Mechanistic Toxicology of Triethyl Citrate in Cultured Mammalian Cells by Cinematography

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The role of triethyl citrate in growth inhibition of mouse fibroblast cells (strain L-929) in culture has been studied by the use of phase-contrast microscopy and time-lapse microphotography. Concentrations of the ester studied ranged from 7.9 to 48 m*M* of growth medium. It was found that at concentrations below 12 m*M*, the primary site of action of the toxicant was mitochondria resulting in mitochondrial swelling, inhibiting the function of this organelle, and growth inhibition. At higher dose levels, a second mechanism masks the mitochondrial involvement. The cytological picture is characterized by cytoplasmic gelation, plasma membrane contraction, and eventual lysis of the cell. The mechanism proposed was protein denaturation, but further studies are necessary for its definition. The method is a valuable tool for observing the effects of toxicants on living cells. When coupled with biochemical procedures, it offers a workable technique for the interpretation of mechanisms of actions of toxicants.

TRIETHYL CITRATE and other esters of citric acid are commonly used as plasticizers in polyvinyl chloride and cellulosic formulations. The *in vitro* toxicity of triethyl citrate has received some attention in the past as evidenced by previous studies from this laboratory (1). In these studies, Rosenbluth demonstrated the inhibitory effect of a homologous series of citric acid esters on the growth of mammalian cells in culture, and characterized triethyl citrate as being a nonspecific physical toxicant. As a physical toxicant, its biological effects could be related to the physical properties of the compound by mathematical relationships derived by Ferguson (2). In the homologous series studied, triethyl citrate represented the lowest number of the series exhibiting this phenomenon of physical toxicity since trimethyl citrate toxicity was shown to be of a chemical nature.

Compounds exhibiting physical toxicity are generally thought to accumulate in the lipoprotein of the cell due to their specific physical properties. Previous work on the mechanism of action of depressants, which fall into the category of physical action, have yielded two proposed mechanisms of action. Albert (3) suggested that the accumulation of a depressant drug caused a swelling of the plasma membrane. This swelling results in the mechanical separation of the enzyme systems necessary for active transport. Also, this swelling could result in a physical blocking of the pores in the membrane, thus again blocking the passage of material into and out of the cell. On the other hand, in 1961, Pauling (4) suggested that depressants exert their activity by forming clathrates through localized complexation of crystalline water in the cell.

A different mechanism of action for depressants was proposed by Warburg in 1921 (5). He suggested that they act by an inhibition of respiration of the cell at the level of the mitochondria. Later biochemical evidence by Butler (6), demonstrated that amobarbital specifically interfered with the cytochrome reductase system resulting in a decreased cellular respiration.

In contrast to these above noted modes of action, compounds exhibiting chemical specificity are in numerous reports in the literature. In the cases of these chemical toxicants, the biological activity cannot be related to the physical characteristics of the compound in question by mathematical relationships as previously mentioned. They are able to evoke a biological response at a much lower concentration than is predicted in Ferguson's relationships.

As a continuation of the *in vitro* studies previously mentioned, the present study was undertaken to elucidate a mechanism or mechanisms by which triethyl citrate acts at the subcellular level to inhibit cell growth. The approach taken was primarily based on the fact that dynamic and morphological changes induced by a toxicant in cellular and subcellular structures are directly related to biochemical changes which have occurred previous to the time of the noted change. Through the establishment of a sequence of events which lead to growth inhibition and possibly cell death, it was felt that the deduction of general biochemical changes which occur preceding these events might be possible and a mechanism of action obtained.

In normal cellular functions and activities,

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the cell maintains a high degree of integrity. This maintenance is part of a dynamic system and requires a continuous source of energy. The subcellular organelle, the mitochondrion, supplies this energy in the form of adenosine triphosphate (ATP), which it manufactures through a series of metabolic reactions. Since the mitochondria are responsible for maintaining the energy system of the cell, any interference or inhibition of their functions would result in a significant effect upon cellular activity and should be manifested in altered cellular morphology (7).

The mitochondria have been shown to be quite sensitive to unfavorable conditions for cell life (8) yet at times quite resistant (9). However, other cellular organelles and cellular substructures such as the nucleus, plasma membrane, nuclear membrane, *etc.*, play a key role in maintaining the cellular integrity, and their responses to a toxicant should be evaluated in the interpretation of any of the biochemical reactions which occurred preceding the noted morphological change.

In order to obtain these data, the system of choice was the phase contrast microscope since it enabled observations to be made on the living cell. Their morphological alterations were noted under the influence of triethyl citrate administration and recorded with aid of time-lapse cinematography.

MATERIALS AND METHODS

Mouse fibroblast cells (Earle's NCTC929), a typical mixture of cell types maintained in static culture in this laboratory, were used exclusively. The cell line was last cloned in 1963 and has undergone an unknown number of passages since that time. The cells were cultured in Eagle's basal medium (10) plus 4.0 ml. of calf serum, 1.0 ml. of a 200 mM solution of L-glutamine, and 5.0 mg. of streptomycin/100 ml. of growth medium in a 5% CO_2–air atmosphere maintained at 35°. Routine subcultures were made every 4 days using a 0.05% trypsin solution.

Sykes-Moore tissue culture chambers (Belco Glass, Inc.), fitted with 2.5-mm. working distance rubber "O"-rings, were used for all microscopic work. Upon the preparation of the culture chambers at the time of subculturing, a cell suspension of approximately 10^4 cells per ml. was added to the chambers using the procedures of Sykes and Moore (11). After the chambers were filled with the cell suspension, they were placed right side up in an incubator at 35° and allowed to remain in that position for 12 hr. to allow the cells to settle to the bottom and attach to the lower coverslip.

Time-lapse experiments were begun 24 to 36 hr. after the chambers were filled. The purpose of this lapse in time after filling was to insure that the cells had recovered from any of the trauma induced by trypsinization during the subculturing procedures.

Prior to infusion of the test chemicals, study cells were observed by photography for a minimum of 30 min. for normal activity, thus serving as an internal control. Repeated media change induced no cellular alterations during control periods of study.

Concentrations of the triethyl citrate (Chas. Pfizer and Co., Inc.) used were determined by weighing the ester and dissolving it in the required amount of growth medium. The concentrations used in this study were 7.9 mM, 9 mM, 12 mM, 24 mM, and 48 mM and were prepared immediately before use. Approximately 6 mM and 12 mM represent the ID_{50} and the ID_{100} for the 72-hr. cultures previously reported (1). The introduction of the medium plus chemical was accomplished by perfusing the chamber with exactly 1.0 ml. of medium containing the drug. This resulted in a concentration in the chamber of 0.65 times the concentration of the perfusion medium (11, 12). Prior to infusion, all fluids were prewarmed to 35° and the pH of the medium was adjusted by aeration with an air mixture containing 5% carbon dioxide. In this communication, all concentrations indicated are those of the medium in the chamber and not the perfusion medium. It should also be noted that all procedures discussed to this point were conducted using aseptic techniques.

For observation, the chambers were placed on the stage of a Nikon model M inverted phase-contrast microscope housed in an electric incubator maintained at 35°. The illuminator was a low voltage lamp (6 v., 15 w.) equipped with both a green filter and a heat reflecting filter. A 100× oil immersion phase-contrast objective was chiefly used. The time-lapse equipment consisted of a Bolex SH 44 16 mm. movie camera with Nikon's connecting hardware and a time control panel with camera drive mechanism (R. J. Matthias and Associates). Other photomicrographic equipment consisted of a Nikormat FS 35 mm. camera with Nikon adaptor connections.

The individual cells for time-lapse observations were selected by scanning the culture for a cell undergoing mitosis, then waiting 8 hr. after anaphase before the administration of the triethyl citrate. At this time, all cells were well into interphase and had taken on their normal interphase characteristics and certain subcellular structures were readily visible. After the administration of the triethyl citrate, the cells were observed for 4 hr. with exposures taken every 3.6 sec.

Kodak 16 mm. Tri-X reversal movie film and Kodak 35 mm. Plus-X panatomic film were used exclusively. The processed movie film and prints from the 35 mm. film were analyzed for morphological alterations. The movie film was analyzed not only at normal projection speeds which accelerated movements in the cells by about 70× but also through the use of a single frame projection editor made in the laboratory. Replicate experiments were conducted at each dose level.

RESULTS AND DISCUSSION

The normal L-cell during interphase (Fig. 1) in a static culture is firmly attached to the glass surface of the container in which it is growing. It may be found in one of many configurations, ranging from almost circular to greatly elongated, such that its length may be 8 to 10 times its width.

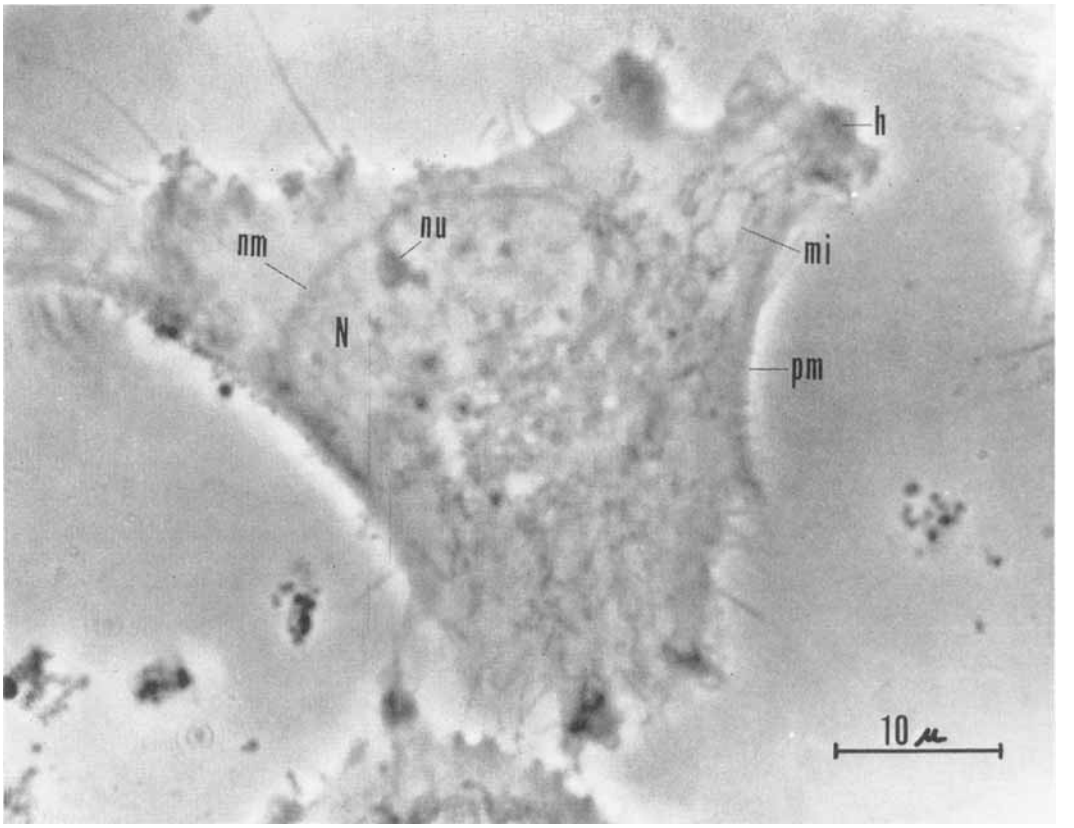

Fig. 1—Normal interphase cell illustrating the nucleus (N), *nuclear membrane* (nm), *nucleolus* (nu), *plasma membrane* (pm), *mitochondria* (mi), *and hyaloplasmic zone* (h).

Control experiments showed that in general, the cells appeared somewhat elongated (Fig. 1), such that the length was two to three times the width. The plasma membrane, which is responsible for the maintenance of concentration gradients between the cytoplasm and the exterior medium appears very irregular with many filament-like extensions. These are actual extensions of the membrane and are filled with cytoplasm. On time-lapse photography, these zones of the plasma membrane show very little movement.

Active areas of movement were seen in zones of the plasma membrane where the membrane had extended beyond the points of attachment to the glass. The entire zone, known as the hyaloplasmic zone, is noted to undergo wavelike motions when the cine records are observed. This hyaloplasmic zone is generally associated with pinocytosis and quite frequently, a small vesicle arising from pinocytosis is seen in the adjacent cytoplasm.

The normal cytoplasm is filled with small particulate material, most of which is distinguishable only as ground substance due to the limit of resolution of the light microscope. This ground substance is more concentrated in the perinuclear area with the peripheral cytoplasm being relatively free of visible particulate matter. However, among this ground substance, one major subcellular structure, the mitochondria, is readily discernible. The mitochondria generally take on long rod-shaped configurations which are especially identifiable in the peripheral cytoplasm. Other than vesicles, these mitochondria are generally the only subcellular structure which are readily identifiable in the hyaloplasmic zones.

Located near the middle of the cytoplasm, the nucleus of the normal 8-hr. cell was readily visible. It generally has a kidney-shaped cross section, the indentation being due to the presence of the rigidly fixed centrioles in the cytoplasm. The membrane boundary of the nucleus was sharply outlined with smoothly curving contours. The nucleoplasm appeared homogeneous at magnifications used in this study with one to five nucleoli spread throughout it. The nucleoli appeared as darker condensed masses with fairly well-defined peripheries.

Control experiments revealed the following additional facts about the normal cell from 8 to 12 hr. after anaphase. As has been previously indicated, in the 4-hr. observation period, there is little movement of the plasma membrane, except for the wavelike movements in the hyaloplasmic zones. The vesicles arising in these areas migrated toward the center of the cell and during the migration increased in absorbance while they decreased in size. As they joined the ground substance in the perinuclear zone, they became indistinguishable.

The mitochondria in the peripheral areas of the cytoplasma undergo active movements. These movements appeared to be associated with the localized movements of the cytoplasm. The ground substance in the perinuclear zone was observed to

migrate both in and out of the area as there is a constant transfer of material to the various locales within the cell.

The nucleus retained its general orientation with the rest of the cell. However, with time it often rotates about the centrioles. This rotation may or may not occur, since in some controls it rotated and in others it did not. When rotations did occur, the maximum angle of rotation was observed to be about 30°.

When a normal healthy cell was exposed to a medium change while under observation, there was no change in the morphological characteristics previously mentioned. However, when various concentrations of triethyl citrate were present in the infusion medium, the following responses of the individual subcellular units were noted.

Plasma Membrane—In early response to the toxicant, the plasma membrane retracted as indicated by the disappearance of the hyaloplasmic zones (Fig. 2, *A* and *B*). At all dose levels this response was observable. The onset times ranged from 2 to 3 min. at the lower concentrations to a matter of seconds at the higher doses. The initial contraction was followed by a general contraction of the plasma membrane with a decrease in cross-sectional area of the cell and an accompanying decrease in surface area of the plasma membrane. This general contraction was a gradual process as indicated by the changes in cross-sectional area of the cell as illustrated in Fig. 2, *C* and *D*. The general retraction of the plasma membrane was observable on the cine records within 15 min. at all dose levels except 7.9 m*M*. In this cine record, membrane activity appeared normal until 3 hr. after dosing. At this time, slight retraction was noticeable.

At doses of 24 m*M* and 48 m*M* the general retraction of the membrane appeared to be continuous with the retraction of the hyaloplasmic zones. Also at the 48-m*M* dose level, the retraction was followed by the appearance of superficial vacuoles (Fig. 3) at 2 hr. Lysis of the cell followed within a few minutes. Cultures at other dose levels were not observed continuously until death. However, the 12-m*M* and 24-m*M* cultures were dead within 24 hr. Their mode of death also appeared to be lysis with the superficial vacuoles still present.

Cytoplasm—As indicated above, the ground substance and the particulate matter in the cytoplasm moved about in definite patterns in the normally functioning cell. In general, triethyl citrate caused a slowing of movements of the particles through the cytoplasm. This phenomenon, called cytoplasmic gelation was seen in the cine records at higher dose levels, but appeared to be dose dependent. The onset of gelation occurs simultaneously with the general retraction of the plasma membrane. At dose levels of 9 m*M* and 12 m*M*, the gelation appeared to start from the peripheral portions of the cell and migrate toward the nucleus. At the higher concentration levels, the onset was so rapid that such a migration was not observable. Total cessation of movement was observed only at the 48-m*M* level. Immediately prior to lysis, all cellular movements ceased. Also only at this dose level did the movements of the ground substance ever appear to be inhibited in the region of the centrioles and Golgi complex.

Appearance of Dense Granules in the Cytoplasm—At all dose levels with the exception of 48 m*M*, within 20 to 30 min. after the infusion of the medium containing the drug, small dark spheres of 0.5 to 1 μ in diameter appeared in the cytoplasm (Fig. 2, *C* and *D*). At the higher dose level, lysis occurred so rapidly that this response was apparently not observed. Associated with the appearance of these spherical granules was the disappearance of the rod-shaped mitochondria. Examination of the cine records revealed that the spherical granules appeared to arise from the mitochondria. Thus these dense particles are apparently swollen mitochondria. Their appearance in different areas of the cytoplasm followed no set pattern, nor was it a totally dose-related phenomenon. However, it was uncommon to see them in the region of the centriole, the area least affected by gelation. They were generally confined to other portions of the cytoplasm.

Nucleus—The nucleus appeared to be unaffected during the period of observation at dose levels below 12 m*M*. However, at this dose and at 24 m*M*, the nuclear membrane did appear to increase in optical density as if some materials were being deposited on or in it. This nuclear involvement occurred at 3.5 hr. and 3 hr., respectively. The same response was noted at the 48-m*M* dose level at 1.5 hr. Associated with this response, the nuclear membrane lost its smooth contour and became very irregular with a scalloped configuration. However, at 48 m*M*, there followed a constriction of the nucleus with an increase in the optical density of the nuclear membrane and a decrease in the optical density of the nucleoplasm. The nucleoli also increased in their optical density (Fig. 3). Upon lysis of the cell, the nucleus remained in this fixed configuration. The same fixed nuclear morphology was noted in the lysed cells dosed at 12 m*M* and 24 m*M* which were discussed above.

DISCUSSION

In the light of the previously described results, it is concluded that there are at least two possible mechanisms of action by which triethyl citrate inhibits cell growth and results in cell death. At the lower dose levels, where growth inhibition has been noted (1), triethyl citrate acts specifically on the mitochondria, eventually resulting in a decrease in the production of ATP, and thus a decrease in the growth rate of the cultures. As the dose level was increased to 12 m*M*, another mechanism probably comes into play which at higher concentrations (48 m*M*) completely masks any mitochondrial involvement. In such situations, the triethyl citrate precipitated the soluble proteinaceous materials in the cytoplasm resulting in osmotic changes and cell lysis.

If the toxicant is to act intracellularly, it must cross the plasma membrane by one of several mechanisms. Different mechanisms by which a compound may enter the cell are pinocytosis, phagocytosis, active transport across the membrane, and simple diffusion across the membrane. Triethyl citrate does cross the cell membrane and acts intracellularly as evidenced by the various morphological alterations noted. The exact mechanism of the penetration was of course not determined in this study but it was concluded that the

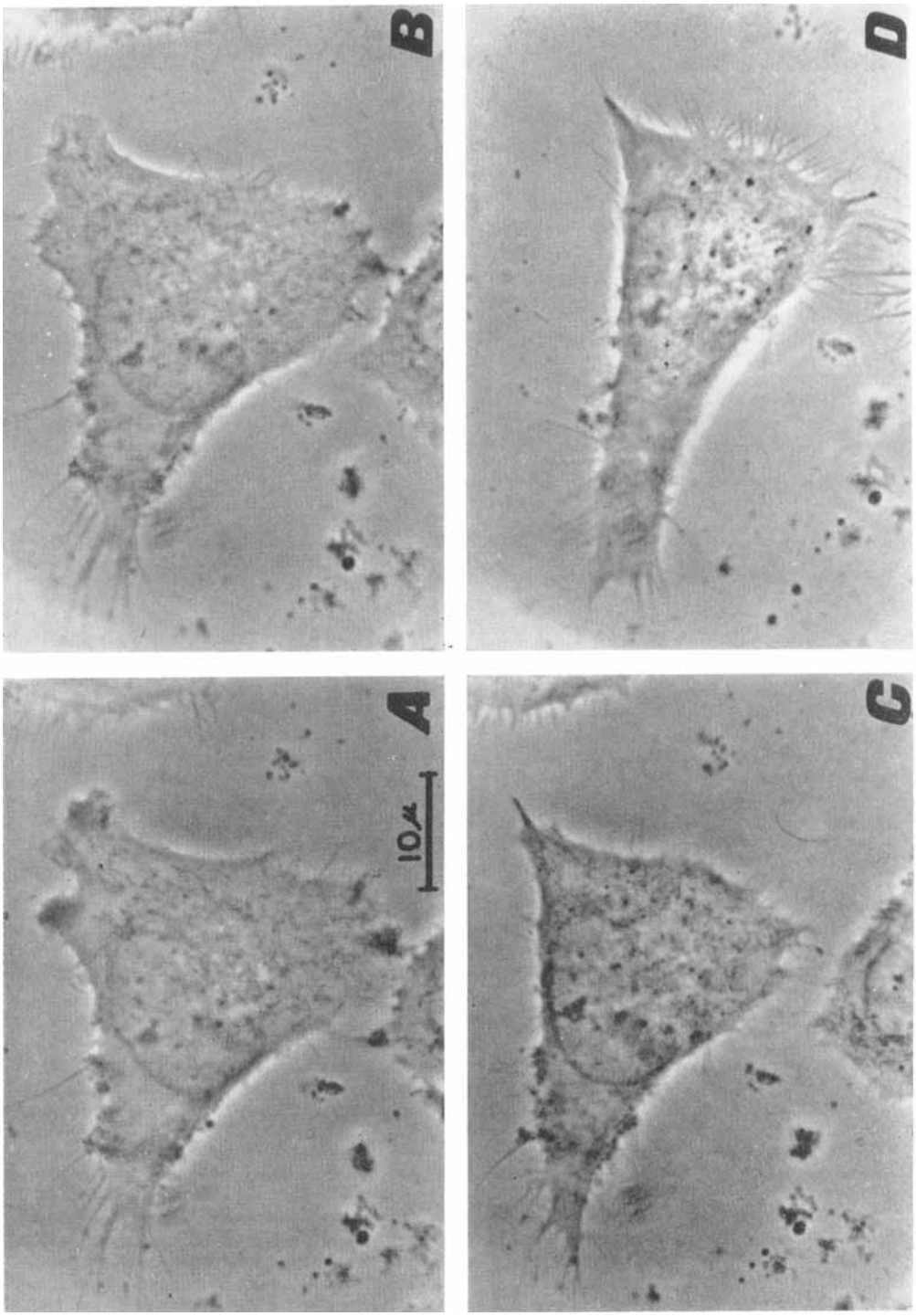

Fig. 2—Responses of a cell when dosed with a 16-mM solution of triethyl citrate. Key: A, prior to dose; B, 2 min. after dose; C, 1 hr. after dose; D, 5 hr. after dose. (See text for discussion.)

compound crossed by simple diffusion or active transport, since under the experimental conditions used with the mouse fibroblast cells, only an occasional pinocytotic vesicle appeared and no phagocytosis was observed.

The primary result of the intracellular activity of triethyl citrate at the lower concentrations (7.9 m*M* and 9 m*M*) was eventual mitochondrial swelling, since this reponse was observed well before any other responses. Similar morphological alterations in mitochondria have been noted by other workers. Biesele (8) observed this phenomenon in the treatment of embryonic mouse skin fibroblasts and mouse sarcoma 180 with 6-mercaptopurine, and Chevremont *et al.*, (13), noted it with trihydroxy-*N*-methylindole when treating primary embryonic chick

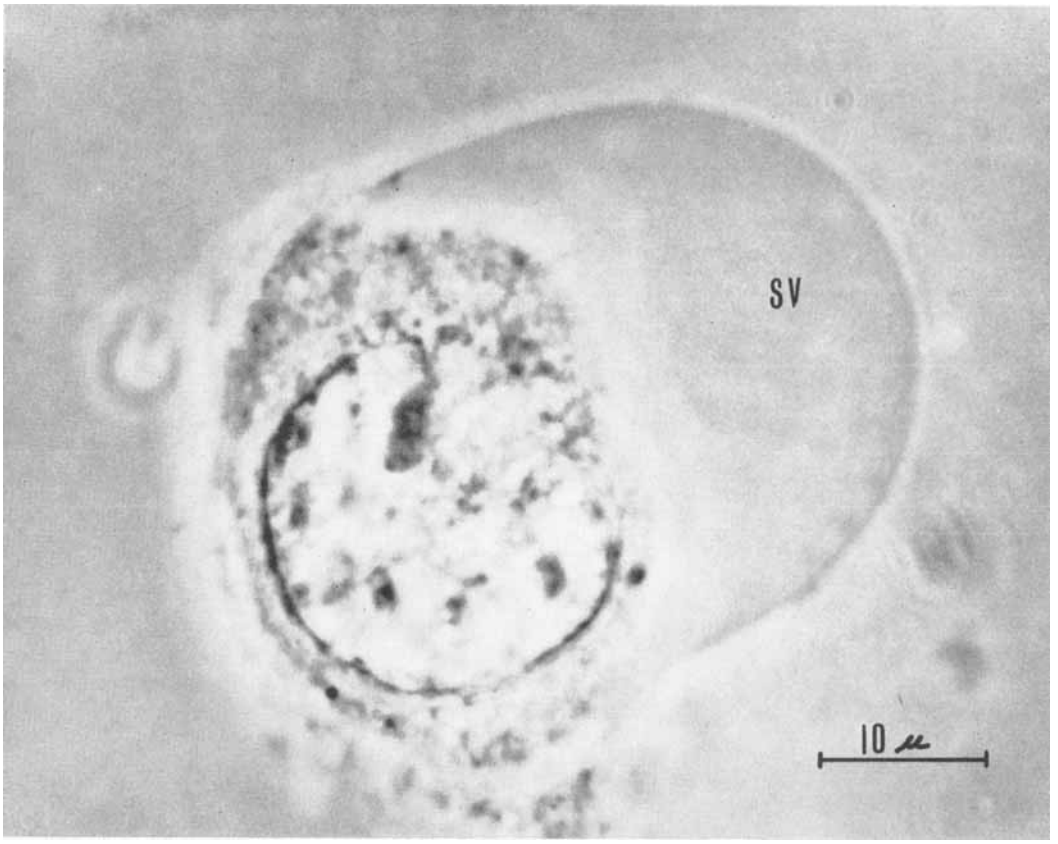

Fig. 3—Cell prior to lysis. Note superficial vacoule (sv), *rounded configuration, irregular nuclear membrane, and condensed nucleoli.*

muscle cultures. As Biesele indicated, mitochondria are notoriously sensitive to unfavorable conditions of cell life and the specificity of any morphological change in them is therefore open to question when this parameter is considered alone.

In studies with L-cells, Danes and Struthers (14), demonstrated that an iron–dextran complex interfered with the respiratory system (the mitochondria) of the cell and with only a slightly reduced respiratory activity, there was a greatly reduced growth. In similar studies with L-cells, Golaz (15) has demonstrated a decrease in the ATP production with 9m*M* and 12m*M* concentrations of triethyl citrate. These decreases were noted after 1.5 hr. of treatment.

Since ATP is produced in the mitochondria and observation of cytological changes showed mitochondrial swelling, it is concluded that this organelle is a specific point of attack for the triethyl citrate. As indicated above, only a slight depression in the respiratory system is necessary for growth inhibition. Thus, it is believed that triethyl citrate causes mitochondrial swelling and interferes with ATP production and respiration of the cell, whereby growth inhibition results.

On the other hand, at dose levels above 12 m*M*, additional response noted cannot be attributed to mitochondrial involvement. As has been indicated, plasma membrane contraction and cytoplasmic gelation are apparent at these dose levels prior to mitochondrial swelling. It is well known that many compounds of a chemical nature are capable of precipitating proteins. Pure triethyl citrate, when added to calf serum, causes an immediate precipitation of serum proteins. However, in concentrations used in the growth medium, no noticeable precipitation was noted. This points to a possible complexation of triethyl citrate with proteins in the cell, resulting in the observed progressive gelation. With high concentrations in the medium, the triethyl citrate rapidly penetrated the cell membrane. In doing so, some complexation with membrane lipoprotein could occur, but the data did not demonstrate this, since the membrane itself is well below the resolution of the phase-contrast microscope.

As the triethyl citrate entered the cytoplasm, and increased in concentration in this phase, there was an apparent interaction with the proteinaceous material there. Specifically, there appeared to be some interaction with the actin-myosin-like proteins in the cytoplasm which, among other things, are associated with the movements of the cytoplasm throughout the cell (16). Such an interaction would account for the rapid cessation of movements in the hyaloplasmic zones. Further interactions would account for the contraction of the membrane and the cessation of movements in other areas of the cytoplasm. In addition to the actin-myosin-like proteins, other cytoplasmic proteins may also be affected and thereby play a role in membrane contraction and the gelation phenomenon.

The decrease in the optical density of the nucleoplasm along with the condensation of the nucleoli and the contraction of the nuclear membrane probably follows this same nonspecific mechanism.

Occurring simultaneously with the nuclear contraction was the appearance of the superficial vacuoles followed by cell lysis. This response, however, was not related to the nuclear involvement but probably due to the complete gelation which was present. With such a gelation along with mitochondrial suppression the active transport system of the membrane was unable to obtain the required ATP necessary for maintaining concentration gradients necessary for normal cell function. The fact that gelation occurred rapidly at the higher dose levels also accounted for the lack of ATP for the membrane since the ATP could not be transported to the membrane through the gel matrix. Thus, the sodium ion diffused across the energyless membrane and obligatory water entered the cell resulting in the appearance of the superficial vacuoles with eventual lysis.

Bessis (17) observed similar sequential events which resulted in cell death and as he and other authors (18, 19) have pointed out, these sequential events can be a great aid in deciding where to look for supportive biochemical data.

SUMMARY

The role of triethyl citrate in growth inhibition of mammalian cells in culture has been studied by the use of phase-contrast microscopy with the following major findings.

(*a*) At dose levels below 12 m*M*, the mitochondria of the cell are initially inhibited in their functions resulting in the growth inhibition of the cell.

(*b*) At dose levels above 12 m*M*, a second mechanism masks the mitochondrial involvement. The cytological picture is characterized by cytoplasmic gelation, plasma membrane contraction, and eventual lysis of the cell. The mechanism proposed was protein denaturation, but further studies are necessary for its definition.

(*c*) Phase-contrast time-lapse cinematography is a valuable tool for determining sequential events occurring in the morphological patterns of living cells under the influence of toxic agents. When coupled with biochemical procedures, it offers a workable technique for the interpretation of mechanisms of action of toxicants, and should play a key role in bridging the gap between *in vitro* and *in vivo* systems.

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Keyphrases

Triethyl citrate toxicity
Tissue culture–mouse fibroblast cells
Eagle's basal medium plus calf serum
Membrane, plasma-toxicant effects
Cytoplasm-toxicant effects
Nucleus-toxicant effects
Phase-contrast microscopy
Time-lapse cinematography