazine, they appeared to attain a normal growth rate. Likewise in heart cell cultures, the aberrant electrical and contractile effects produced by the presence of 1.0 mM hydrazine abated when these cells were washed and bathed in normal medium. In the presence of low hydrazine concentrations, treated heart cells appeared to show some recovery from the observed effects (Figs. 8, 9).

The mechanism whereby treated cells rebound in the presence of fresh hydrazine is unclear. It seems possible that this "tolerance" or "recovery" may be due to an increased capacity of exposed cells to inactivate hydrazine or its effectual metabolite. The production of "tolerant" cells in the presence of hydrazine could also suggest the selection of genetically resistant cells or the alteration of an affected organelle to a hydrazine-refractory state. Regardless of

the mechanism, it appears that hydrazine evokes some form of selection or tolerance. This observation is supported by data showing that entire populations, rather than a few selected cells, seem altered by hydrazine treatment (Figs. 3, 4). Furthermore, Fig. 3 indicates that, in the tissues studied, hydrazine acted in a disruptive but nonmutational manner. It therefore seems logical that hydrazine may elicit these concentration dependent and reversible responses by actively and nonmutagenically interfering with a common cellular site in a wide spectrum of cell types. The SEM and electrophysiological studies, which were done on very different cell types, suggest that the cell surface may be a common target site of hydrazine action. Furthermore, the increase of multinucleation by cell fusion seen in hydrazine-treated cultures also indicates that the cell surface is a major site of this compound's action.

The above observation is further supported by the literature in which hydrazine derivatives have been shown to elicit a wide variety of effects in studies performed on biological membranes (Balduini *et al.*, 1977; Barkla and Tutton, 1977; Braun and Wolfe,

FIG. 10. Effects of exposure to 1.0 mM hydrazine (a) control activity, (b) aberrant electrical activity after 1 hr in 1.0 mM hydrazine solution.

1977; Caroni, 1977; Jain and Subrahmanyam, 1978; Jain *et al.*, 1977, 1978; Katsumata *et al.*, 1977; McLean *et al.*, 1978; Tsau *et al.*, 1977; Walter *et al.*, 1978; Zimmer, 1977). The observations of these numerous studies support the view that the hydrazine-induced cellular effects seen in our data stem from membrane interactions with this compound. Furthermore, it seems logical that such a strong reducing agent as hydrazine would directly affect the cell structure at first contacts.

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