Studies on the Effects of Acetaldehyde on Tissue Cells Cultivated in Vitro*

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SUMMARY

Short-term studies have been made on the effects of acetaldehyde on four different cell lines (clones). When the cells were exposed to relatively high concentrations of acetaldehyde, 2.0 and 8.0 mg/ml, they became fixed. Cells in 1.0 mg. and 0.5 mg. acetaldehyde per ml. did not become fixed but rounded up and detached within a few hours.

In 0.05 and 0.01 mg. acetaldehyde per ml. no definite effects were noted within the limits of the short-term testing, and hence these concentrations were used in the long-term studies. Mouse liver cells exposed to 0.05 mg. acetaldehyde per ml. for 23 days were all dead within 7 days following return to control medium. During the first 37 days in 0.01 mg/ml, the liver cells proliferated to a greater extent than those in control medium, but after this period the rate of increase was lower than in the controls.

Basically, the results obtained with Strain L cells and HeLa cells were similar to those with the mouse liver cells.

There is at present considerable interest in the possible toxicity, carcinogenicity, or cocarcinogenicity of the constituents of tobacco smoke. It has been known for many years that acetaldehyde is one of the aldehydes present in tobacco smoke (8, 10, 17). It occurs in comparatively high concentrations—from 0.98 to 1.31 mg. per cigarette, depending upon the kind of tobacco smoked (9). Although Hartwell (5), and Shubik and Hartwell (15), reported that there was no evidence of carcinogenicity in acetaldehyde, Watanabe and Sugimoto (16) observed four cases of sarcomas in rats in areas where repeated subcutaneous injections of acetaldehyde were made.

Very little work has been reported on the effects of acetaldehyde upon cells grown in vitro. Investigations which have been reported were primarily concerned with the effects of this and related substances upon growth and metabolism of various tissue explants maintained in vitro (1, 12, 13, 18).

Therefore, since acetaldehyde occurs in comparatively high concentration in cigarette smoke, and since little is known concerning its action, an investigation was undertaken on the short- and long-term effects of this substance upon several types of tissue cells cultivated in vitro.

MATERIALS AND METHODS

Four cell lines were used in these investigations. They were Earle's strain L cells (NCTC clone 929), mouse liver epithelial cells (NCTC clone 1469), human skin cells (NCTC clone 1769), and HeLa cells. Stock cultures of these cells were maintained at 37.5° C. in T-60 flasks in Earle's NCTC #109 medium supplemented with 10 per cent horse serum (3, 4, 7). The medium was changed three times weekly. The liver cells and L cells were subcultured routinely every 3 or 4 days. Skin cells and HeLa cells were sometimes subcultured as often as every 3 or 4 days. Each culture throughout was gassed with a filtered 5 per cent CO₂-air mixture for 10 seconds.

Observations on the toxicity and possible influence of acetaldehyde upon rate of proliferation were made periodically, by a modification of the replicate method of culture as described by Pace and Aftonomos (9). During the preparation of the stock cultures for replication, cells from cultures 7–10 days old were scraped from the bottom
Experimental cultures were maintained in T-60 flasks. A quantity of fresh nutrient medium was added. The cells in the experimental cultures were usually exposed to the medium containing acetaldehyde 48 hours after replication. This allowed time for the cells to become attached to the floor of the culture flasks. Each culture flask was gassed for approximately 3 seconds at every medium change throughout the experiments.

Two or 3 times a week, three Carrel flasks from the control group and from each group of experimental cultures were selected at random and sacrificed for counting, as described by Sanford et al. (14). Counts were made by means of the Coulter Electronic Counter (6).

RESULTS

Short-Term Experiments

All four cell types previously mentioned were used. In these experiments observations were made over periods ranging from 15 minutes to 14 days. Six different concentrations of acetaldehyde were prepared, two concentrations for each of three series of experiments; 8.0 mg. and 2.0 mg. per ml.; 1.0 mg. and 0.01 mg. per ml.; and 0.5 mg. and 0.05 mg. per ml.

Series 1.—Strain L cells, liver cells, and skin cells were exposed to media containing either 2.0 mg. or 8.0 mg. acetaldehyde per ml., respectively. A photomicrograph of the cells of a typical control culture is presented in Figure 1.

In cultures exposed to 2.0 mg. acetaldehyde per ml., the cells seemed to resemble those of the control, except for a definite granulation and vacuolization. However, they appeared to be dead since they failed to attach and proliferate on subculturing. Careful microscopic examination and time-lapse photography gave further evidence that they were dead, apparently "fixed" by the acetaldehyde (Fig. 2). In general, this was also true for liver cells and skin cells.

When exposed to 8.0 mg. acetaldehyde per ml., the cells began to round up and detach within ½ hour (Fig. 3).

Thus, these two concentrations, 2.0 mg. and 8.0 mg., of acetaldehyde proved toxic to the cells in a matter of hours in the former concentration and minutes in the latter. The toxicity may have been due at least partly to an osmotic effect, especially in the 8.0 mg/ml cultures.

Series 2.—In two experiments, skin cells, HeLa cells, and liver cells were exposed to media containing 1.0 and 0.01 mg. acetaldehyde per ml., respectively; strain L cells were used in only one experiment.

In 1.0 mg/ml the cells soon rounded and detached; liver cells within 1½—2 hours, HeLa and skin cells within 3½ hours. Photomicrographs of HeLa cells showing the effects of acetaldehyde are presented in Figures 4—6, inclusive.

Series 3.—One experiment using concentrations of 0.5 and 0.05 mg. acetaldehyde per ml., respectively, was made with each of the four cell strains.

Cells exposed to 0.5 mg/ml were not killed immediately but rounded and detached within a few hours. All the L cells were killed within 48—96 hours' exposure; only 50 per cent of the HeLa cells still survived after 48 hours; all the liver cells died within 24 and 48 hours, the greatest death rate occurring within 4—24 hours' exposure.

The results obtained with the cells exposed to 0.05 mg/ml were similar to those obtained in medium containing 0.01 mg/ml. No changes could be detected by the usual microscopical methods.

Long-Term Experiments

These experiments were designed in order to ascertain the long-time effects of acetaldehyde in concentrations sufficiently low that no immediate effects are noticed.
Mouse liver, strain L, and HeLa cells were used in the experiments. Nine replicate cultures for each cell type were set up in T-60 flasks. Four days later, at the time of the second medium change, the cells had attached and proliferated, and the nine flasks were randomly divided into three groups. One of the groups was maintained on the control medium; another received the control medium plus 0.01 mg. of acetaldehyde per ml.; and a third group was exposed to the control medium plus 0.05 mg. of acetaldehyde per ml.

These three sets of cultures were kept at a constant temperature of 37.5°C; the medium was changed routinely every 48 hours, and after removal of the old medium each flask received 12 ml. of fresh medium. The liver cells and L cells were subcultured weekly. HeLa cells were often subcultured more frequently.

The T-60 flasks used for new replicate cultures received 12 ml. of cell suspension from 7-day-old cultures. Replicate cultures were also set up in Carrel D3.5 flasks for more accurate observation, each receiving 1 ml. of cell suspension. Following the first series of tests with mouse liver cells, the percentage of viable cells used for replicating the Carrel flask cultures and new T-60 stock cultures, was ascertained. For this purpose, a sample of the suspended cells was collected at the time of replication, although this was not done with series 1.

Beginning with the second series of each of the long-term experiments, one-half of the Carrel flask cultures in which the cells were exposed to 0.01 mg. or to 0.05 mg. acetaldehyde per ml. of medium were returned to the control medium at the first medium change following replication.

Three series of replicate cultures of each type of cell were made to check on the possible effects of these concentrations of acetaldehyde upon the rate of proliferation and gross population morphology. Each series represents a different exposure time as designated in the several tables.

Mouse Liver Cells

Series 1.—Twenty-three days after the three parent stock cultures (controls, cells in 0.01 mg., and cells in 0.05 mg. acetaldehyde) had been established in T-60 flasks, replicate cultures were made in Carrel flasks with cells from the control and each of the two experimental groups. These cultures were carried for 14 days (Table 1, Series 1). Rate and extent of proliferation in the control cells, as well as those in 0.01 mg. acetaldehyde, were similar. Microscopic observation revealed apparently healthy, flourishing cells in both.

Although the cells in 0.05 mg. acetaldehyde appeared normal during the first day or two, some had become detached by the 6th day, and by the 7th day all were dead or dying.

Series 2.—A second series of replicate cultures of liver cells in Carrel flasks was begun following 72 days' exposure to acetaldehyde in T-60 flasks. Table 1, series 2, shows both the initial cell density for the three sets and the percentage of viable cells. Part of the replicated cells in 0.01 mg. and those in 0.05 mg. acetaldehyde in Carrel flasks were returned to the regular control medium at the first medium change following replication.

The cells exposed to 0.01 mg. acetaldehyde continuously, and those that had been returned to control medium, appeared to proliferate rapidly during the first 6 days, after which there was an actual decrease in numbers. However, the cells appeared to be perfectly healthy throughout the experiment (Figs. 7, 8, and 9).

The cells in 0.05 mg. acetaldehyde never became established in Carrel flasks, and, for the most part, they remained rounded and floating (Fig. 10). The replicate cultures in T-60 flasks had to be discarded after 89 days' exposure; the cells would not attach to the flasks.

However, cells exposed to 0.05 mg. acetaldehyde and then returned to control medium recovered and proliferated rapidly after 3 days. The percentage increase for these cells exceeded the percentage increase for the others. They appeared normal and healthy, as indicated in Figure 11, taken 7 days, and in Figure 12, taken 17 days after return to control medium.

Series 3.—In this series of experiments, the mouse liver cells were exposed to acetaldehyde for 148 days before some of the cultures were returned to control medium (Table 1, Series 3). The cells exposed to 0.01 mg. acetaldehyde grew well. Figures 13–15, inclusive, are photomicrographs of some of the cultures in this series taken 5 days after replication (following 148 days' exposure). The cultures became so dense that many of the cells detached and were lost previous to the time of counting. Thus, the counts obtained and recorded were undoubtedly below the true number.

The cells which were returned to control medium following exposure to 0.01 mg. did not proliferate but gradually decreased in number.

Strain L Cells

The same procedures were followed for Strain L cells as for the mouse liver cells. Three different series of cultures exposed for three different periods of time were studied.

Series 1.—The cells were exposed to acetalde-
TABLE 1

A COMPARISON OF GROWTH OF MOUSE LIVER CELLS IN CONTROL MEDIUM AND IN MEDIA CONTAINING 0.01 AND 0.05 MG. ACETALDEHYDE (Ac.) PER ML., RESPECTIVELY

Some of the cultures so exposed were returned to control medium at first replication into Carrel flasks. Each series represents a different exposure time to acetaldehyde before starting replicate cultures in Carrel flasks in order to observe day-to-day effects. Three Carrel cultures were counted for each condition and for each day. Cell numbers are based on counts made on each of three cultures. RTCM, returned to control medium.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Initial cell no. (millions/mL)</th>
<th>No. viable cells (millions/mL)</th>
<th>Per cent viable</th>
<th>No. cells (millions/mL) at designated times in days following replication in Carrel flasks</th>
<th>Maximum cell no./Initial no. of viable cells</th>
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<td></td>
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<td></td>
<td>2</td>
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<td>Control cells</td>
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<td>0.42</td>
<td>0.88</td>
<td>2.27</td>
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<tr>
<td>Cells exposed to 0.01 mg. Ac.</td>
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<td>0.48</td>
<td>1.18</td>
<td>2.72</td>
<td>5.29</td>
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<td>Cells exposed to 0.05 mg. Ac.</td>
<td>0.32</td>
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<td>0.24</td>
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<td><strong>Series 2:</strong></td>
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<tr>
<td>Control cells</td>
<td>0.59</td>
<td>0.48</td>
<td>81</td>
<td>0.61</td>
<td>1.06</td>
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<td>Cells exposed to 0.01 mg. Ac.</td>
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<td>0.51</td>
<td>89</td>
<td>0.98</td>
<td>2.28</td>
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<tr>
<td>0.01 cells, RTCM</td>
<td>0.57</td>
<td>0.51</td>
<td>89</td>
<td>0.98</td>
<td>2.84</td>
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<td>Cells exposed to 0.05 mg. Ac.</td>
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<td>0.05 cells, RTCM</td>
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<td>56</td>
<td>0.20</td>
<td>0.24</td>
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<tr>
<td>Control cells</td>
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<td>0.26</td>
<td>90</td>
<td>0.51</td>
<td>1.18</td>
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<td>Cells exposed to 0.01 mg. Ac.</td>
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<td>86</td>
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<tr>
<td>0.01 cells, RTCM</td>
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<td>0.21</td>
<td>86</td>
<td>0.23</td>
<td>1.16</td>
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* Cells in T-60 flasks were exposed to acetaldehyde 83 days before starting replicate cultures in Carrel flasks.

† Based on initial cell number.

‡ Cells did not survive.

§ Cells in T-60 flasks were exposed to acetaldehyde 72 days before starting replicate cultures in Carrel flasks.

¶ Cells in T-60 flasks were exposed to acetaldehyde 148 days before starting replicate cultures in Carrel flasks.
hyde (0.01 mg and 0.05 mg/ml, respectively) for 40 days in T-60 flasks. Three groups of replicate cultures were set up in Carrel flasks, one group exposed to 0.05 mg. acetaldehyde per ml., one to 0.01 mg., and a third unexposed (control). The results are presented in Table 2, Series 1. The cells of all three groups appeared healthy throughout the experiments. The maximum cell numbers at the end of the experiment were not significantly different in either experimental or control cultures.

Series 2.—In this series after 110 days' exposure to acetaldehyde, the cultures were also divided into three groups—one containing 0.05 mg. acetaldehyde per ml., another 0.01 mg., and the third, none. Again, as in the liver cell series, part of the L cells in 0.01 mg. and part of those in 0.05 mg. acetaldehyde were returned to control medium at the first medium change. The results are presented in Table 2, Series 2. At the end of the experiment the percentage increase was greatest for the cells in 0.05 mg., next for the controls, and least for the cells in 0.01 mg. Those that had been returned to control medium died. All cells appeared to be in healthy condition (Figs. 16-20, inclusive). Although maximum numbers reached by the experimental cultures were somewhat greater than those of the controls, acetaldehyde showed no significant stimulatory or inhibitory effect.

Series 3.—The L cells were exposed to 0.01 mg. and 0.05 mg. acetaldehyde per ml., respectively, for 160 days before replicate cultures were started in Carrel flasks. The conditions and the results of these experiments are given in Table 2, Series 3. The same procedures were followed as in previous experiments. The results differ slightly from those obtained in Series 2, but, except for the cells exposed to 0.05 mg. (Fig. 23), no detectable changes were observed by the usual microscopic examination, and the cells appeared to be normal (Figs. 21, 22, and 24).

**HeLa Cells**

Three series of experiments were also carried out with HeLa cells. In Series 1, the cells were exposed to acetaldehyde for 45 days; in Series 2, for 125 days; and in Series 3, for 160 days.

The conditions of the experiments and the procedures followed are presented in Table 3. The results indicate that, basically, acetaldehyde has about the same general effect on HeLa cells as on liver and Strain L cells: a concentration of 0.05 mg/ml may effect an initial stimulation in proliferation; its long-time effect is usually inhibitory. The same is true for those experiments in which 0.05 mg. acetaldehyde per ml. was used.

**DISCUSSION**

There are many substances found in tobacco smoke, some of which are known to be harmful and others thought to be harmless. The effects produced by any of them would naturally depend upon concentration.

For the present study, acetaldehyde was chosen chiefly because of its rather high concentration in cigarette smoke (2).

Short-term experiments were carried out in order to ascertain concentrations of acetaldehyde which are not immediately toxic to the cells. Concentrations of 8.0 mg. or 2.0 mg/ml were toxic, and the cells became "fixed." On the basis of these short-term findings, 0.01 mg. and 0.05 mg. acetaldehyde per ml. were chosen as the concentrations for the long-term studies. At these concentrations cell proliferation appeared to be normal or was actually stimulated (except for the liver cells in 0.05 mg/ml), leading to these questions: Does this stimulation continue as long as the cells are exposed? Do the cells return to their normal rate of growth and then continue to proliferate as though no acetaldehyde were present? Is there a gradual accumulative effect of the substance that may later hinder growth?

For the long-term experiments, the mouse liver cells, strain L cells, and HeLa cells were used.

From the results it is evident that 0.05 mg. acetaldehyde per ml. is toxic to the liver cells—the cultures, following a 23-day exposure to acetaldehyde, never actually became established when replicated. In fact, the cells were dead within 3–7 days following replication. At this concentration, Strain L and HeLa cells proliferated well, at least for the first few weeks. However, cells in this concentration never increased as much as those in the controls. Why this great difference should exist between liver cells and other strains is at present unknown.

Although the liver cells did very well for the first 23 days (their number even surpassed that of the control cells) in 0.01 mg. acetaldehyde per ml., they began to show effects of this substance after 82 days.

When liver cells in 0.01 mg/ml for 72 days were returned to control medium they seemed to do no better than those left in acetaldehyde; all the cells exposed to this concentration for 148 days died within 12 days when returned to control medium. Thus, although acetaldehyde at this concentration was still somewhat toxic (inhibitory), the liver cells evidently became partly adjusted to the substance, especially after 148
A Comparison of Growth of Strain L Cells in Control Medium and in Media Containing 0.01 and 0.05 mg. Acetaldehyde (Ac.) per mL, Respectively

Some of the cultures so exposed were returned to control medium at first replication into Carrel flasks. Each series represents a different exposure time to acetaldehyde before starting replicate cultures in Carrel flasks to observe day-to-day effects. Three Carrel cultures were counted for each condition and for each day. Cell numbers are based on counts made on each of three cultures. RTCM, returned to control medium.

<table>
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<th>Cultures</th>
<th>Initial cell no. (millions/mL)</th>
<th>No. viable cells (millions/mL)</th>
<th>Per cent viable</th>
<th>No. cells (millions/mL) at designated times in days following replication in Carrel flasks</th>
<th>Maximum cell no./initial no. of viable cells</th>
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<td><strong>Series 1:</strong></td>
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<tr>
<td>Control cells</td>
<td>0.59</td>
<td>0.50</td>
<td>85</td>
<td>0.54, 1.28, 1.82, 2.10, 2.86, 5.96, 4.70, 4.76, 6.73</td>
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<tr>
<td>Cells exposed to 0.01 mg. Ac.</td>
<td>0.47</td>
<td>0.40</td>
<td>85</td>
<td>0.66, 1.48, 1.78, 2.80, 4.16, 5.92, 5.70, 5.98, 6.50</td>
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<td>0.42</td>
<td>84</td>
<td>0.48, 1.02, 1.42, 1.72, 3.24, 4.38, 4.90, 5.64, 6.26</td>
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<td>Cells exposed to 0.01 mg. Ac.</td>
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<td>Cells exposed to 0.05 mg. Ac.</td>
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<td>0.05 cells, RTCM</td>
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<tr>
<td>Control cells</td>
<td>0.48</td>
<td>0.45</td>
<td>94</td>
<td>0.62, 2.54, 3.12, 5.42, 5.92, 6.66</td>
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<tr>
<td>Cells exposed to 0.01 mg. Ac.</td>
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<td>0.48</td>
<td>89</td>
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<td>0.01 cells, RTCM</td>
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<td>0.48</td>
<td>89</td>
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<td>Cells exposed to 0.05 mg. Ac.</td>
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<td>0.05 cells, RTCM</td>
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<td>82</td>
<td>0.30, 1.72, 2.42, 4.42, 5.01, 5.84</td>
<td>15.8</td>
</tr>
</tbody>
</table>

* Cells in T-60 flasks were exposed to acetaldehyde 40 days before replicate cultures in Carrel flasks were started.
† Cells in T-60 flasks were exposed to acetaldehyde 100 days before replicate cultures in Carrel flasks were started.
‡ Cells in T-60 flasks were exposed to acetaldehyde 110 days before replicate cultures in Carrel flasks were started.
FIGS. 1-3.—Short-term experiments on the effects of acetaldehyde on strain L cells after 16 hours' exposure. X200.

Fig. 1.—Photomicrographs of cells in control medium—NCTC #109 plus 10 per cent horse serum.

Fig. 2.—L cells in medium containing 2 mg. acetaldehyde per ml.

Fig. 3.—L cells in medium containing 8 mg. acetaldehyde per ml.

FIGS. 4-6.—Short-term experiments on the effects of acetaldehyde on HeLa cells after 2 days' exposure. X200.

Fig. 4.—Photomicrograph of cells in control medium—NCTC #109 plus 10 per cent horse serum.

Fig. 5.—Cells exposed to 0.01 mg. acetaldehyde per ml. of medium.

Fig. 6.—Cells exposed to 1.0 mg. acetaldehyde per ml. of medium.

FIGS. 7-15.—Long-term experiments on the effects of acetaldehyde on mouse liver cells. X200.

Fig. 7.—Photomicrograph of cells in control medium—NCTC #109 plus horse serum—7 days after replication.

Fig. 8.—Cells in medium containing 0.01 mg. acetaldehyde per ml. (total exposure, 78 days)—7 days after replication.

Fig. 9.—Cells previously exposed to 0.01 mg. acetaldehyde per ml. but returned to normal medium (total exposure, 75 days followed by 3 days in control medium)—7 days after replication.

Fig. 10.—Cells in medium containing 0.05 mg. acetaldehyde per ml. (total exposure 78 days)—7 days after replication.

Fig. 11.—Cells previously exposed to 0.05 mg. acetaldehyde per ml. but returned to normal medium (total exposure 75 days, followed by 8 days in control medium)—7 days after replication.

Fig. 12.—Liver cells in control medium 17 days following a 75-day exposure to a medium containing 0.05 mg. acetaldehyde per ml.

Fig. 13.—Cells in control medium, 5 days after replication.

Fig. 14.—Cells in medium containing 0.01 mg. acetaldehyde per ml., 5 days after replication. (Total exposure to acetaldehyde, 138 days.)

Fig. 15.—Cells previously exposed to 0.01 mg. acetaldehyde per ml. but returned to control medium 5 days after replication. (Total exposure, 150 days to acetaldehyde, 3 days in normal.)

FIGS. 16-24.—Long-term experiments on the effects of acetaldehyde on strain L cells. The photomicrographs in each case were taken 3 days following replication. X200.

Fig. 16.—Strain L cells in control medium 3 days after replication.

Fig. 17.—Cells in medium containing 0.01 mg. acetaldehyde per ml. Total exposure to acetaldehyde, 113 days.

Fig. 18.—Cells previously exposed for 112 days to medium containing 0.01 mg. acetaldehyde per ml., after which they were returned to control medium.

Fig. 19.—Cells in medium containing 0.05 mg. acetaldehyde per ml. for 113 days.

Fig. 20.—Cells previously exposed for 112 days to medium containing 0.05 mg. acetaldehyde per ml., after which they were returned to control medium.

Fig. 21.—Cells in medium containing 0.01 mg. acetaldehyde per ml. for 169 days.

Fig. 22.—Cells previously exposed to medium containing 0.01 mg. acetaldehyde per ml. for 166 days, after which they were returned to control medium for 7 days.

Fig. 23.—Cells in medium containing 0.05 mg. acetaldehyde per ml. for 169 days.

Fig. 24.—Cells previously exposed to medium containing 0.05 mg. acetaldehyde per ml. for 162 days, after which they were returned to control medium for 7 days.
days, and the return to normal medium appeared to be too great a change for them.

Results with Strain L cells were somewhat different. Acetaldehyde had very little effect on those exposed for 70 days to 0.01 and 0.05 mg/ml, except perhaps a stimulating effect in 0.01 mg. However, growth in the experimental cells showed a downward trend after 110 days. This was especially noticeable in cells exposed to 0.05 mg acetaldehyde for 160 days. Although there was variation in the results obtained when cells that had been exposed to acetaldehyde were placed in control medium, the chief effect seemed to be one of shock. Strain L cells, just as was true of the liver cells, appeared to adapt to these concentrations of acetaldehyde, and though the results may indicate an accumulative toxic effect over a period of time, sudden removal of the acetaldehyde had a great deleterious effect, at least for the first week or so.

Thus, it appears from the results reported here that acetaldehyde (0.01 mg and 0.05 mg/ml) may, at first, enhance proliferation. After a period of several months, however, it becomes inhibitory. In fact, the cultures may die out eventually because of its presence.

It also appears as though those cells which showed the effects of acetaldehyde accumulation recovered if placed in normal nutrient medium unless the time of exposure to this substance had been excessive.

REFERENCES
Studies on the Effects of Acetaldehyde on Tissue Cells Cultivated \textit{in Vitro}

Donald M. Pace and Alice Elliott