Recently Wagner and his co-workers (10) studied the effect of dosage form on serum levels of indoxole. They found that the drug administered as a suspension produced a greater serum level response than did the hard gelatin capsules, although both were prepared from the same lot of drug with identical small particle size. They state that the most likely explanation is that the indoxole particles were less agglomerated when administered in suspension form than when given in capsules, and the surface area of indoxole presented for dissolution in the gastrointestinal fluids at the absorption sites must have been greater with the suspension.

In a recent publication, Higuchi *et al.* (6) cited various other examples where the deaggregation rates were the controlling factors in absorption.

It would appear that in dealing with insoluble drugs which are difficult to wet and disperse, the rate of deaggregation from different dosage forms should be routinely determined, just as the rate of solution, particle size, and polymorphic forms are investigated.

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Mechanistic Toxicology of Triethyl Citrate in Mouse Fibroblast Cells by Liquid Scintillation Techniques

By MARILYN GOLAZ, W. L. GUESS, and JOHN AUTIAN*

Mouse fibroblast cells, strain L-929, in static tube culture were exposed to 6, 9, and 12 mmole doses of triethyl citrate (TEC) for periods up to 78 hr. At 1.5, 48, and 78 hr. after TEC exposure, the cells were pulsed with ^{14}C-labeled purine and pyrimidine metabolic precursors in an attempt to differentiate the site of purine–pyrimidine metabolism interference. U.V. spectrophotometric analyses were run concurrently with liquid scintillation analyses. The mechanism of inhibition appeared to be initial mitochondrial involvement, with secondary DNA repression.

SINCE ALL pharmacology and toxicology begins ultimately at the cellular level, it seems only logical to elucidate mechanisms involving basic biochemical pathways by way of cellular techniques, and project or apply these findings to the whole organ or body. The advantages of cell culture in a variety of applications are many. Highly specialized cells, differentiating cells, or abnormal cells may be used so that drug effects may be seen under widely varying circumstances. Another convenience is that quantitative evaluation of activity is possible, whereas pharmacodynamic quantitization in the intact animal is often impossible due to the nature of the compound or of the system. Using cell culture techniques, one can test solids, liquids, and gases, and evaluate changes both to the cells and to the growth medium (1–3).

Recently in this laboratory, Rosenbluth *et al.* (2), found that triethyl citrate (TEC), a commonly used plasticizer for polyvinyl chloride resin, was a growth inhibitor to mouse fibroblast cells, strain L-929, in static tube culture at 35° at several dose levels. Through a series of simple experiments, he was able to eliminate chelation of calcium ions as the mechanism of triethyl citrate toxicity. By correlating parachor and other physiochemical values assigned to triethyl citrate, it was postulated that TEC is probably a physically toxic compound. Triethyl citrate is relatively lipid soluble. Being

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soluble in water only to the extent of 6.25% w/v, it would be classified according to Ferguson (4) on the basis of its thermodynamic activity as a physical toxicant as opposed to a chemically specific toxicant. Thermodynamic activity is an expression of threshold activity concentration relative to its solubility in water. Because of its lipid solubility and molecular volume, triethyl citrate should have a certain affinity for proteins, nucleic acids, lipoproteins, enzymes, and other macromolecules normally found within the cell and in the cell membranes.

From the previous studies, it was known that triethyl citrate in some way interfered with purine and pyrimidine metabolism to the extent that cell growth was inhibited relative to the dose. The interference need not be a direct effect on mitotic cells, since purines and pyrimidines are indispensable in energy metabolism and cytoplasmic synthetic activities as well as chromosomal replication.

In the above-mentioned study, the method of analysis did not differentiate the site of interference, but merely represented total activity. Since it is widely accepted that thymidine and uridine are precursors of the nucleic acids, DNA and RNA (5), respectively, their uptake could easily be adapted to the original method of McIntire and Smith (6). If adenine, a constituent of the mitochondrial enzymes, adenosine triphosphate (ATP), and the nucleic acids were assayed simultaneously within the same cell population, a more complete profile of purine and pyrimidine metabolism could be easily obtained.

Consequently, it was decided to follow purine and pyrimidine utilization with ^{14}C-labeled precursors and liquid scintillation techniques in an attempt to explain the mechanism of triethyl citrate toxicity. The liquid scintillation study was designed to analyze the parameters of exposure time to TEC, concentration effects, and precursor utilization within each time period and concentration.

MATERIALS AND METHODS

Cell Culture—Mouse fibroblast cells, strain L-929[1] were maintained in static culture in 120 ml. prescription bottles or in Blake culture bottles at 35° in a 5% carbon dioxide atmosphere. Eagle's basal medium 1X (1) supplemented with 1% L-glutamine,[1] 4% calf serum, and 0.5% streptomycin sulfate (Pfizer) per milliliter was used routinely. At regular intervals, the cells were subcultured with the aid of a 0.05% trypsin solution.

Static Tube Culture—The mouse fibroblast cells used in the study were placed in Eagle's basal medium (BME 1X) with 10% calf serum and 1% L-glutamine. Cell population was determined by hemocytometer count and volume adjustments made to provide 3.0×10^5 cells per 1.5 ml. The cells were delivered into sterile disposable culture tubes by a Cornwall automatic pipet. An additional 1.0 ml. of medium (BME 1X) was added to control tubes for assay at 48 and 78 hr. To the test sample tubes was added 1.0 ml. of triethyl citrate[2] at the desired concentrations dissolved in BME 1X. The tubes were covered with sterile aluminum or plastic closures, placed upright in racks in the incubator, and withdrawn at the specified time intervals for pulse-labeling and assay procedures. All tubes were left in the incubator for 1.5 hr. prior to initial assay to allow for resumption of normal cellular activities which are disturbed during the subculture and inoculation techniques.

The tubes to be used in liquid scintillation studies were pulsed by the addition of 1.0 ml. of ^{14}C-purine and pyrimidine precursors[3] dissolved in sterile deionized water to give an activity of 0.15 μc./ml. One milliliter of Eagle's basal medium 2X (7) was added to ensure tonicity of the radioisotope solution. The tubes were then incubated for 1 hr., at the end of which time they were assayed for total purine–pyrimidine content (2).

Assay Procedure for Absorbance Readings—Tubes for initial assay were centrifuged at 2000 r.p.m. for 15 min. No others were centrifuged unless the cells appeared to be loose or had the tendency to be washed from the test tube walls. The supernatant medium was decanted, and the cells were washed twice with 2.5-ml. portions of phosphate buffered saline solution (PBS 1X) (2), to remove color interference. Cells were then covered with 5.0 ml. of 0.3 N H_2SO_4 in 20% NaCl. The closures were replaced and the tubes placed in a sterilizer, covered, and maintained at 100° for 30 min. The tubes were quickly removed to a 4° refrigerator for 20 min. At the end of this period, all tubes were centrifuged at 2000 r.p.m. for 15 min. The resulting supernatant solution was drawn off with a bulb-tipped capillary pipet and read in a quartz cell against the acid-salt blank in a Beckman DB spectrophotometer at 268 mμ (3).

Assay Procedure for Liquid Scintillation—The supernatant medium was decanted after the 1-hr. incubation period, and the cells were washed twice with 2.5-ml. portions of PBS 1X to remove all unbound, extracellular, radioactive material. Centrifugation was carried out only when cells appeared loose from the test tube walls. The cells were then covered with 0.5 ml. of 0.25% trypsin[4] to remove the monolayer from the glass. After trypsinization, the cells were covered with 1.0 ml. of 0.3 N H_2SO_4. Tubes were recapped and boiled at 100° for 30 min., then quick-cooled in a 4° refrigerator for 20 min. After centrifuging at 2000 r.p.m. for 15 min., the supernatant solution was decanted into 15 ml. of the liquid scintillation solvent mixture. A 1.0-ml. quantity of 0.3 N sulfuric

[1] All media supplements obtained from Microbiological Associates, Baltimore, Md.

[2] Citroflex 2, Chas. Pfizer & Co.

[3] Adenine was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Thymidine and uridine were obtained from International Chemical and Nuclear Corp., City of Industry, Calif.

[4] Trypsin, 2.5%, 10%; PBS 1X, 15%; sterile deionized water, 75%.

acid wash was added to the scintillation cocktail to make a total of 17.5 ml. The scintillation cocktail, a modified Bray's solution (8), was composed of dioxane–ethylene glycol monomethyl ether–xylene[5] (12:2:1) with an additional 4.2% of a premix solution (50 Gm. PPO and 0.625 Gm. POPOP/L. of toluene).[6] Each Wheaton snapcap potassium-free counting vial was appropriately labeled, placed in the refrigerated detection chamber, and allowed to cool to −7.0° before counting. Control vials contained 15.0 ml. of scintillation solvent, 0.5 ml. of 0.25% trypsin solution, 1.0 ml. of 0.6 *N* H_2SO_4, and 1.0 ml. of radioisotope with an activity of 0.15 μc./ml.

Counting Technique—Counting was performed with a Nuclear-Chicago, series 720, liquid scintillation spectrometer. The channels ratio method (9) was used to correct for quenching with an efficiency of 49%.

RESULTS

All data were compiled by summing the means of four replicate experiments and obtaining the ratios of treated to control cells. Variance analysis was used to determine statistical significance within the parameters of time, concentration, and precursor utilization. Unless otherwise mentioned, all changes referred to as significant were at the 95% confidence level or greater.

Liquid scintillation data showed that within all concentrations of TEC used, adenine utilization was decreased significantly (C.L. >90%) at the initial time period, with respect to controls, and to the other precursors. At a TEC dose level of 6 mmoles, however, adenine uptake was apparently resumed after 48 hr. (Fig. 1.) At a dose level of 9 mmoles, adenine uptake was decreased at 1.5 hr. and at 48 hr., but had resumed normal utilization at 78 hr. (Fig. 2). At a dose level of 12 mmoles, adenine uptake was more significantly reduced than at other dose levels and continued to decrease throughout the 78-hr. period. (Fig. 3.)

Thymidine incorporation was not significantly affected by a dose of 6 mmoles over a time period of 78 hr. However, at 9 mmoles, it decreased significantly at 48 hr. and began an upward trend again at 78 hr. The same pattern was true of the 12-mmoles TEC dose, except that decreased uptake was more pronounced at all time periods.

At a TEC dose level of 6 mmoles, uridine incorporation was decreased only with respect to controls, and by 78 hr. had exceeded control uptake. It was more significantly decreased at the 9-mmole dose level at 1.5 hr., but again resumed uptake by 48 hr. and exceeded that of controls at 78 hr. There was little difference between 9 and 12 mmole TEC dose effects on uridine uptake at all time periods.

Adenine uptake levels remained significantly lower than thymidine and uridine in all TEC dose levels. Uridine uptake increased significantly over adenine and thymidine at 48 to 78 hr., but only at the 9 and 12 mmoles TEC dose levels.

From the standpoint of time of exposure of the cells to TEC, the initial assay revealed a more significant decrease in uridine and adenine utilization than in thymidine, regardless of dose. Adenine

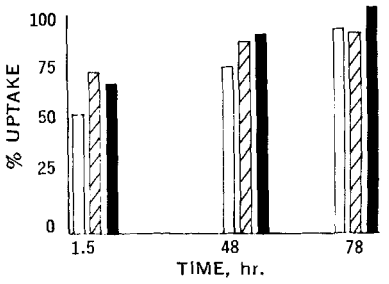

Fig. 1—Percentage uptake of ^{14}C-labeled precursors after a 1-hr. pulse, as a function of 6-mmole dose of TEC. Key: □, adenine; ▨, thymidine; ■, uridine.

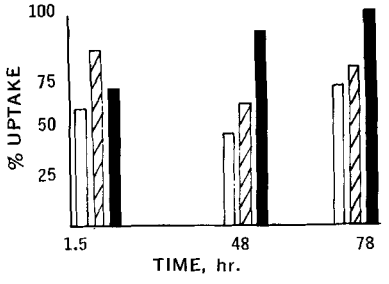

Fig. 2—Percentage uptake of ^{14}C-labeled precursors after a 1-hr. pulse, as a function of 9-mmole dose of TEC. Key: □, adenine; ▨, thymidine; ■, uridine.

uptake was most depressed at the 12 mmole TEC level at 48 hr., while thymidine did not vary appreciably among 9 and 12 mmole TEC doses during this time period. At all time periods, uridine uptake was significantly higher at 9 and 12 mmole dose levels than at a 6-mmole dose with respect to other precursors. Of all three precursors, adenine incorporation into the cell was lowered to the greatest extent at all dose levels. The decreased adenine uptake continued with time only at 12 mmoles, while at the other dose levels, it recovered and approached normal values by 78 hr. Thymidine remained relatively unchanged with respect to TEC dose level except at 48 hr. where a significant decrease in uptake was observed. Uridine uptake increased with both time and dose, and was therefore not inhibited in its incorporation into the cell by TEC.

Spectrophotometric data yielded results similar to those obtained in previous studies (Fig. 4). Liquid scintillation data were compared to spectrophotometric data to ascertain what fractions each of the labeled purines and pyrimidines occupied in the total cell extract. It should be realized at this point that the cell extracts for both spectrophotometry and liquid scintillation contained total purines and pyrimidines, but that the extract for liquid scintillation "read out" only the ^{14}C-labeled purines and pyrimidines. The differences between readings on the two instruments can be accounted for by guanine and cytosine, the base pairs found in both RNA and DNA, as well as other areas of intermediary metabolism.

Ratios of uptake percentages taken from counts per minute and absorbance (CPM/*A*) showed the rel-

[5] All solvents were Fisher, reagent quality.
[6] Liquifluor, Nuclear-Chicago Corp.

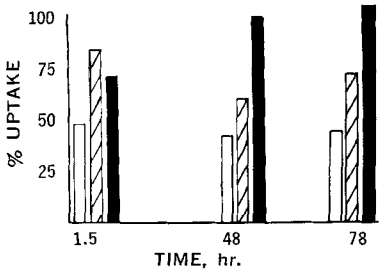

Fig. 3—Percentage uptake of ^{14}C-labeled precursors after a 1-hr. pulse, as a function of 12-mmole dose of TEC. Key: □, adenine; ▨, thymidine; ■, uridine.

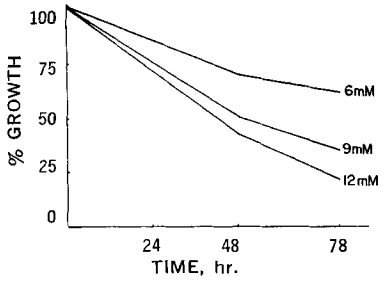

Fig. 4—Percentage growth of L cells after exposure to TEC, assayed for total purine–pyrimidine content by U.V. spectrophotometry at 268 mμ.

ative quantities of each precursor in the total purine and pyrimidine content. These ratios are higher than true values because labeled guanine and cytosine were not introduced into the system in this study.

Assuming there is no significant change between initial absorbance values and those at 1.5 hr., the zero-time value was derived by taking the ratio of the individual precursor in question to total precursors as expressed by counts per minute under each of the three initial assay conditions and obtaining an average value. Since there is no real number, *per se*, for initial assay by absorbance, the absorbance values were representative of 100% growth.

At all dose levels, adenine had an approximate CPM/*A* ratio of 0.24 initially, 0.63 at 48 hr., and 1.45 at 78 hr. Thymidine had a CPM/*A* ratio of about 0.66 initially, 0.51 at 48 hr., and 0.91 at 78 hr. Uridine gave a ratio of about 0.13 initially, 0.82 at 48 hr., and 1.25 at 78 hr. There occurs, then, the general tendency for uridine/adenine and adenine/thymidine ratios to increase significantly with time. These data essentially substantiated the liquid scintillation data, indicating a major, over-all increase in uridine content when compared to the other purines and pyrimidines. (Table I.)

In general then, only the 9 and 12-mmole dose levels of TEC showed significant effects on cellular utilization of the test precursors except at initial assay. At both the 9 and 12-mmole dose levels, however, adenine was apparently inhibited preferentially and did not recover to normal values.

TABLE I—FRACTION OF EACH ^{14}C-LABELED PRECURSOR IN TOTAL PURINE–PYRIMIDINE EXTRACT AS EXPRESSED BY ABSORBANCE AT 268 mμ

^{14}C-Labeled Precursor	CPM/*A* 268 mμ Time of Assay After Exposure to TEC, hr. 1.5	48	78
Adenine	0.24	0.63	1.45
Thymidine	0.66	0.51	0.91
Uridine	0.13	0.82	1.25

DISCUSSION

The immediate and sustained adenine decrease was one of the most noticeable effects of treatment of mouse fibroblasts by triethyl citrate. With this in mind, one should first look into the possibility of mitochondrial involvement since generation of adenosine triphosphate (ATP) occurs within this particular organelle. The energy processes involving mitochondria are electron transport, oxidative phosphorylation, and the citric acid cycle. The substrates oxygen, adenosine diphosphate, and inorganic phosphate enter the mitochondria and leave as carbon dioxide, water, and ATP. ATP is a high energy phosphate-ester moiety which mediates reactions such as cleavage of molecules, synthesis and coupling of molecules, transfer of materials across cell membranes, protein synthesis, enzyme synthesis, lipid metabolism, and nucleic acid metabolism. Without ATP, there would occur an increase in entropy and an alteration in balance of endergonic biological processes (10).

Adenine is also involved in the coenzymes participating in the electron transport system within the mitochondria, and in acetyl coenzyme A, which mediates glucose, and fatty acid metabolism. Blockage of either the electron transport system or proper metabolism of coenzyme A would favor inhibition of ATP production, accumulation of polysaccharides and lipids, and decreases in synthetic processes such as DNA and RNA polymerization.

It is of interest that uridine uptake increased while there was a concurrent decrease in adenine utilization and a relative decrease in thymidine uptake. The fact that thymidine decreased proportional to the increase in uridine, but tended to remain fairly constant within a 78-hr. period indicated that there was little or no chromosomal replication and cell duplication (11, 12, 15, 17). This could in part account for the observed inhibitory effect on cellular growth by TEC. It is, however, still possible for RNA to be synthesized as evidenced by the now common knowledge that RNA synthesis continues in the G_2 phase after DNA synthesis has stopped (14) and that "heterochromatin" of a nongenetic nature is apparently the source of a large amount of RNA which functions in the metabolism of the cell (12, 13). As long as there is DNA template available, RNA, and particularly messenger RNA, will be synthesized. It is quite possible, then, that as long as sufficient precursors, primers, and ATP are available, RNA synthesis will continue (14, 16). However, it seemed that in this study, the amount of uridine accounted for by liquid scintillation far exceeded that of adenine, and was so disproportionate that

synthesis of RNA after 48 hr. would be impossible. This is only reasonable since adenine not only is a major constituent of RNA, but is necessary in the form of ATP for the polymerization of RNA. By referring to Figs. 2 and 3, it can be seen that uridine appeared to be involved in a cyclic relationship with thymidine. Initially, at a TEC dose level of 12 mmoles, thymidine was not depressed significantly, indicating that some DNA synthesis was still occurring. At 48 hr., the decreased thymidine uptake could be representative of the RNA depression which resulted from 1.5-hr. exposure to TEC. If there is an insufficient quantity of RNA for synthesis of proteins and enzymes, a secondary depression in DNA will ensue, and it appears quite likely that this is happening at 48 and 78 hr. with triethyl citrate (18). The uridine increase at 48 and 78 hr. could then be explained by the presence of pre-existing DNA which functions only as a template.

It is also possible, but highly unlikely, that uridine was being aminated *via* a small proportion of available ATP and glutamine to form cytidine; in this way, the same level of radioactivity would be maintained and the amount of adenine consumed would probably be less than half of that required to phosphorylate and polymerize new ribonucleic acid.

There is yet another possibility that triethyl citrate might serve as a substrate to enzymes mediating citrate transformations into and out of the citric acid cycle (19). This possibility is feasible only if the ethyl groups of TEC do not sterically hinder "active sites" of the enzymes. If such were the case, however, inhibition of ATP would occur.

After reviewing individual possibilities, there still remains the question of TEC exerting its effects by multiphasic involvement. This would include accumulation of TEC in membranes of a lipoprotein nature, including those of the mitochondria, or clathrate formation with macromolecules in a nonspecific manner (22). Such a mechanism would make nucleic acids, enzymes, and other proteins virtually unavailable for cellular metabolism (23). Since there is a rapid labeling, degeneration, and turnover of soluble RNA normally occurring with the cell (20), it is more plausible that the increased uridine count is actually the result of pooled degradation products caught in the cytoplasmic gel which forms at the higher dose levels studied in this paper (21).

CONCLUSION

At the TEC dose levels reported in this paper, it might be concluded that the action of triethyl citrate is fairly specific. Its apparent initial site of action is in the mitochondria which generate energy necessary for other cellular processes to occur. Secondary cyclic functions are then noticeably altered at 48 and 78 hr., especially at 9 and 12 mmole dose levels. The DNA content of the cell does not increase sufficiently to support duplication, but does serve as a template for some further RNA synthesis at the expense of adenine before the 48-hr. assay. Finally, that RNA which was built up between initial assay and 48 hr. is probably degraded, pooled, and kept within the cell membranes by gelation (21), accounting for the resulting uridine levels as revealed by the liquid scintillation techniques. Other sites of action are possible but were not investigated in this study.

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