Preparation and Characterization of Liver Cells Made Permeable to Macromolecules by Treatment with Toluene*

(Received for publication, December 27, 1974)

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Isolated individual liver cells were made permeable to charged molecules and macromolecules by treatment with toluene, and the properties of such cells were examined in detail. The optimal conditions of toluene treatment, as determined by assay of aminoacyl-tRNA synthetase activities, was 7 to 9% toluene for 2 min at 0°. Conditions are also described for maintaining the treated cells for periods up to 1 hour. Toluene treatment was found to be as efficient as various disruptive methods for making internal enzymes accessible to exogenous substrates, and this was true for enzymes in the cytosol, membrane, or organelle fractions. Electron microscopic and biochemical examination of toluene-treated cells indicated that they were relatively intact and lost only small amounts of cellular constituents to the suspension medium. The data in this paper suggest that toluene treatment of individual cells might prove useful for studies of macromolecular synthesis in liver.

In recent years there has been considerable interest in the isolation of individual liver cells to study a variety of cellular processes in vitro, including protein synthesis (1), gluconeogenesis (2), lipogenesis (3, 4), and fatty acid synthesis (5, 6). One limitation to many of these studies is the inability to get charged or large exogenous molecules into the cells, making many experiments impossible. In a previous communication we have shown that individual liver cells could be made permeable to an exogenous source of ATP and tRNA by treatment with toluene, and that it was possible to examine aminoacyl-tRNA synthesis within such treated cells (7). In bacteria, toluene treatment has been used to study DNA synthesis (8-10), RNA synthesis (11), and the regulation of enzymes (12). In addition, toluene treatment has also been used to investigate DNA synthesis in the lower eukaryotes, Chlamydomonas reinhardi (13), Saccharomyces cerevisiae, and Ustilago maydis (14). Recently, Atherly described a method of cold-shocking bacterial cells which made them reversibly permeable to exogenous substrates (15).

In the present paper we describe conditions for the toluene treatment of liver cells which render them permeable to exogenous molecules, and examine some properties of these cells. In addition, we have determined the structural integrity of toluene treated cells by both electron microscopy and the distribution of various marker enzymes. Finally, we have analyzed the material that is extracted from liver cells by toluene treatment. In a subsequent paper, 1 we will describe the usefulness of toluene treatment for the study of DNA, RNA, and protein biosynthesis in isolated liver cells.

EXPERIMENTAL PROCEDURES

Materials

Male Long-Evans rats weighing 125 to 200 g were used. Collagenase (121 units/mg), hyaluronidase (360 NF units/mg), D-glucose 6-phosphate, p-nitrophenylphosphate, cytochrome c, and digitonin were purchased from Sigma Chemical Company. [14C]ATP and [32P]phosphate were obtained from Schwarz/Mann. All salts and buffers were reagent grade.

Preparation of Individual Hepatocytes

Hepatocytes were isolated by enzymatic digestion using a modification of the method of Berry and Friend (16). Rats were fed ad libitum and killed by decapitation. Livers were perfused in situ at room temperature with CaCl₂-free Hanks' basic salt solution (17) containing 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, (Buffer A), for 10 min at a flow rate of about 13 ml/min. The liver was removed from the animal and placed in a 150-mm sterile Petri dish. Each lobe of the liver was perfused separately with about 10 ml of Buffer B for 10 min by recycling the buffer. Each lobe was minced, and when the perfusion was completed the minced liver was dispersed in 10 volumes of Buffer B by twice drawing it through a large bore 25-ml pipette. The liver suspension was incubated with shaking (100 oscillations/min) at 19° for 3 hours. During the incubation period the pH was kept at 7.5 ± 0.2 by the addition of a few drops of 1 M NaOH. This time and temperature of incubation was chosen because these conditions gave the highest aminoacyl-tRNA synthetase activities per g of isolated cells. After the 3 hours of incubation the resulting cells were filtered through a layer of cheesecloth and the filtrate was centrifuged at 100 x g for 2 min. The cell pellet was resuspended in 10 volumes (based on the original weight of liver) of Buffer A containing 0.15 M NaCl and centrifuged at 50 x g for 30 s. Hepatocytes were purified by suspension
three times in Buffer A and centrifugation at 50 x g for 30 s. These cells, as judged by light and electron microscopy, were 80 to 90% hepatocytes. The yield of hepatocytes in different preparations varied from 3 to 7 x 10^7 cells per 5 g of liver, and 85 to 90% of these cells were viable as judged by their ability to exclude trypan blue. Individual hepatocytes retained all their aminocyl-tRNA synthetase activity when kept for 8 hours in Buffer A in ice. Bacterial contamination of the purified liver cells amounted to less than 50 cells per ml.

Electron Microscopy

Small cubes of intact liver perfused in Buffer A (prior to enzymatic digestion) were minced in 4% glutaraldehyde buffered with 0.1 M cacodylate buffer and fixed at 0-4° for 2 hours. Isolated hepatocytes, removed from Buffer B after different periods of digestion, as well as purified hepatocytes before and after toluene treatment, were also fixed in 2% OsO_4 buffered with 2% glutaraldehyde (18). All samples were dehydrated in graded alcohols and embedded in Epon-Araldite mixture as described previously (19). Blocks for thin sectioning were selected from 1-μm-thick sections stained with toluidine blue. Thin sections were cut on a Reichert OMU_1 microtome, picked up on uncoated 300 mesh copper grids, stained with uranyl acetate (20) and lead citrate (21), and examined in a Philips EM 300 electron microscope at 60 kV.

Chemical Analyses

Protein was determined by the method of Lowry et al. (22). RNA was determined by the orcinol procedure, and DNA by the diphenylamine method according to Schneider (23). Phospholipid analysis was by the method of Chen et al. (24) after extraction with chloroform-methanol (2:1).

Preparation of [32P]RNA and Phospholipids

Inorganic [32P]phosphate (0.1 mCi) was injected into rats intraperitoneally and after 16 hours the animals were killed by decapitation. Hepatocytes were isolated and toluene treatment was performed as described in the text. Phospholipids were extracted from the toluene-treated cells or supernatant fraction with 5 volumes of chloroform-methanol (2:1). Samples were shaken on a Vortex mixer, placed in ice for 15 min, and then centrifuged at 19,100 x g for 10 min. The aqueous and organic layers were separated and the aqueous layer was extracted 2 more times. The combined organic layers were used for determination of [32P] in lipid. RNA was precipitated from the aqueous layer with 6 volumes of ethanol after addition of 1 M NaCl and 1 mg/ml of carrier protein was determined by the method of Lowry et al. (22). Phospholipid analysis was by the method of Chen et al. (24) after extraction with chloroform-methanol (2:1).

Acid Phosphatase—Activity was determined by measuring the release of inorganic phosphate from p-nitrophenylphosphate (30).

Cytochrome c Oxidase—Activity was assayed by measuring the oxidation of reduced cytochrome c (cytochrome c reduced with dithionite) at 550 nm (31). One unit is defined as the amount of enzyme which causes a change of 1 A_550/min.

RESULTS

Optimal Conditions of Toluene Treatment—Since we were interested in using the permeable cells in studies of the aminocyl-tRNA synthetase complex (see Ref. 7), the optimal conditions for toluene treatment were determined by assaying aminocyl-tRNA synthetase activity for four different amino acids (lysine, arginine, threonine, and alanine). These included two (arginine and lysine) which are tightly bound in the synthetase complex and two (threonine and alanine) which are easily dissociated. Fig. 1 shows the effect of toluene concentration and temperature of treatment on aminocyl-tRNA synthetase activity. Negligible activity was found in the absence of toluene treatment, but increasing synthetase activity was detectable as the toluene concentration was raised. Optimal activity was observed at 0° and 7 to 9% toluene. Under these conditions about 90% of the total aminocyl-tRNA synthetase activity remained associated with the cell (7). At higher temperatures a greater amount of activity was found in the supernatant fraction, and less in the cell pellet. The time course of toluene treatment at 0° is shown in Fig. 2. By 2 min of incubation the maximum amount of aminocyl-tRNA synthetase activity had been made accessible. Periods of incubation longer than about 10 min led to some decrease in the activity associated with the cell pellet. Unless indicated otherwise the toluene-treated cells used in this paper were prepared by incubation with 9% toluene for 2 min at 0°.

Stability of Toluene-treated cells—The data in Figs. 1 and 2 suggested that prolonged incubation and higher incubation temperatures decreased the stability of toluene-treated cells.

In order to determine the efficiency of toluene treatment for making cells permeable, the level of aminocyl-tRNA synthetases assayable after this procedure was compared with those assayed by other methods of opening cells.

Comparison of Toluene Treatment to Other Methods of Opening Cells—In order to determine the efficiency of toluene treatment for making cells permeable, the level of aminocyl-tRNA synthetases assayable after this procedure was compared with those assayed by other methods of opening cells.
FIG. 1. Effect of toluene concentration and temperature of treatment on aminoacyl-tRNA synthetase activities in purified hepatocytes. The purified hepatocytes were suspended in 5 volumes of Buffer A and various amounts of toluene were added to 200 μl aliquots of the cell suspensions to give the final concentrations indicated. The samples were incubated at 0, 20 or 37° for 2 min with gentle shaking by hand. The treated cells were centrifuged and resuspended in 200 μl of Buffer A containing 20% glycerol. Ten microliters of the cell pellet or supernatant fractions were then assayed for aminoacyl-tRNA synthetase activities as described previously (7). Solid lines represent pellet activity and dashed lines represent supernatant activity.

FIG. 2. Effect of time of toluene treatment on aminoacyl-tRNA synthetase activities in purified hepatocytes. The purified hepatocytes were suspended in 5 volumes of Buffer A and toluene (20 μl) was added to 200-μl aliquots. The samples were incubated at 0° with gentle shaking for the indicated times. The treated cells were centrifuged and suspended in 5 volumes of Buffer A containing 20% glycerol. Ten microliters of the cell pellet or supernatant fraction were then assayed for aminoacyl-tRNA synthetase activities. Solid lines represent pellet activity and dashed lines represent supernatant activity.

FIG. 3. Stability of toluene-treated cells at 0°. After toluene treatment the cells were suspended in 5 volumes of Buffer A containing 20% glycerol. Aliquots of 200 μl were placed in ice for various lengths of time, centrifuged, and the cell pellet resuspended in 200 μl of the same buffer. Ten microliters of the cell pellet or supernatant fraction were then assayed for aminoacyl-tRNA synthetase activities.

FIG. 4. Stability of toluene-treated cells in modified suspension medium. The new suspension medium (Buffer C) contains: 25 mM Tris-acetate, pH 7.0; 0.5 mM MgCl₂; 10 μg/ml of bovine serum albumin; 0.02 mM EDTA; and glycerol at a final concentration of 20%. After toluene treatment the cells were suspended in 5 volumes of Buffer C and divided into 200-μl aliquots. The aliquots were placed in ice for various lengths of time, centrifuged, and the cell pellet resuspended in 200 μl of Buffer C. Ten microliters of the cell pellet or supernatant fraction were then assayed for aminoacyl-tRNA synthetase activities.

Electron Microscopic Comparison of Hepatocytes Before and After Toluene Treatment—Our studies of the distribution of aminoacyl-tRNA synthetases suggested that toluene-treated cells were relatively intact, although they had become permeable to both charged molecules and macromolecules. In order to examine the structure of these cells further they were compared to that in cells opened by a variety of other methods. The data in Table I show that the total units of aminoacyl-tRNA synthetase detectable after toluene treatment of cells was somewhat greater than that found by homogenization of an equivalent weight of intact liver, but slightly lower than that obtained by sonication or Nonidet P-40 treatment of individual liver cells. This lower activity in toluene-treated cells was not due to enzyme inhibition or inactivation since the same concentration of toluene had no effect on aminoacyl-tRNA synthetase activity in a homogenate of intact liver. Likewise, remixing of toluene supernatant and pellet fractions gave only additive results suggesting that the lower activity was not due to removal of a factor from the cells. In addition, since Nonidet P-40 treatment of toluene-treated cells did not further increase the total units of enzyme detectable, toluene did appear to make all of the activity accessible. Although the reason for the slightly lower aminoacyl-tRNA synthetase activity in toluene treated cells is not known, these data indicate this procedure is nevertheless quite efficient for making cells permeable.
pared to untreated cells by electron microscopy. Samples of liver fixed after perfusion and the purified liver cells were examined first, and both appeared to be similar to preparations reported by other authors (16, 32). The percentage of individual hepatocytes varied from 80 to 90% in blocks selected at random. Red blood cells, leukocytes, lymphocytes, and macrophages were the principal contaminants. In most instances, the hepatocytes were present as single cells, although some clumps of two or three cells were seen which were still adherent by the junctional complexes surrounding the bile canaliculus. The majority of cells were intact and appeared to have survived the dissociation procedure with little evidence of injury (Fig. 5A). They were generally round or oval with some microvilli. Adherent portions of membrane from adjacent cells were usually present on these cells, and at high magnification these appeared to be tight junctions. A peripheral zone (ectoplasm) which was devoid of organelles and highly vacuolated was also usually present. However, the remainder of the cytoplasmic organelles and nuclear detail were similar to intact liver. In particular, the nuclear chromatin was dispersed, nucleoli had a well preserved nucleoloenema, and mitochondria had an elongated profile and dense matrix granules. The endoplasmic reticulum was dispersed somewhat, but parallel stacks of endoplasmic membranes, and cochlear arrays of ribosomes in association with these membranes were easily seen. A variable percentage (10 to 30%) of cells, however, showed some response to the preparation procedure. Such cells usually had breaks in the plasma membrane, and in some there were two populations of mitochondria; some of the mitochondria were elongated and dense, whereas others were rounded and lucent, suggesting an altered state of respiration (33).

In general, toluene-treated cells appeared relatively normal when compared to the untreated cells (Fig. 5B). As noted above, some cells displayed the mitochondrial swelling and other alterations of dissociated cells. The most obvious change in the toluene-treated cells involved the endoplasmic reticulum. This structure appeared dilated, the parallel arrays were usually absent, and the ribosomal granules tended to be scattered in the hyaloplasm rather than arranged in spirals. There was also a somewhat greater degree of chromatin clumping and vacuolization of lysosomes in addition to the

### Table I

Comparison of aminoacyl-tRNA synthetase activity in cells made permeable by toluene treatment or opened by other methods

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Homogenization of intact liver</th>
<th>Sonication of individual liver cells</th>
<th>Nonidet P-40 treatment of individual liver cells</th>
<th>Toluene treatment of individual liver cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.51</td>
<td>0.74</td>
<td>14.8</td>
<td>0.83</td>
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<tr>
<td>Lysine</td>
<td>9.08</td>
<td>0.13</td>
<td>11.3</td>
<td>0.19</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.15</td>
<td>0.75</td>
<td>6.21</td>
<td>0.54</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.63</td>
<td>0.86</td>
<td>11.9</td>
<td>0.48</td>
</tr>
</tbody>
</table>

![Fig. 5A](http://example.com/image1.png)  ![Fig. 5B](http://example.com/image2.png)

**Fig. 5.** Electron micrographs of hepatocytes before and after toluene treatment. The hepatocytes were prepared and purified as described under “Experimental Procedures.” A, Untreated hepatocyte; B, hepatocyte subjected to standard conditions of toluene treatment, centrifuged, and resuspended in 5 volumes of Buffer A. Magnification: A, 5700; B, 7000.
peripheral vacuoles noted above. The plasma membrane appeared as intact as that in untreated cells. From these results we conclude that toluene treatment is a relatively gentle procedure which leaves much cell structure intact.

Effect of Toluene Treatment on Marker Enzymes—Our studies with aminoacyl-tRNA synthetases indicated that toluene treatment of liver cells could render a presumed cytoplasmic enzyme accessible to exogenous substrates. However, it was of interest to determine whether this treatment was also useful for the study of enzymes with other subcellular localization. Accordingly, we measured a variety of marker enzymes to ascertain whether they became accessible to substrates, and whether they remained with the cells after toluene treatment. These included tRNA nucleotidyltransferase, presumably located in the cytosol; 5'-nucleotidase, localized in the plasma membrane (29); glucose-6-phosphatase which is found in the endoplasmic reticulum (34); acid phosphatase, located in the lysosomes (35, 36); and cytochrome c oxidase, a mitochondrial enzyme (37). The data in Table II indicate that each of the marker enzymes could be assayed in toluene-treated cells, and that the level of activities made accessible to exogenous substrates were similar to those found by sonication. The data also show that greater than 80% of each enzyme remained associated with the cells. Only tRNA nucleotidyltransferase, a cytoplasmic enzyme, and 5'-nucleotidase, a membrane protein, leaked out of the treated cells to any significant extent. The results indicate that toluene treatment is a useful procedure for enzymes with various subcellular localizations. However, although these marker enzymes remain associated with the treated cells, we do not yet know whether they maintain their original subcellular localization after exposure to toluene. Studies are in progress to clarify this point.

Analysis of Material Extracted from Cells by Toluene Treatment Although cells after toluene treatment appeared relatively intact, it was important to determine whether any cellular material was extracted from the cells during their preparation. For this purpose we chemically measured the amount of various cell constituents in the untreated cells, and in the pellet and supernatant fractions after toluene treatment. The data in Table III show that no DNA and only about 5% of the cellular RNA were extracted by the toluene treatment. On the other hand, about 20% of the protein and 30% of the phospholipids were no longer associated with the cells. The protein released by toluene treatment was examined further by chromatography on Sephadex. Almost all of the protein found in the toluene supernatant fraction had a molecular weight of 140,000 to 180,000, there being relatively few lower molecular weight components. Since a large fraction of the enzymes examined remained with the treated cells (see above), we do not yet know which part of the cell is the source for the released protein.

The results of the chemical analyses were confirmed by studies with cells isolated from rats injected with inorganic $^{32}$P phosphate (see "Experimental Procedures"). Thus, about 15% of the total $^{32}$P radioactivity in isolated liver cells was extracted by toluene treatment, as was about 20% of the acid-precipitable $^{32}$P. The toluene-treated cells and supernatant fractions were fractionated further by extraction with chloroform-methanol (2:1) to obtain phospholipids. In agreement with the chemical analysis, we found that 28% of the $^{32}$P radioactivity extractable by the organic solvents was released from the cells by the toluene treatment. RNA was determined in the aqueous layer remaining after chloroform-methanol extraction by measurement of the $^{32}$P radioactivity solubilized by pancreatic RNAse. From these experiments it was shown that about 10% of the cellular RNA was released by toluene. Finally, in experiments using cells labeled with $[^14]C$ orotic acid, we also found that about 10% of the cellular RNA was released by toluene. In these latter experiments the released RNA was analyzed on Sephadex G-150, and greater than 90% of the labeled material co-chromatographed with carrier rabbit liver transfer RNA.

These results support our previous conclusions that toluene-treated cells retain a high degree of structural integrity. However, the appreciable release of phospholipids probably indicates some alterations in the plasma and endoplasmic reticulum membranes. In addition, a small percentage of the isolated cells may have been damaged during their isolation which could have led to their complete disruption during toluene treatment.

Discussion

The studies presented here indicate that toluene treatment is an efficient method for rendering liver cells permeable to charged molecules and macromolecules, while keeping cellular structure relatively intact. Enzymes present in the cytosol or in various organelles can be assayed within the cell. The levels of enzyme activity made accessible by this procedure are similar...
to those found by vigorous disruption of cells. Electron microscopic and biochemical examination of the toluene-treated cells indicated that cell structures were not destroyed by the toluene treatment and that most cell constituents remained within the cell. The greatest effect of toluene treatment appeared to be on membrane components since higher amounts of 5’-nucleotidase and phospholipid were extracted than other cellular components, and the normal structure of the endoplasmic reticulum was disturbed. Although the exact mechanism of the effect of toluene treatment on cells is unknown, the lipid-solubilizing properties of this solvent undoubtedly play a role, as suggested by the effects on membranes and the altered permeability properties of the cell.

Toluene treatment of bacterial cells has already found wide use in studies of nucleic acid synthesis (8-11), regulation (12) and isolation of mutant enzymes (38). In a subsequent paper we will demonstrate that toluene-treated liver cells can be used for studying various macromolecular biosynthetic processes.

Toluene treatment of bacterial cells has been shown to cause considerable leakage of RNA and disruption of ribosomes (39). We have not observed such damage in the liver cell system, and in addition, these cells can be used to examine protein synthesis, which is not feasible in permeable bacterial cells.

Acknowledgments—The authors are extremely grateful for the expert technical assistance of Sherry Perrie and Catherine Eastwood.

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