Cytotoxic Effects of Low Levels of $^3$H-, $^{14}$C-, and $^{35}$S-labeled Amino Acids*

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Tissue injury by radiolabeled amino acids may severely affect experimental results. In this report, $^3$H-, $^{14}$C-, and/or $^{35}$S-labeled proline, serine, lysine, and methionine at concentrations of 1 and 5 pCi/ml were shown to cause severe injury to organ cultures of embryonic rat lungs. This injury was evident by 6 h and was amplified by 4 days of culture. This injury was characterized with light and electron microscopy, with morphometric analysis of growth, and with quantitation of the total protein and DNA/lung. After 6 h with 5 pCi/ml of $^{14}$C- or $^{35}$S-amino-acid there were more signs of cell degeneration, and by 24 h the labeled lungs were smaller, showed more signs of cell degeneration and death, and contained 30 to 60% less new protein and DNA than control lungs. After 24 h with 5 pCi/ml of $^{14}$C- or $^{35}$S-amino-acid the total protein and DNA/lung began to decrease. This toxicity was directly proportional to the amount of intracellular decay of each isotope. With $^{14}$C- and $^{35}$S-amino-acids, lung growth slowed with 100 disintegrations/cell/day ($d/c/d$), growth stopped with $<$200 $d/c/d$, and atrophy occurred with $>$300 $d/c/d$. Cell proliferation, cell differentiation, and bronchial branching continued through 4 days even though atrophy occurred with $>$200 $d/c/d$. With $^3$H-amino-acids, growth slowed with $>$200 $d/c/d$ and stopped with $>$400 $d/c/d$. However, no toxicity was evident with $<$80 $d/c/d$ of $^{14}$C- or $^{35}$S, or with $<$90 $d/c/d$ of $^3$H. These data suggest that the amounts of intracellular decay of these weak $\beta$-emitting isotopes should be strictly limited. Increasing amounts of tissue injury occurred with $^{14}$C or $^{35}$S at $>$10,000 dpm/µg of DNA, and with $^3$H at $>$20,000 dpm/µg of DNA.

Since the low energy $\beta$-emitting radioisotopes $^3$H, $^{14}$C, and $^{35}$S are widely used in biomedical research, it is important to know of the toxic effects of these isotopes on cells and tissues (3-7). Each of these sources of radiation cause both single and double strand breaks in DNA, and this is one of the major mechanisms of radiation-induced cell injury (4-7). Base alterations and interstrand cross-links also occur in DNA, but the strand breaks are more important (6, 7). These breaks in DNA occur most frequently when $^3$H-nucleosides are incorporated into DNA; with more than two breaks occurring per disintegration of $^3$H (4). This damage of DNA results in a slowing or block of cell proliferation (4-10). For example, with cultures of HeLa cells, 0.2 pCi/ml of $[^3]$H-thymidine reduced cell proliferation by $\sim$10% and 0.4 pCi/ml reduced cell proliferation by $\sim$99% (9). Similarly, with cultures of WI-38 cells, a 0.1 pCi/ml concentration of $[^3]$H-thymidine slowed cell proliferation by $\sim$50% and 0.25 pCi/ml stopped cell proliferation completely (10). This block of cell proliferation is most efficient with $^3$H in DNA, less efficient with $^3$H in nuclear RNA, and least efficient with $^3$H-labeled amino acids (11). This is due to the fact that the path length of 90% of the $\beta$-electrons of $^3$H decay is less than 1 µm (12). Nevertheless, cell proliferation is blocked by the incorporation of $[^3]$H-$\alpha$-aminoisobutyric acid, which is transported into the cytosol as a small neutral amino acid but is not incorporated into cellular components (13). This phenomenon is referred to as reproductive cell suicide.

A review of numerous radiotracer studies of protein synthesis and other metabolic processes shows that many investigators routinely use high concentrations of radiolabeled amino acids for periods from 6 h to a few days. Furthermore, the corresponding unlabeled amino acid is often omitted from the culture medium. It appears that many investigators are not aware that $^3$H-, $^{14}$C-, and $^{35}$S-amino-acids quickly result in the type of tissue injury that is described in this report. For example, we were quite surprised when 5 pCi/ml of $[^3]$H- and $[^4]$C-proline were severely toxic in cultures of embryonic chick somites mesoderm and in cultures of both embryonic rat and chick lungs (3).

Since radiolabeled amino acids are widely used at toxic concentrations it is important to show how trace levels of these labeled precursors can affect tissue function. Therefore, these studies were designed to learn 1) whether the inhibition of tissue growth is due to the radiolabel or a toxic contaminant in the labeled amino acid preparation, 2) whether different types of labeled amino acids are equally toxic, 3) whether the toxicity is reduced by dilution with increasing concentrations of L-amino acids in the nutrient medium, or 4) whether the toxicity is proportional to the transition energy of the isotope, to the concentration of radioactivity in the nutrient medium, to the amount of labeled amino acid that is incorporated, or to the function of the isotopic atom or its transition product.

**MATERIALS AND METHODS**

**Lung Bud Isolation and Culture—**Timed pregnant, 200 g, BLU Sprague-Dawley rats were obtained from Blue Spruce Farms, Alta.

**Unpublished experiments.**
stem bronchus was cut at the hilum of each lung, and the buds were transferred to the surface of a nutrient agar substrate.

The initial experiments in these studies utilized a nutrient agar substrate consisting of 1% agar in a mixture of 10% SBSS, 4% fetal bovine serum, and 80% F-12C. The F-12C was Coon's modification of Ham's F-12 containing 8 X amino acids, 8 X sodium pyruvate, 4 X hypoxanthine, and 0.1 X thymidine (Table I and Ref. 16). This F-12C was provided by Grand Island Biological Co. without pyridoxine, lysine, valine, or glutamine (formula No. 78-0429). When fresh media was prepared these amino acids were added to a final concentration of 2.4 mM L-proline, 1.6 mM L-lysine, 0.8 mM L-valine, and 8.0 mM L-glutamine (Table I). To increase the specific activity of radiolabeled proline or asparagine the concentrations of proline and lysine were reduced to 0.8 mM. This modified nutrient mixture was referred to as F-12mc (Table I). In selected experiments proline was omitted from the F-12C and this mixture was referred to as F-12C(-pro).

During the course of this study it was found that the 1% nutrient agar substrate was much easier to prepare and results were more consistent with a mixture of 20% SBSS, 10% FBS, and 70% F-12C. This mixture was used for subsequent experiments.

In all experiments, the nutrient agar and liquid nutrient feeding media were prepared as described previously (10). Kanamycin solution (GIBCO No. 516) and penicillin-streptomycin solution (GIBCO No. 507) were added to a final concentration of 1%, and a radiolabeled amino acid was added to the same concentration in both the nutrient agar and liquid medium. Ascorbic acid was added to a concentration of 200 μg/ml in the liquid medium. Aliquots of 100-200 μl of liquid medium were stored at −20 °C for each daily feeding. One ml of nutrient agar was placed in the well of each organ culture dish (Falcon No. 3010), and 8 or 10 lung buds, with equal numbers of right and left lungs, were explanted to each dish. Each dish was fed 50 μl of the same liquid nutrient medium as that in the nutrient agar, and the lungs on a representative dish were photographed. One dish of lung buds was then collected for analysis of the zero time protein and DNA as described below. After feeding, the remaining dishes were incubated in a humified atmosphere of 5% CO₂ in air at 37 °C. At daily intervals the dishes were photographed and, each dish was fed 50 μl of liquid nutrient medium.

Radiolabeled Amino Acids—A typical experiment in these studies consisted of one dish of lung buds for analysis of the freshly isolated tissues at zero time, one set of three dishes of control cultures without a label, one set of three dishes containing 1 μCi/ml of radiolabeled amino acid, and one set of three dishes containing 5 μCi/ml of the same radiolabeled amino acid. Most of the radiolabeled amino acids were purchased from New England Nuclear. This included L-[U-¹³C]proline (NEC 285), L-[U-¹⁴C]-serine (NEC 256), L-[U-¹⁴C]lysine (NEC 298), L-[2-³H]-proline (NET 329), and L-[³H]-lysine (NET 248). These radiolabeled amino acids were all in 0.1% HCl. It also included L-[³⁵S]-methionine (225) in 1:1 ethanol HCl solution and L-[³⁵S]-methylmethionine (NEG 90011) in water containing 10% glycerol and 2-mercaptoethanol/ml. The specific activity of each of these preparations is listed in Table II. In addition, two batches of L-[¹⁴C]proline in 2% ethanol (CFB 111) were obtained from Amersham. In some experiments the radiolabeled amino acids were dried and resuspended in SBSS, but in no case was there a detectable difference between lung buds growing in the presence of U-[¹⁴C]-amino acid preparation in 0.01 N HCl, in 2% ethanol, or in SBSS. Since the [³⁵S]-methionine contained 2-mercaptoethanol, the amount of reducing agent in a 5-μl aliquot of labeled amino acid was added to each milliliter of nutrient medium for control cultures in experiments labeled methionine.

Analysis of Cultured Lungs—After 12 h of labeling, the cultured lungs were examined for visible evidence of toxicity. At the end of 1, 2, and 4 days of culture, the lungs on one dish from each 0, 1, and 5 μCi set were photographed and collected for analysis of the total protein, DNA, and dpm of radioactivity was always determined as an average amount per lung bud in four to six pairs of right and left buds. For analysis, thawed samples were uniformly dispersed by sonication with a Branson Model 185 cell disruptor with a special microtip. Duplicate aliquots were taken for total protein, total DNA, and total dpm of radioactivity per lung. The total protein in each aliquot was determined by the procedure of Lowry et al. (18) as modified by Schacterle and Pollock (19). The total DNA in each aliquot was determined by the fluorometric procedure of Kissane and Axelrod (20) as modified by Schacterle and Ayala (21). In some previous studies have shown that diploid rat cells contain about 7 pg of DNA (22), each microgram of DNA was estimated to represent ~143,000 cells.

The total incorporation of radioactivity/lung (dpm/lung), and the proportion of this radioactivity that was precipitable with 5% cold trichloroacetic acid was estimated by the following procedure. A 300-μl aliquot of the sonicate of washed lungs was diluted to 1 ml and 100-μl aliquots were counted in Liquidscint (National Diagnostics Corp.). Bovine serum albumin (0.5 mg) was added as a carrier and
TABLE II

Characteristics of the labeled precursor preparations

|                  | Specific activity | No. heavy atoms/μCi | Total No. molecules/μCi | Average No. heavy atoms/molecule | No. labeled molecules/μCi |
|------------------|-------------------|----------------------|-------------------------|----------------------------------|---------------------------|
| [14C]Proline     |                   |                      |                         |                                  |                           |
| Batch 1          | 0.291             | 9.41 × 10^{15}       | 2.07 × 10^{15}          | 4.55                             | 2.07 × 10^{15}            |
| Batch 2          | 0.255             | 9.41 × 10^{15}       | 2.36 × 10^{15}          | 3.99                             | 2.38 × 10^{15}            |
| [14C]Serine      |                   |                      |                         |                                  |                           |
| Batch 1          | 0.159             | 9.41 × 10^{15}       | 3.79 × 10^{15}          | 2.48                             | 3.79 × 10^{15}            |
| Batch 2          | 0.178             | 9.41 × 10^{15}       | 3.38 × 10^{15}          | 2.78                             | 3.38 × 10^{15}            |
| [14C]Lysine      | 0.338             | 9.41 × 10^{15}       | 1.78 × 10^{15}          | 5.29                             | 1.78 × 10^{15}            |
| [14C]Methionine  | 0.291             | 9.41 × 10^{15}       | 2.07 × 10^{15}          | 3.52                             | 2.07 × 10^{15}            |
| Batch 1          | 928               | 4.06 × 10^{11}       | 6.49 × 10^{11}          | 0.64                             | 4.06 × 10^{11}            |
| Batch 2          | 1118              | 4.06 × 10^{11}       | 5.38 × 10^{11}          | 0.75                             | 5.38 × 10^{11}            |
| [3H]Proline      | 23                | 2.09 × 10^{13}       | 2.62 × 10^{13}          | 0.80                             | 2.09 × 10^{13}            |
| [3H]Serine       | 16                | 2.09 × 10^{13}       | 2.76 × 10^{13}          | 0.76                             | 2.09 × 10^{13}            |
| [12C]-proline    |                   |                      |                         |                                  |                           |
| Batch 1          | 23                | 2.09 × 10^{13}       | 0.80                    |                                  |                           |
| Batch 2          | 1118              | 4.06 × 10^{11}       | 5.38 × 10^{11}          | 0.75                             | 5.38 × 10^{11}            |

Trichloroacetic acid was added to a concentration of 5%. The sample was incubated 2 h at 4 °C, and centrifuged at 2000 × g for 30 min at 4 °C. The supernatant was removed, the pellet was dispersed by sonication in 0.5 ml of H2O, and 100-μl aliquots of both the supernatant and suspended pellet were counted in Liquiscent. These analyses showed that with each labeled precursor ~90% of the radioactivity in the washed lungs was precipitated by 5% cold trichloroacetic acid.

The disintegrations per cell per day (d/c/d) were estimated with the formula: dpm/lung × (μg of DNA/lung)^{-1} × 1440 min/day × (143,000 cells/μg of DNA)^{-1} = estimated d/c/d. The d/c/d were then used with the following formula from Casarett (23) to estimate the rads/cell/day.
**TABLE III**

| [\(^{14}\text{C}\)] Proline | Final dilution | Culture day | Protein | DNA | Incorporation of \(^{14}\text{C}\) | Estimated d/c/d |
|--------------------------|----------------|-------------|---------|-----|-----------------|----------------|
| \(\mu\text{Ci/ml}\)    |                |             | \(\mu\text{g/lung}\) | \(\mu\text{g/lung}\) | \(10^{-3} \times \text{dpm/lung}\) | \(10^{-3} \times \text{dpm/\mu g DNA}\) |       |
| 0                        | \(\infty\)     | 0           | 24.3 ± 1.1 | 2.2 ± 0.3 |                 |                 |       |
|                           | 1              | 1           | 42.0 ± 0.6 | 3.5 ± 0.7 |                 |                 |       |
|                           | 2              | 2           | 52.7 ± 3.2 | 5.1 ± 0.1 |                 |                 |       |
|                           | 4              | 4           | 65.2 ± 6.8 | 5.8 ± 0.7 |                 |                 |       |
| 1                        | 11             | 1           | 30.3 ± 6.9 | 2.6 ± 0.6 | 25.8 ± 12 | 9.9 | 106 |
|                           |                | 2           | 34.7 ± 1.8 | 2.8 ± 0.1 | 48.5 ± 12 | 17.3 | 174 |
|                           |                | 4           | 42.3 ± 1.9 | 3.4 ± 0.5 | 58.0 ± 12 | 17.0 | 71  |
| 5                        | 2.2            | 1           | 30.3 ± 5.2 | 2.2 ± 0.4 | 103.5 ± 16 | 47.0 | 473 |
|                           |                | 2           | 21.7 ± 1.3 | 1.3 ± 0.1 | 118.7 ± 12 | 91.3 | 919 |
|                           |                | 4           | 12.3 ± 4.8 | 0.6 ± 0.2 | 62.4 ± 0.2 | 104.0 | 1047 |

**TABLE IV**

| 1-[\(^{14}\text{C}\)] Proline stock (17.24 \(\mu\text{M/ml}\)) | Total Protein | Total DNA | Protein to DNA ratio |
|-------------------------------------------------------------|---------------|-----------|----------------------|
|                                                             | \(\mu\text{g/lung}\) | \(\mu\text{g/lung}\) |                        |
| 1. [\(^{14}\text{C}\)] Proline stock                        | 69.4 ± 5.9    | 7.65 ± 0.6 | 9.1                  |
| 2. [\(^{14}\text{C}\)] Proline, 0.01 \(\text{N HCl}\) as per Sample 4 | 68.4 ± 4.7    | 6.88 ± 0.6 | 9.9                  |
| 3. [\(^{14}\text{C}\)] Proline chromatographed as per Sample 4 | 69.1 ± 8.9    | 7.55 ± 1.1 | 9.2                  |
| 4. [\(^{14}\text{C}\)] Proline, 5 \(\mu\text{Ci/ml}\)        | 18.6 ± 3.5    | 1.38 ± 0.5 | 13.5                 |

Estimated rads/cell/day

\[
\text{d/c/d} \times E \text{ (MeV)} = 7 \times 10^{-10} \text{ (g/cell)} \times 6.24 \times 10^{7} \text{ (MeV/erg)} \times 100 \text{ (ergs/g/rad)}
\]

The average transition energy per disintegration (\(E\)) for \(^{3}\text{H}\) is 0.0065 MeV, and for \(^{14}\text{C}\) \(E\) is 0.065 MeV (17). The estimate of \(7 \times 10^{-10}\) MeV was obtained with the following formula: \(10 \times g \text{ of protein/} \mu\text{g } \times \) (estimated number of cells/lung) \(^{-1}\) \(= \) estimated g/cell. This assumes that the average cell is \(\sim\) 10% protein. These values are obviously only rough estimates but they facilitated the comparison of the toxic levels of radioactivity in the cultured lung buds with data from other experimental systems.

Two methods of estimating cell volume were used to estimate the concentrations of incorporated isotope in lung buds. One method assumed that the average cell was a 10-μm sphere and this gave an average cell volume of 5.236 \(\times \times 10^{-3}\) ml. Comparable values were obtained if one assumed that the average cell in these lung buds was 10% protein. The average protein:DNA ratio was \(\sim\) 10 (Tables III–VI) and the average diploid rat nucleus contains \(\sim 7\) pg of DNA (8). Therefore, the average cell was estimated to contain \(\sim 70\) pg of protein and have an average cell weight of 700 pg. With a specific density of 1.10 this weight would occupy a volume of 6.36 \(\times \times 10^{-3}\) ml. Thus, the average cell was estimated to have a volume of 5.8 \(\times \times 10^{-3}\) ml and this volume was used to estimate the intracellular concentration of each isotope.

The amount of radioactivity remaining in the nutrient agar was also determined by counting aliquots of the nutrient agar that was dissolved in 3 ml of warmed water. These analyses showed that 5 to 10% of the radioactivity in each dish was incorporated by five pairs of 14.5-day-old rat embryo lung buds.

For microscopic analysis the lungs were fixed for 24 h at 4 °C in 1% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4, and post-fixed for 1 h at 4 °C in 1% osmium tetroxide in the same buffer. In some experiments the tissues were stained en bloc with ruthenium red as described previously (24). The fixed lungs were stained en bloc with uranyl acetate (24), and were cut transversely into three pieces that included the proximal, intermediate, and distal airways, respectively. These pieces were dehydrated in ethanol, followed by propylene oxide, and were embedded in Epon:Araldite. One micron sections of lungs were deplastcized and stained as described previously (24). Thin sections, stained with uranyl acetate and lead citrate, were examined with a Phillips 301 electron microscope.

**RESULTS**

**Effects of \(^{14}\text{C}\) Proline**

Lung growth, bronchial morphogenesis, and cytodifferentiation were identical in these studies with that described previously (3). The original goal of these experiments was to study the synthesis and deposition of collagen during the period of bronchial morphogenesis. Surprisingly, there was
Table V

Lung growth with or without [14C]proline at high levels of dilution with L-proline

The values represent the mean and standard deviations obtained in three experiments with cultures of 14.5-day-old embryo lung buds that were grown for 1, 2, or 4 days on 1% agar in a 20:10:70 mixture of SBSS:FBS:F-12C containing either 0, 1, or 5 µCi/ml of [14C]proline with a specific activity of 291 mCi/mmol.

| µCi/ml | Final dilution | Culture day | Protein | DNA |
|--------|----------------|-------------|---------|-----|
|        | µg/lung        | µg/lung     |         |     |
| 0      | ∞              | 16.9 ± 0.7  | 1.8 ± 0.4 |
| 1      | 500            | 29.5 ± 1.5  | 3.0 ± 0.3 |
| 5      | 100            | 29.6 ± 9.9  | 2.9 ± 0.3 |
|        |                | 38.7 ± 6.5  | 3.7 ± 0.4 |
|        |                | 47.3 ± 3.8  | 4.0 ± 0.1 |
|        |                | 30.5 ± 8.4  | 2.9 ± 0.3 |
|        |                | 22.3 ± 8.2  | 1.9 ± 0.1 |
|        |                | 52.2 ± 7.9  | 17.8 ± 179 |
|        |                | 66.5 ± 3.4  | 23.0 ± 232 |
|        |                | 57.8 ± 3.7  | 30.6 ± 308 |

Consistently a marked decrease in growth when 5 µCi/ml of [14C]proline were added to the culture system. This slowing of growth began during the first 12 h of culture with both the 10:10:80 and 20:10:70 mixtures of SBSS:FBS:F-12C(-pro) nutrient media. After 12 h of culture, the explants with [14C]proline appeared darker and more granular than control explants. This density and granularity corresponded to an increase in cell death and an accumulation of debris filled secondary lysosomes as described below. At 24 h this difference in translucency and granularity was more pronounced and the labeled lungs were smaller than control lungs (Figs. 1 and 2). These differences were most pronounced in cultures of 13.5-day-old lungs (Fig. 2). This difference in size was accentuated after the 2nd day of culture when the labeled lungs began to atrophy while the control lungs continued to grow (Figs. 1 and 2). Thus, bronchial branching continued but growth stopped and atrophy occurred in the presence of 5 µCi of [14C]proline.

The visible differences in living cultures consistently corresponded to a marked decrease in the rate of accumulation of both protein and DNA in explants growing in the presence of [14C]proline (Tables III and IV). During the first 24 h approximately 66% less new protein and DNA accumulated, and after 24 h the protein and DNA began to decline in explants with 5 µCi of [14C]proline. This toxicity of the [14C]proline was unchanged when the stock of [14C]proline in 0.01 N HCl (NEC 285) was lyophilized and resuspended in SBSS, or when the product was rechromatographed on Dowex and resuspended in SBSS (data not shown). Similar results were obtained with 5 µCi/ml of [14C]proline from Amersham (CFB 71) (data not shown). It was not known, however, whether this inhibition of growth was due to the [14C]proline or to a toxic contaminant.

The next group of experiments was designed to determine whether a toxic contaminant was copurifying with the [14C]proline from New England Nuclear. Each of these experiments consisted of four sets of duplicate dishes with 8 lung buds/dish. Each set of duplicate dishes contained one of the samples of L-proline from New England Nuclear. Sample 1 was the L-[14C]proline stock (Calbiochem 5370), Sample 2 was the [14C]proline dissolved in the HCl in water that was used to resuspend the [14C]proline, Sample 3 was the [15C]proline that was chromatographed on the same system as the [14C]proline, and Sample 4 was cold L-proline.
and Sample 4 was 5 μCi of [14C]proline (NEC 285) with a specific activity of 290 mCi/mmol. These samples were added to a final concentration of 17.24 μmol/ml of L-proline in 10% SBSS:10% FBS:80% F-12C(-pro) nutrient medium. Each dish was photographed and fed 50 μl of the same nutrient medium daily. In each of these experiments the lungs grew well in all of the dishes with [14C]proline samples, while growth slowed and stopped in all dishes containing [12C]proline (Fig. 3). During 4 days of culture there was essentially no net growth of lungs in dishes containing 5 μCi/ml of [14C]proline while there was approximately a 3-fold increase in protein and DNA/lung in dishes containing the different samples of [14C]proline (Table IV). Consequently, these results indicated that the [14C]proline itself rather than a toxic contaminant was responsible for the inhibition of lung growth.

The next group of experiments was designed to determine whether the toxic effect of [14C]proline was affected by differences in the quantities of L-proline in the nutrient medium. To answer this question dishes of 8 lung buds were cultured in the presence of 1 or 5 μCi/ml of [14C]proline in a 20:10:70 mixture of SBSS:FBS and either F-12C or F-12mC. L-Proline was assumed to be 0.374 mM in the FBS and it was 2.4 mM in the F-12C and 0.8 mM in the F-12mC (Table I and Ref. 25). As shown in Table V the toxicity of the [14C]proline was still present but there was less inhibition of growth as the incorporation of [14C]proline was reduced by dilution with the increased amounts of L-proline in the F-12C (cf. Tables III and V). With a 2.2 × dilution of 5 μCi of [14C]proline, atrophy occurred after culture day 1 (Table III), but atrophy did not occur until the 3rd or 4th day of culture with 5 μCi/ml of [14C]proline diluted 100 × with 70% F-12C (Table V). The inhibition of growth in cultures with F-12mC was intermediate between that with F-12C(-pro) and F-12C (data not shown). Thus, the inhibition of growth varied inversely with dilution and directly with the amount of incorporation of [14C]proline.

Effects of [14C]Serine

Once it was clear that the incorporation of [14C]proline was the cause of the inhibition of lung growth, it became important to know whether other 14C-amino-acids have similar effects. To answer this question 14.5-day-old lung buds were cultured with either 0, 1, or 5 μCi of [14C]serine with a specific activity of 159 or 178 mCi/mmol. This experiment was repeated three times and in each case the inhibition of lung growth was very severe with 5 μCi/ml and moderately severe with 1 μCi/ml of [14C]serine (Figs. 4 and 5 and Table VI). During the 1st day of culture there was an increase of 12 μg of protein/lung in control cultures, 8 μg/lung in cultures with 1 μCi/ml, and only 6 μg/lung in cultures with 5 μCi/ml of [14C]serine (Table VI). This indicated that during a 24-h pulse with 1 μCi of [14C]serine there was 33% and with 5 μCi there was 50% less new protein accumulation than in lungs growing without a labeled amino acid. By the end of 4 days of culture, the total protein/lung had increased approximately 300% in the control cultures and approximately 100% in cultures containing 1 μCi/ml. However, with 5 μCi/ml the total protein and DNA decreased after the 1st day of culture, and with 1 μCi/ml there was no increase in DNA after day 1 (Table VI). Nevertheless, the dpm of 14C/μg of DNA continued to increase (Table VI), suggesting that protein synthesis continued through 4 days of culture.

As shown in Table VI there was approximately 58 d/c/d after 24 h of culture with 1 μCi/ml of [14C]serine. At 48 h this value had increased to ~173 d/c/d, at this time there was a notable decrease in lung growth. With 5 μCi/ml there were ~213 d/c/d at 24 h, and during the 2nd day of culture lung growth stopped and atrophy began. This indicated that the rate of cell death exceeded the rate of cell proliferation when

![Graph](image)

**Fig. 5.** The average area per lung was measured in photographs of living lungs in the [14C]serine experiment shown in Fig. 4. The nutrient agar contained no label (●—●), 1 μCi/ml (■—■), or 5 μCi/ml (○—○) of [14C]serine. The changes in average area corresponded directly with the changes in total protein and DNA per lung (Table V).

| [14C]Serine Final dilution Culture day Protein DNA | 10⁻³ × dpm/lung  | 10⁻² × dpm/μg DNA | Estimated d/c/d |
|---|---|---|---|
| μCi/ml | | | |
| 0 | | | |
| 1 | 91 | 1 | 271.7 ± 0.7 | 3.4 ± 0.3 | 19.3 ± 1.8 | 5.7 | 58 |
| 2 | | 39.2 ± 0.4 | 2.4 ± 0.1 | 26.8 ± 3.3 | 11.2 | 113 |
| 4 | | 43.0 ± 7.2 | 3.2 ± 0.9 | 36.4 ± 8.0 | 11.4 | 115 |
| 5 | 18 | 1 | 24.9 ± 3.6 | 3.0 ± 1.5 | 63.4 ± 11.7 | 21.1 | 213 |
| | | 2 | 19.2 ± 1.1 | 2.3 ± 0.8 | 77.6 ± 26.2 | 33.7 | 340 |
| | | 4 | 16.7 ± 3.6 | 1.6 ± 0.6 | 61.8 ± 6.1 | 38.6 | 389 |
the rate of intracellular decay of $^{14}$C was $>200$ d/c/d or $\sim 20,000$ dpm/µg of DNA. This is equivalent to $\sim 230$ rads/cell/day.

Microscopic studies showed that there was a consistent increase in the signs of cell degeneration and death in labeled cultures (Figs. 6 to 8). After 6 h of culture with $5 \mu$Ci/ml of $[^{14}C]$serine, increased numbers of secondary lysosomes or digestive vacuoles had begun to accumulate in both epithelial and mesenchymal cells (Fig. 6). By 24 h large complexes of digestive vacuoles were present in many mesenchymal cells, and pycnocytic cells and cell debris were present in the blood islands (Fig. 6). In addition, an extensive accumulation and hydrolysis of glycogen resulted in the appearance of localized cytoplasmic vacuolation in nearly all epithelial cells in labeled lungs (Figs. 7 and 8). This increased accumulation of digestive vacuoles and cell debris in the mesenchyme and the accumulation of glycogen in the epithelium continued through 4 days of culture (Figs. 7 and 8). These signs of cell degeneration and death were directly related to the increased density and granularity of living cultures, and to the slowing of growth and onset of atrophy in labeled lungs.

Mitotic figures were present in both epithelial and mesenchymal cells in both labeled and unlabeled cultures throughout 4 days of culture (Figs. 7 and 8). Thus, cell proliferation and bronchial branching both continued through 4 days in the presence of $5 \mu$Ci/ml of $[^{14}C]$serine.

Effects of $[^{14}C]$Lysine

These experiments were designed to ask whether $[^{14}C]$lysine is more toxic than $[^{14}C]$proline or serine. Since lysine constitutes 17% of the residues in histone proteins, the proportion of intranuclear decay should be increased with this label. The fact that it is both cationic and an essential amino acid might also affect the toxicity of $[^{14}C]$lysine. Three sets of dishes
Cytotoxicity of $^3H$, $^{14}C$, and $^{35}S$

These results were inconsistent with the hypothesis that the toxicity would be increased by enrichment of $^{14}C$-labeling of nucleoproteins.

$[^{14}C]$- versus $[^{35}S]$Methionine

$[^{14}C]$Methionine—In three identical experiments, 8 or 10 lung buds were grown on 1 ml of agar in a 10:10:80 mixture of SBSS:FBS:F-12 containing either 0, 1, or 5 μCi of $[^{14}C]$ methionine. The results of these experiments were essentially identical with those with other $^{14}C$-amino-acids (cf. Tables III, VI, VII, and IX). In each case 5 μCi/ml were severely toxic and 1 μCi/ml was moderately toxic (Fig. 10 and Table IX). After 12 h the lungs with 5 μCi/ml were more dense and granular, and by 24 h the labeled lungs were smaller and contained 20 to 25% less protein and DNA than control lungs.
As shown in Fig. 10 and Table IX the inhibition of growth with $[^{14}C]$methionine was directly related to the disintegrations/cell/day of incorporated $^{14}C$. At 24 h with 1 $\mu$Ci/ml there were ~61 d/c/d and growth was only slightly slower than in control lungs. After 48 h with 1 $\mu$Ci/ml there were >100 d/c/d and growth was noticeably reduced but had not stopped. With 400 d/c/d, however, growth stopped and the lungs began to atrophy (Table IX). These d/c/d were estimated to represent doses of 70, 114, and 458 rads/cell/day.

$[^{35}S]$Methionine—One of the major purposes of these studies was to learn whether the number of heavy atoms incorporated or the position or function of the isotopic atom(s) affected the toxicity of the radiolabeled amino acids. To help answer this question the previous experiment was repeated four times with 0, 1, and 5 $\mu$Ci/ml of $[^{35}S]$methionine. A 10:10:80 mixture of SBSS:FBS:F-12C was used in the first two experiments and a 20:10:70 mixture was used in the others. Consequently, there were either 0.1736 or 0.1976 $\mu$mol of L-methionine/ml of nutrient medium (Table I). Each microcurie aliquot contained 4.06 $\times$ 10$^7$ labeled molecules or 6.74 $\times$ 10$^7$ $\mu$mol of $[^{35}S]$methionine (Table II). This gave a minimum final dilution of 257,800 with 1 $\mu$Ci/ml and 51,500 with 5 $\mu$Ci/ml (Table X). These dilutions of the $[^{35}S]$methionine were ~4,500 times that of the $[^{14}C]$methionine in the previous experiments (cf. Tables IX and X).

The results of these experiments showed that in spite of the large dilution there was a severe inhibition of lung growth in all cultures with 5 $\mu$Ci/ml and a moderate inhibition in those with 1 $\mu$Ci/ml (Fig. 11 and Table X). At 12 h the labeled lungs were more dense and granular, and at 24 h the labeled lungs were smaller and contained ~60% less new protein than control lungs. After 24 h with 5 $\mu$Ci/ml the lungs began to atrophy but bronchial branching continued through 4 days. As in previous experiments the atrophy was evidenced by a progressive decrease in the average area, total protein, and DNA/lung (Fig. 11 and Table X).

Light and electron microscopic examination showed that the morphologic differences between labeled and control lungs were identical with those seen previously in experiments with $[^{14}C]$serine (Figs. 6 to 8). The bronchial epithelial cells accumulated large amounts of glycogen in their cytosol, debris-filled secondary lysosomes accumulated in mesenchymal cells and dead cells, and cell fragments accumulated in blood islands. After 4 days of culture, type II pneumocytes begin to differentiate in all cultures but there were fewer lamellar bodies in the glycogen laden epithelial cells in cultures with 5 $\mu$Ci/ml. Mitotic figures were also present in both the epithelium and mesenchyme and there were many morphologically normal cells in all cultures. This showed that there was much more evidence of cell degeneration and death, but there was not a generalized killing of cells in the labeled lungs (data not shown).

The toxic effects of $[^{35}S]$methionine were equal to or greater than those of $[^{14}C]$methionine (cf. Tables IX and X). With both labels the rate of accumulation of protein and DNA was markedly lowered by 1 $\mu$Ci/ml and there was a net loss of protein and DNA after a short period of growth with 5 $\mu$Ci/ml. Furthermore, the amount of radioactivity per lung with $[^{35}S]$methionine was equal to or slightly greater than that with $[^{14}C]$methionine (Tables IX and X). This equivalence of incorporated radioactivity resulted from the fact that the decay constant of $^{35}S$ is ~2.3 $\times$ 10$^{-2}$ larger than that of $^{14}C$. Even though ~4500 molecules of $[^{14}C]$methionine containing ~2.3 $\times$ 10$^7$ atoms of $^{14}C$ were incorporated for each molecule of $[^{35}S]$methionine, a much larger proportion of the $^{35}S$ disintegrated during the culture period. This was interesting for it showed that the inhibition of growth was more directly related

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**Cytotoxicity of $^3H$, $^{14}C$, and $^{35}S$**

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The toxic effects of $[^{35}S]$methionine were equal to or greater than those of $[^{14}C]$methionine (cf. Tables IX and X). With both labels the rate of accumulation of protein and DNA was marked lowered by 1 $\mu$Ci/ml and there was a net loss of protein and DNA after a short period of growth with 5 $\mu$Ci/ml. Furthermore, the amount of radioactivity per lung with $[^{35}S]$methionine was equal to or slightly greater than that with $[^{14}C]$methionine (Tables IX and X). This equivalence of incorporated radioactivity resulted from the fact that the decay constant of $^{35}S$ is ~2.3 $\times$ 10$^{-2}$ larger than that of $^{14}C$. Even though ~4500 molecules of $[^{14}C]$methionine containing ~2.3 $\times$ 10$^7$ atoms of $^{14}C$ were incorporated for each molecule of $[^{35}S]$methionine, a much larger proportion of the $^{35}S$ disintegrated during the culture period. This was interesting for it showed that the inhibition of growth was more directly related...
Cytotoxicity of $^{3}$H, $^{14}$C, and $^{35}$S

TABLE VII

Lung growth with or without $[^{14}$C]lysine at higher levels of dilution with l-lysine

The values represent the mean and standard deviations obtained in two experiments with cultures of 14.5-day-old embryo lung buds that were grown for 1, 2, or 4 days on 1% agar in a 20:10:70 mixture of SBSS:FBS:F-12 containing either 0, 1, or 5 μCi/ml of $[^{14}$C]lysine with a specific activity of 238 mCi/mmol.

| $[^{14}$C]lysine | Final dilution | Culture day | Protein | DNA | $^{10^{-3}} \times$ | $^{10^{-5}} \times$ | Estimated d/c/d |
|------------------|----------------|-------------|---------|-----|-----------------|-----------------|----------------|
|                  | μCi/ml         |             |         |     | dpm/μg DNA      | dpm/lung        |                  |
| 0                | ∞              | 0           | 21.5    | 1   | 2.1 ± 0.3       | 6.0 ± 0.1       | 50              |
| 1                | 384            | 1           | 35.6 ± 4.2 | 2.7 ± 0.7 | 25.7 ± 3.1     | 64.6 ± 9.0      | 137             |
| 2                | 78             | 1           | 36.8 ± 6.0 | 2.5 ± 0.2 | 42.6 ± 4.9     | 102.2 ± 24.4    | 412             |
| 4                | 5              | 1           | 28.7 ± 2.5 | 2.5 ± 0.4 | 23.0 ± 1.5     | 75.8 ± 1.5      | 694             |

TABLE VIII

Lung growth with or without $[^{14}$C]lysine at lower levels of dilution with l-lysine

The values represent the mean and standard deviations obtained in two experiments with cultures of 14.5-day-old embryo lung buds that were grown for 1, 2, or 4 days on 1% agar in a 20:10:70 mixture of SBSS:FBS:F-12 containing either 0, 1, or 5 μCi/ml of $[^{14}$C]lysine with a specific activity of 238 mCi/mmol.

| $[^{14}$C]lysine | Final dilution | Culture day | Protein | DNA | $^{10^{-3}} \times$ | $^{10^{-5}} \times$ | Estimated d/c/d |
|------------------|----------------|-------------|---------|-----|-----------------|-----------------|----------------|
|                  | μCi/ml         |             |         |     | dpm/μg DNA      | dpm/lung        |                  |
| 0                | ∞              | 0           | 20.8 ± 2.9 | 2.1 ± 0.3 | 23.0 ± 1.5     | 75.8 ± 1.5      | 694             |
| 1                | 57.5           | 1           | 30.2 ± 1.5 | 2.94 ± 0.5 | 30.6 ± 1.7     | 63.3 ± 1.9      | 111             |
| 2                | 11.5           | 1           | 30.8 ± 4.7 | 2.21 ± 0.4 | 85.1 ± 12.0    | 38.5 ± 3.8      | 388             |
| 3                |                | 2           | 30.6 ± 0.8 | 1.86 ± 0.3 | 103.3 ± 8.6    | 55.5 ± 5.9      | 559             |
| 4                |                | 4           | 21.0 ± 1.7 | 1.0 ± 0.3 | 76.2 ± 14.0    | 69.3 ± 6.9      | 698             |

TABLE IX

Lung growth with or without $[^{14}$C]methionine

The values represent the mean and standard deviations obtained in three experiments with cultures of 14.5-day-old embryo lung buds that were grown for 1, 2, or 4 days on 1% agar in a 10:10:80 mixture of SBSS:FBS:F-12 containing either 0, 1, or 5 μCi/ml of $[^{14}$C]methionine with a specific activity of 291 mCi/mmol.

| $[^{14}$C]methionine | Final dilution | Culture day | Protein | DNA | $^{10^{-3}} \times$ | $^{10^{-5}} \times$ | Estimated d/c/d |
|----------------------|----------------|-------------|---------|-----|-----------------|-----------------|----------------|
| 0                    | ∞              | 0           | 20.8 ± 2.9 | 2.1 ± 0.3 | 23.0 ± 1.5     | 75.8 ± 1.5      | 694             |
| 1                    | 57.5           | 1           | 30.2 ± 1.5 | 2.94 ± 0.5 | 30.6 ± 1.7     | 63.3 ± 1.9      | 111             |
| 2                    | 11.5           | 1           | 30.8 ± 4.7 | 2.21 ± 0.4 | 85.1 ± 12.0    | 38.5 ± 3.8      | 388             |
| 3                    |                | 2           | 30.6 ± 0.8 | 1.86 ± 0.3 | 103.3 ± 8.6    | 55.5 ± 5.9      | 559             |
| 4                    |                | 4           | 21.0 ± 1.7 | 1.0 ± 0.3 | 76.2 ± 14.0    | 69.3 ± 6.9      | 698             |

to the amount of intracellular decay than to the number of labeled molecules or the number of heavy atoms incorporated.

The amount of inhibition of growth with $[^{35}$S]methionine also corresponded to the estimated number of disintegrations/cell/day of incorporated $^{35}$S (Table X). With 1 μCi/ml there were ~100 d/c/d and growth slowed but did not stop. However, with 5 μCi/ml there were >400 d/c/d and atrophy occurred. These data also corresponded to the previous data
that showed that growth slowed with ~100 d/c/d, and atrophy began with >200 d/c/d of incorporated 14C (cf. Tables IX and X). This suggested that the toxic effects of these compounds was not directly related to the position, function, or transformation of the isotopic atoms in the labeled amino acids.

[14C] versus [3H] Proline

In the initial experiments with tritiated amino acids the effects of [3H]proline were compared to those of [14C]proline. Five sets of duplicate dishes of 10 lung buds were used with a 10:10:80 mixture of SBSS:FBS:F-12C(-pro). Three sets of duplicate dishes contained either 0, 2.5, or 5 pCi/ml of [14C]proline (NET 323) with a specific activity of 23 Ci/mmol, and two sets of dishes contained either 1 or 2.5 pCi/ml of [14C] proline (NEC 285) with a specific activity of 255 mCi/mmol. After 4 days of culture the lungs were assayed for total protein and DNA. As shown in Fig. 12 and Table XI, the inhibition of growth with 5 pCi/ml of [14C]proline was intermediate between that with 1.0 and 2.5 pCi/ml of [14C]proline. The accumulation of protein was reduced by 46% and 78% by 1 and 2.5 pCi/ml of [14C]proline and 58% with 5 pCi/ml of [3H]proline (Table XI).

At equal concentrations of radioactivity (2.5 pCi/ml), there was ~60% less inhibition of lung growth with [3H]proline than with [14C]proline (Table XI). This suggested that 3H with an average transition energy of 0.0055 MeV was less toxic than 14C with an average transition energy of 0.05 MeV/disintegration. This suggestion was complicated, however, by the differences in dilution that resulted from the differences in specific activity, by the differences in the decay constants of 3H and 14C, and by the differences in the number of heavy atoms/molecule in these labeled precursors. For example, at a concentration of 25 pCi/ml there were 78.4 x more molecules of [14C]proline than [3H]proline, and ~455 atoms of 14C for each atom of 3H. This suggested that the greater dilution of [3H]proline contributed to the decrease in the inhibition of lung growth with this precursor. Furthermore, with 1 pCi/ml of

![Fig. 10. Average area per lung in photographs of cultures with no label ( ), 1 pCi/ml ( ), or 5 pCi/ml ( ) of [14C]methionine.](image)

![Fig. 11. Average area per lung in photographs of cultures with no label ( ), 1 pCi/ml ( ), or 5 pCi/ml ( ) of [35S]methionine in a 20:10:70 nutrient mixture of SBSS:FBS:F-12C.](image)

**Table X**

| [35S]Methionine | Final dilution | Culture day | Protein | DNA | Incorporation of [35S] | Estimated d/c/d |
|-----------------|----------------|-------------|---------|-----|-----------------------|-----------------|
|                 | µCi/ml         |             |         |     | 10⁻³ x dpm/lung       | 10⁻⁴ x dpm/µg DNA |
| 0               | ∞              | 0           | 22.6 ± 3.1 | 2.12 ± 0.35 |                       |                 |
|                 |                | 1           | 31.4 ± 3.3 | 2.47 ± 0.32 |                       |                 |
|                 |                | 2           | 48.2 ± 1.6 | 4.38 ± 0.37 |                       |                 |
|                 |                | 4           | 58.6 ± 4.9 | 5.82 ± 0.95 |                       |                 |
| 1               | 257,600        | 1           | 25.9 ± 3.2 | 3.06 ± 0.13 | 29.5 ± 6.1             | 9.7             |
|                 |                | 2           | 34.6 ± 0.8 | 3.30 ± 0.16 | 46.7 ± 5.2             | 14.2            |
|                 |                | 4           | 41.1 ± 3.0 | 3.75 ± 0.08 | 65.0 ± 3.5             | 17.3            |
| 5               | 57,500         | 1           | 26.7 ± 2.0 | 2.72 ± 0.17 | 119.2 ± 2.7            | 43.8            |
|                 |                | 2           | 20.6 ± 0.4 | 1.78 ± 0.26 | 99.1 ± 3.2             | 55.7            |
|                 |                | 4           | 11.6 ± 6.5 | 0.68 ± 0.19 | 63.4 ± 1.7             | 93.2            |
[\textsuperscript{14}C]proline there were \( \sim 47,000 \text{ dpm} \) or \( \sim 2,350 \text{ MeV/min/lung} \) and with 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]proline there were \( \sim 124,000 \text{ dpm} \) or \( \sim 682 \text{ MeV/min/lung} \) (Table XI). Since 682 MeV of [\textsuperscript{3}H] was more toxic this suggested that the ionizing energy of [\textsuperscript{3}H] was considerably more toxic than that of [\textsuperscript{14}C]. This may be due to the fact that most of the energy of [\textsuperscript{3}H] decay would be absorbed within the cell of origin while much of the energy of [\textsuperscript{14}C] decay would be released outside of the cell of origin.

In the next experiments in this series the effects of dilution of [\textsuperscript{3}H]proline were studied by using 1 and 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]proline with nutrient medium containing either F-12C, F-12mC, or F-12C(-pro) (Table I). This gave final dilutions of 3,900, 19,700, and 112 with 1 \( \mu \text{Ci/ml} \) and dilutions of 300 d/c/d growth stopped (Table XII). This is equivalent to \( \sim 30,000 \text{ dpm/\mu g of DNA} \) or \( \sim 38 \text{ rads/cell/day} \).

**Effects of [\textsuperscript{3}H]Serine**

The next series of experiments was designed to determine whether other [\textsuperscript{3}H]-labeled amino acids also inhibit tissue growth. In this series, three sets of dishes containing 0, 1, or 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]serine with a specific activity of 16 Ci/mmol were used with a 20:10:70 mixture of SBSS:FBS:F12C. As shown in Fig. 13 and Table XIII these experiments showed that the inhibition of growth with 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]serine was comparable to that with 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{14}C]proline. Furthermore, with 1\( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]serine at a dilution of 10,790 there was

In 4-day cultures of 14.5-day-old embryo lungs, the total protein/lung increased 300\% in lungs grown without [\textsuperscript{3}H]proline, and 222\% in lungs grown with 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]proline that was diluted 11,400 \( \times \) with l-proline in the nutrient medium. Similar results were obtained with duplicate cultures of 13.5-day-old lung buds (data not shown), and this suggested that lung growth was only reduced by about one-third when 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]proline is diluted by large amounts of l-proline. However, when the intracellular decay of [\textsuperscript{3}H] reached 300 d/c/d growth stopped (Table XII). This is equivalent to

\[ \text{Estimated d/c/d} = \text{Incorporation of } \text{dpm/\mu g DNA} \times 10^{-3} \]

**TABLE XI**

*Comparison of the effects of [\textsuperscript{14}C] and [\textsuperscript{3}H]proline on lung growth*

The values represent the means and standard deviations obtained in three experiments in which 14.5-day-old lung buds were grown for 4 days on 1\% agar in a 20:10:70 mixture of SBSS:FBS:F12C containing one of the concentrations of labeled proline listed. Analyses of 4 pair of lung buds at the onset of each experiment gave an average value of 21.4 \( \mu \text{g of protein/lung} \). The average area/lung bud on each day of culture in one of these experiments is shown in Fig. 10.

**TABLE XII**

*Lung growth with or without [\textsuperscript{3}H]proline at intermediate dilutions with l-proline*

The values represent the mean and standard deviations obtained in two experiments with cultures of 14.5-day-old embryo lung buds that were grown for 1, 2, or 4 days on 1\% agar in a 20:10:70 mixture of SBSS:FBS:F-12mC containing either 0, 1, or 5 \( \mu \text{Ci/ml} \) of [\textsuperscript{3}H]proline at a specific activity of 23Ci/mmol.
The values represent the mean and standard deviations obtained in three experiments with cultures of 14.5-day-old embryo lung buds that were grown for 1, 2, or 4 days on 1% agar in a 20:10:70 mixture of BESSELMBS/P12C containing either 0, 1, or 5 μCi/ml of [3H]serine with a specific activity of 16 Ci/mmol. Additional data from these experiments are shown in Fig. 11.

| [3H]Serine | Final dilution | Culture day | Protein | DNA |
|------------|----------------|-------------|---------|-----|
| μCi/ml     |                |             | pg/lung | pg/lung | dpm/lung | dpm/μg DNA | Estimated d/c/d |
| 0          | ∞              | 0           | 25.6 ± 2.0 | 2.0 ± 0.4 |           |           |           |
|            |                | 1           | 37.4 ± 0.3 | 3.5 ± 0.6 |           |           |           |
|            |                | 2           | 48.2 ± 3.4 | 4.3 ± 0.4 |           |           |           |
|            |                | 4           | 62.8 ± 4.3 | 5.8 ± 0.8 |           |           |           |
| 1          | 100,800        | 1           | 32.4 ± 2.5 | 3.3 ± 0.7 | 13.5 ± 3.5 | 4.1 | 41 |
|            |                | 2           | 41.4 ± 6.0 | 4.0 ± 0.4 | 22.2 ± 6.6 | 5.5 | 55 |
|            |                | 4           | 57.7 ± 7.0 | 5.5 ± 0.5 | 38.0 ± 7.7 | 6.9 | 70 |
| 5          | 2,200          | 1           | 32.5 ± 0.8 | 3.5 ± 0.2 | 61.5 ± 10.8 | 17.6 | 177 |
|            |                | 2           | 39.8 ± 6.5 | 3.7 ± 0.3 | 114.9 ± 20.2 | 31.1 | 313 |
|            |                | 4           | 44.7 ± 1.4 | 4.0 ± 0.6 | 134.1 ± 32.9 | 33.5 | 397 |

**FIG. 13. Average area per lung in cultures of 14.5-day-old embryo lungs with no label (O——O), 1 μCi/ml (■——■), or 5 μCi/ml (C——C) of [3H]serine.** The results of chemical analyses of those lungs are shown in Table X.

little inhibition of lung growth during 4 days of culture (Fig. 13).

**DISCUSSION**

The observation that the toxic effects of 5 μCi/ml of 14C- and 35S-labeled amino acids were evident after only 6 h of culture suggests 1) that the injury resulting from the intracellular decay of these isotopes is more severe than was previously anticipated, 2) that the injury occurs during the first few hours of labeling and is amplified by 4 days of culture, and 3) that the injury occurs before a block of cell proliferation would be evident. These results also indicate that the time and amount of exposure of cells to the intracellular decay of these isotopes should be strictly limited.

Atrophy is a decrease in tissue mass and it is the product of a negative anabolic-catabolic balance. The atrophy in the labeled lungs may have resulted from a decrease in cell proliferation and protein synthesis and/or from an increase in protein degradation and cell death. There was no evidence in these studies to quantitate the contribution of each of these processes but a number of observations suggest that an increase in protein degradation and cell death may have been most important. For example, microscopy consistently showed that there were many more cells containing debris-filled secondary lysosome, there were more dead cells, and atrophy occurred in the labeled cultures. At the same time bronchial branching continued, mitotic figures were consistently present, and the dpm/μg of DNA continued to increase, suggesting that cell proliferation and protein synthesis continued through 4 days of culture. Furthermore, these results suggest that if the decay of 3H, 14C, and 35S accelerates protein degradation, radiotracer studies of protein turnover should be carefully controlled.

The consistency of these results suggests that this may be a general toxic effect of radiolabeled amino acids. The data also suggest that the toxicity is directly related to the amount of incorporation of radiolabel. The toxicity was proportional to the concentration of radiolabel and inversely proportional to the amount of dilution of labeled precursors by unlabeled amino acid in the nutrient medium. The specific involvement of the radiolabel is strongly supported by the results of experiments comparing the effects of [14C]proline and [14C]serine with [3H]serine. The data also suggest that the toxicity is related to the amount of intracellular decay of these isotopes. No signs of injury were evident with <50 d/c/d of 14C or with <100 d/c/d of 3H. These values were estimated to represent an absorbed dose of ~60 rads/cell/day from 14C or 35S or with <90 d/c/d of 3H. These values were estimated to represent an absorbed dose of ~90 rads/cell/day from 14C or 35S, or 11.3 rads/cell/day from 3H. In contrast, growth slowed with ~100 d/c/d, growth stopped with ~200 d/c/d, and atrophy occurred with >300 d/c/d of 14C or 35S. This indicates that the rate of cell death equaled or exceeded the rate of cell proliferation when there were >200 d/c/d of 14C or 35S or >400 d/c/d of 3H. With embryonic rat tissues 60 d/c/d equals ~6,000 dpm/μg of DNA while 100 d/c/d is equivalent to ~10,000 dpm/μg. Therefore, with these embryonic lung tissues <6,000 dpm of intracellular decay/μg of DNA might be considered as a "safe dose" while >10,000 dpm/μg of DNA would severely injure tissues during the first 12 h of labeling. It will now be important to learn whether similar concentrations of intracellular decay of these isotopes have similar effects on other cell populations.

As noted above, the average cell in the lung buds was estimated to occupy a volume of 5.8 x 10^-10 ml. With 60 d/c/d this volume would contain ~1.9 x 10^-10 μCi or ~32 μCi/ml. With 200 d/c/d the intracellular concentrations would be ~108 μCi/ml. These values are respectively 3,200 and 10,800 x the maximum permissible concentration of 14C in water for...
man (Table 24.1 in Ref. 17). However, these concentrations are less than those achieved in many experimental studies of the synthesis or degradation of tissue components.

It is not clear whether the toxicity of any of these precursors was selective for different cell populations but the signs of injury were distinctly different in epithelial, mesenchymal, and hematopoietic cells in the cultured lung buds. There was an excessive accumulation of glycogen and hydropic degeneration in epithelial cells, accumulation of secondary lysosomes and residual bodies in mesenchymal cells, and a condensation and fragmentation of hematopoietic cells in labeled lungs (Figs. 6 to 8). These differences suggest that additional studies should be done to determine whether this injury results in a change in the relative proportions of different types of cells, or in the relative proportions of different proteins or other cell products that accumulate in labeled and unlabeled tissues.

Toxic effects of short exposures to radiolabeled amino acids may be masked by a number of variables of tissue isolation and culture. These variables include 1) differences in the amounts of tissue injury resulting from the procedures of tissue isolation and explanation, 2) variations in the age, stage of development, or functional state of the tissues, 3) variations in the amounts of protein or DNA per unit mass of freshly isolated tissue, or 4) the presence of other toxic or deleterious factors in the culture system. In this study the effects of these variables were minimized by the fact that whole lung buds could be isolated and cultured without exposure to proteolytic enzymes or the trauma of slicing or mincing. Furthermore, many variables in the culture system were tested to select favorable conditions for the maintenance of tissue growth and differentiation and to minimize cell degeneration and death (3). Finally, since the presence or absence of the radiolabeled amino acid was the only variable in each experiment, the reduction of growth in response to this variable was easily quantitated. This may answer the question of why the toxicity that was so obvious in this report has not been described previously.

The results described in this report have serious implications for experimental systems using >1 μCi/ml concentrations of labeled amino acids to study the synthesis and turnover of protein. Biologists may be injuring tissues rather severely in radiolabeling experiments, even though the tissues continue to incorporate radiolabeled precursors and undergo cell differentiation and morphogenesis.

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