The recent development of $^{99m}$Tc-tetracycline in our laboratory and its successful use in delineating myocardial infarcts have encouraged further investigation into the mechanism of its uptake and its localisation on a subcellular level. The affinity of $^{99m}$Tc for cells and subcellular constituents when incubated with $^{99m}$Tc-tetracycline was evaluated in our recently developed necrosis model of tissue culture. Live cells obtained from exponentially growing cultures or dead cells obtained from the media of nutritionally depleted plateau-phase cultures were incubated with $^{99m}$Tc-tetracycline at 37°C for various times. After incubation, the cells were washed and the total cellular activity as well as the subcellular localisation of $^{99m}$Tc determined. About 60 times more $^{99m}$Tc remained associated with the dead cells than with the live cells. This affinity was not seen when the dead cells were incubated with the pertechnetate ion. On the subcellular level, at least 50% of the $^{99m}$Tc appeared to be associated with the DNA and protein fractions of the cell. This system may provide a rapid and economical means of screening radioactive compounds currently being prepared to detect necrotic tissue.

The fluorescence of tetracycline and its analogs in necrotic myocardial, pleural, and renal tissue, whether due to ischemia or neoplasia, has been observed by previous investigators (1–6). The mechanism of action of tetracycline in bacteria (7–14) as well as its localization within the bacterial cell (15–17) has been well documented also. Yet, little information is available concerning the action and localization of this compound in mammalian cells (18).

The recent development of $^{99m}$Tc-labeled tetracycline in our laboratory (19) and its successful use in delineating myocardial infarcts in dogs (20) generated the development of an in vitro cellular model in culture to investigate the uptake and localization of $^{99m}$Tc-tetracycline in both living and dead cells.

Because many pathological lesions (whether they are caused by infarction or carcinoma) often involve necrosis at some stage, this study has been designed to aid in understanding the mechanism of uptake of certain $^{99m}$Tc-labeled compounds and to evaluate the potential of this model for the screening of other radiopharmaceuticals.

**MATERIALS AND METHODS**

Chang liver cells (Lich) were grown in monolayer culture at 37°C in an atmosphere of 5% CO$_2$ in 75 cm$^2$ plastic flasks using a modified Eagle's basal media (MEM) (21) and supplemented with 10% heat-inactivated calf serum. The cultures were divided into two groups: live cells, or those maintained in exponential growth, and dead cells, or those harvested from the media of plateau-phase cultures whose media had not been changed for 5 days prior to this experiment.

**Cell labeling.** The live cells were labeled by removing the growth media from exponentially growing cultures and replacing it with 10 ml of media, pH 7.3, containing 10 μCi of $^{99m}$Tc-pertechnetate or $^{99m}$Tc-tetracycline in isotonic saline. After incubation at 37°C for 15, 30, or 60 min with the radioactive media, the media was removed and a 3-ml aliquot was retained for later counting. The cells were then washed twice with calcium-free salts (CFS) and removed from the surface of the flask by exposure to a 0.25% trypsin solution for 2 min. Aliquots of the salt solution and trypsin were retained for counting. After trypsinization, the cells

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were resuspended in CFS and counted; the activity per million cells was determined using a gamma scintillation counter with a NaI(Tl) crystal.

The dead cells were removed from the culture medium by centrifugation at 1,000 rpm for 5 min. They were then resuspended in 10 ml of MEM containing 10 μCl of 99mTc-pertechnetate or 99mTc-tetracycline in isotonic saline and incubated at 37°C for 15, 30, or 60 min. After incubation, the cells were removed from the radioactive media by centrifugation and a 3-ml aliquot of the supernatant was retained for counting. The cells were washed twice with CFS and 3-ml aliquots of the supernatant were retained for determination of the unbound activity. The cells were counted and the amount of radioactivity per million cells was calculated as described previously.

**Determination of activity distribution at subcellular levels.** A Schmidt-Thannhauser procedure (22) with the following modifications was used to separate the acid-soluble and acid-insoluble cell fractions. Fractions of labeled, washed live or dead cells were centrifuged and the supernatant was removed. Three milliliters of freshly prepared, cold 13.3% trichloroacetic acid (TCA) was added to the cell pellet, vortexed for 1 min, and incubated at 4°C for 15 min. The cells were then centrifuged for 3 min at 2,000 rpm and the procedure repeated twice with incubation periods of 3 min each. The supernatant contained the acid-soluble fraction. Four milliliters of 95% ethyl alcohol saturated with sodium acetate were added to the remaining precipitate. After vortexing for 1 min, the tube was incubated for 3 min at 4°C and centrifuged; the supernatant containing the lipid-soluble fraction was removed. To hydrolyze the RNA, 1 ml of 0.3 N KOH solution was added to the precipitate. The mixture was incubated at 37°C for 1 hr with vortexing every 15 min. After this incubation period, 1 ml of 20% cold TCA was added to the above mixture which was then centrifuged at 2,000 rpm for 3 min. The supernatant contained the activity associated with the RNA fraction. The remaining precipitate contained the activity associated with the DNA and protein fractions. The precipitate was suspended in 1 ml of 5% TCA. This was followed by incubation at 90–95°C for 30 min with vortexing every 10 min to bring the DNA into solution. The protein precipitate was removed by centrifugation and washed with 5% TCA solution to free it of any contaminating DNA solution. The activities in the different subcellular fractions were determined with gamma NaI(Tl) well detector.

**RESULTS**

On incubation with 99mTc-tetracycline about 60 times more activity is associated with the dead cells in comparison with the live cells (Table 1). This contrast can be seen throughout the washing procedure of the live cells with CFS: almost three times more activity was removed after the second washing in comparison with the dead cells.

When the cells were resuspended after labeling and allowed to remain for various periods of time, the activity remained bound to the dead cells. A typical experiment in which replicate tubes of labeled cells were suspended in CFS and centrifuged after various periods of incubation at 37°C indicated that most of the unbound activity was removed with the previous washings. Very little more was eluted during the subsequent 3 hr. About 60 times more activity remained with the dead cells than with the live cells.

This preferential uptake of 99mTc in dead cells incubated with labeled tetracycline was not seen on incubation with the pertechnetate ion. An experiment in which dead cells were exposed to 99mTc-tetracycline or 99mTc-pertechnetate showed consistently higher relative uptakes of 99mTc in the cells exposed to 99mTc-tetracycline at the three incubation times (Table 2). No preference for either compound was observed in live cells, however. In a typical experiment with 99mTc-pertechnetate in which the cells were exposed to pertechnetate for 30 min, all the activity remained either in the medium or was lost in the washing procedure (Table 3). Essentially no activity remained with either the dead or the live cells and there was no concentration of activity by the dead cells as was seen with tetracycline.

Although considerable work has been done to determine the site at which tetracycline is bound in bacterial cells, there appears to be some controversy over its localization in mammalian cells. In a typical experiment in which the cell fractions were separated to localize the 99mTc activity after labeled tetracycline exposure, very little of the activity was found in the

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**TABLE 1. LABELING OF LIVE AND DEAD CELLS WITH 99mTc-TETRACYCLINE**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dead cells</td>
</tr>
<tr>
<td>Medium (MEM)</td>
<td>1,243,104</td>
</tr>
<tr>
<td>Washing 1 (CFS)</td>
<td>50,160</td>
</tr>
<tr>
<td>Washing 2 (CFS)</td>
<td>2,556</td>
</tr>
<tr>
<td>Trypsin</td>
<td>—</td>
</tr>
<tr>
<td>Washing 3 (CFS)</td>
<td>3,300</td>
</tr>
<tr>
<td>10^6 cells*</td>
<td>4,359</td>
</tr>
</tbody>
</table>

* 14.7 × 10^6 dead cells and 57.5 × 10^6 live cells used for determination.
TABLE 2. THE UPTAKE OF 99mTc-TETRACYCLINE (Tc-T) AND THE PERTECHNETATE ION (TcO4−) IN DEAD CELLS (CPM/10^6 CELLS)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Time of incubation (min)</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dead 99mTc-T</td>
<td>13,824</td>
<td>16,020</td>
<td>15,825</td>
<td></td>
</tr>
<tr>
<td>Dead 99mTcO4−</td>
<td>576</td>
<td>504</td>
<td>536</td>
<td></td>
</tr>
<tr>
<td>Ratio 99mTc-T</td>
<td>24.1</td>
<td>31.8</td>
<td>28.1</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3. LABELING OF LIVE AND DEAD CELLS WITH PERTECHNETATE ION

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium (MEM)</td>
<td>103,728</td>
</tr>
<tr>
<td>Washing 1 (CFS)</td>
<td>4,158</td>
</tr>
<tr>
<td>Washing 2 (CFS)</td>
<td>147</td>
</tr>
<tr>
<td>Trypsin</td>
<td>950</td>
</tr>
<tr>
<td>Washing 3 (CFS)</td>
<td>65</td>
</tr>
<tr>
<td>10^6 cells*</td>
<td>42</td>
</tr>
<tr>
<td>* 15.7 x 10^6 dead cells and 14.7 x 10^6 live cells used for determination.</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 4. ACTIVITY DISTRIBUTIONS OF LABELED LIVE AND DEAD CELLS (CPM/10^6 CELLS)

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Dead cells*</th>
<th>Live cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity Percent</td>
<td>Activity Percent</td>
<td></td>
</tr>
<tr>
<td>Acid soluble (3)</td>
<td>2,100</td>
<td>7</td>
</tr>
<tr>
<td>Lipid soluble</td>
<td>972</td>
<td>3</td>
</tr>
<tr>
<td>RNA (3)</td>
<td>9,306</td>
<td>30</td>
</tr>
<tr>
<td>DNA + protein</td>
<td>18,625</td>
<td>60</td>
</tr>
<tr>
<td>* Five times the number of live cells were used for determinations.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>† Number of combined fractions.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 5. ACTIVITY DISTRIBUTION IN HOMOGENIZED LIVE AND DEAD CELLS (CPM/10^6 CELLS)

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Dead cells</th>
<th>Live cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity Percent</td>
<td>Activity Percent</td>
<td></td>
</tr>
<tr>
<td>Acid soluble (3)*</td>
<td>4,404</td>
<td>14</td>
</tr>
<tr>
<td>Lipid soluble</td>
<td>1,416</td>
<td>4</td>
</tr>
<tr>
<td>RNA (3)*</td>
<td>14,280</td>
<td>44</td>
</tr>
<tr>
<td>DNA + protein</td>
<td>14,292</td>
<td>38</td>
</tr>
</tbody>
</table>

* Number of combined fractions.

TABLE 6. ACTIVITY DISTRIBUTIONS OF DEAD CELLS LABELED WITH DIFFERENT 99mTc-LABELED RADIOPHARMACEUTICALS

<table>
<thead>
<tr>
<th>Activity</th>
<th>Chloro-</th>
<th>Tetra-</th>
<th>Oxy-</th>
<th>Ascorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetra-</td>
<td>tetracycline</td>
<td>tetracycline</td>
<td>tetracycline</td>
<td>DTPA</td>
</tr>
<tr>
<td>Chloro-</td>
<td>5,876</td>
<td>1,303</td>
<td>3,772</td>
<td>4,807</td>
</tr>
<tr>
<td>AscorbateDTPA</td>
<td>4,807</td>
<td>1,303</td>
<td>3,772</td>
<td>4,807</td>
</tr>
</tbody>
</table>

| Activity (percent of total) | Acid soluble (3)* | 15 | 15 | 15 | 16 | 33 |
| Lipid soluble              | 3 | 6 | 2 | 5 | 3 |
| RNA (3)*                   | 27 | 26 | 26 | 27 | 24 |
| DNA                        | 12 | 12 | 12 | 11 | 8 |
| Protein                    | 42 | 41 | 45 | 41 | 32 |

* Number of combined fractions.

99mTc-ascorbate-DTPA (Table 6) and the results compared with tetracycline. After exposure to four tetracycline analogs the cellular activity was similar; whereas after exposure to 99mTc-ascorbate-DTPA much less 99mTc was taken up by the cells. Fractionation of these cells showed very similar patterns of uptake for the four tetracycline analogs but a significant proportion of the activity appeared in the acid-soluble fraction after 99mTc-ascorbate-DTPA incubation. When the DNA and protein fractions were separated, the majority of the activity was associated with the protein fraction.

DISCUSSION

Our data show that 99mTc is readily taken up by dead cells in tissue culture after exposure to 99mTc-tetracycline. These dead cells which in our model were produced by depletion of essential nutrients appeared to incorporate 99mTc into their macromolecular structure whereas live cells appeared to exclude or prevent this compound from becoming incorporated. Although a necrotic cell system could be obtained by other means (such as heat or irradiation), cell death due to depletion of essential nutrients may correspond to the situation seen with lipid and acid-soluble fractions of dead cells whereas about one-fourth of the activity was associated with the acid-soluble fraction in the live cells (Table 4). In both cases, however, at least 50% of the activity was associated with the DNA and protein fractions and 25% with the RNA fraction.

When the cell components were labeled after homogenization, more of the activity was associated with the components of dead cells than of live cells (Table 5). The majority of the activity appeared to be divided equally between the RNA and the DNA and protein fractions.

Dead cells were exposed to four other 99mTc-labeled compounds (99mTc-chlorotetraycline, 99mTc-oxytetracycline, 99mTc-demethylchlorotetraycline, and 99mTc-ascorbate-DTPA) (Table 6) and the results compared with tetracycline. After exposure to four tetracycline analogs the cellular activity was similar; whereas after exposure to 99mTc-ascorbate-DTPA much less 99mTc was taken up by the cells. Fractionation of these cells showed very similar patterns of uptake for the four tetracycline analogs but a significant proportion of the activity appeared in the acid-soluble fraction after 99mTc-ascorbate-DTPA incubation. When the DNA and protein fractions were separated, the majority of the activity was associated with the protein fraction.
myocardial infarction. The uptake of $^{99m}$Tc in canine myocardial infarcts after $^{99m}$Tc-tetracycline administration (20) seems to substantiate this but studies in different modes and stages of cell death, both reversible and irreversible, due to the aforementioned mechanisms have yet to be completed.

This study also sheds light on the localization of $^{99m}$Tc-tetracycline in mammalian cells at the subcellular level. In bacteria, tetracyclines have been reported to bind to cellular RNA. Specifically, bacteriostatic concentrations of tetracycline have been found by some investigators to bind to 30S ribosomes while other investigators (17,19) have found the drug associated with the 50S ribosomal component also.

There appears to be less substantial data on the localization of $^{99m}$Tc-tetracycline in mammalian cells. Our data suggest that at least 50% of the total $^{99m}$Tc-tetracycline activity was associated with the DNA and protein fractions of the Lich cell and 25% with RNA. Separation of the DNA and protein fractions showed that over 75% of the activity in that fraction was associated with protein. Homogenization of the cells before labeling increased the amount of activity associated with the RNA fraction.

The uptake of $^{99m}$Tc in dead cells may be due to structural changes associated with the degeneration of the subcellular components. These changes, including conformational alterations of the macromolecules, may be necessary to allow $^{99m}$Tc to bind to DNA, RNA, or protein. This postulate is supported by certain chemical studies.

Zubay and Doty (23) have found increased binding of tetracycline to heat-denatured DNA, probably due to the availability of new groups such as purine rings. It has been reported that tetracyclines bind to DNA, proteins, soluble RNA, and ribosomes in the presence of cationic species. Kohn (24), studying the binding of native and heat-denatured DNA with tetracycline in the presence of Zn$^{2+}$, Ga$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$, found that tetracycline binding was four to five times higher with denatured DNA in the presence of Zn$^{2+}$

In our system the $^{99m}$Tc-ion was chelated to tetracycline and was probably positively charged in the presence of excess Sn$^{2+}$ ion. Under these conditions it is reasonable to assume that during the process of cell death, changes in the subcellular components occur which open up sites for the binding of $^{99m}$Tc-labeled tetracycline potentiated by the stannous ion.

**ACKNOWLEDGMENTS**

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