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# COLLAGEN PRODUCTION RATES FOLLOWING ACUTE LUNG DAMAGE INDUCED BY BUTYLATED HYDROXYTOLUENE

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**Abstract**—Treatment of mice intraperitoneally with butylated hydroxytoluene (BHT) results in the formation of a diffuse, dose-dependent lung lesion. At high doses of BHT, pulmonary fibrosis becomes evident. The rate of collagen production was measured *in vitro* in normal and BHT-damaged lung tissue by quantitating the formation of acid-insoluble [$^3$H]hydroxyproline from [$^3$H]proline at 1, 2, 3 and 4 hr of incubation. Collagen production was elevated 2 days after BHT (400 mg/kg) and reached a maximum rate of 150 pmoles·(mg dry wt)$^{-1}$·hr$^{-1}$ at day 7. The rate then declined but was still significantly above control levels of 57 pmoles·(mg dry wt)$^{-1}$·hr$^{-1}$ at day 14. Expressing these data as a percentage of total protein synthesis committed to collagen demonstrated a specific stimulation of collagen synthesis. A maximum level of 1.5% of total protein synthesis was committed to collagen 7 days after BHT. Control mice committed 0.6% to collagen synthesis. Both the maximum and control percentages of collagen synthesis were the same as those previously reported *in vivo* following BHT (400 mg/kg). Doses of BHT as low as 200 mg/kg produced a significant increase in both the *in vitro* rate and percentage of pulmonary collagen synthesis. Only at doses of BHT of 300 mg/kg or greater could the deposition of excess collagen be detected as an increase in total lung hydroxyproline. There was a linear dose–response relationship between BHT and the rate of collagen synthesis. Pulmonary DNA synthesis, another index of lung damage, exhibited a steep, non-linear dose–response relationship with BHT. These data show that increases in the rate and percentage of collagen production are readily detected at levels of lung damage which do not result in the deposition of a measurable excess of hydroxyproline and that *in vitro* rates of collagen synthesis are, therefore, a sensitive index of lung damage which may be used to extrapolate to "non-toxic" dosage levels. This study also shows that the percentage of protein synthesis devoted to collagen is the same *in vivo* and *in vitro* in both normal and BHT-damaged lung tissue.

Butylated hydroxytoluene [2,5-di-*t*-butyl-4-hydroxytoluene (BHT)] is a phenolic antioxidant which is capable of producing lung damage in all strains of mice that have been tested [1–3]. This damage occurs diffusely throughout the lung and is initially characterized by damage to the type I alveolar epithelial cells. As these cells become necrotic, they are replaced by proliferating type II alveolar epithelial cells, some of which assume the morphologic characteristics of type I cells [4, 5]. At low doses of BHT, tissue repair processes ultimately result in a return to a normal morphological and biochemical status. At high doses of BHT, however, there is both morphological and biochemical evidence of the deposition of excess collagen characteristic of pulmonary fibrosis [6, 7].

The biochemical variable most indicative of toxic lung damage has not yet been determined although several indices have been proposed. Cyclic GMP has been found to be elevated in BHT-damaged lung tissue and this nucleotide was shown to be necessary for cell proliferation [8]. Assessment of pulmonary cell proliferation by measuring DNA synthesis at various times after the induction of lung damage has been used as a means to quantitate indirectly the initial lung lesion produced by BHT [3, 9, 10]. One recent report has suggested that the *in vitro* rate of collagen production in lung tissue minces may be a useful parameter for quantitating lung damage by fibrosis-inducing pneumotoxins [11]. The rate at which collagen was synthesized in lung tissue *in vitro* was elevated following fibrotic doses of ozone [11], bleomycin [12, 13] and paraquat [14]. A dose–response relationship was shown between the level of ozone exposure and the rate of collagen synthesis [11]. A correlation was also shown to exist between the paraquat-induced increase in collagen synthesis and the morphologic appearance of fibrosis [15], further supporting the use of *in vitro* collagen production rates as an index of toxic lung damage.

Previous work showed that net collagen synthesis was increased *in vivo* in lung tissue damaged by fibrotic doses of BHT [16]. The increased collagen synthesis in all of these studies was postulated as the major cause for the deposition of excess collagen as fibrosis developed. The data presented in this paper demonstrate time-dependent increases in the *in vitro* rate of pulmonary collagen production following the administration of BHT. Doses of BHT which did not result in the deposition of a measurable excess of collagen also increased the rate of collagen production. It is shown that the percentage of protein synthesis devoted to the formation of collagen *in vitro* was the same as that previously reported *in vivo* in both normal and BHT-damaged lung tissue. Finally, data are presented relating the dose of BHT administered to the rate of pulmonary collagen production and the amount of pulmonary DNA syn-

thesis. These data suggest that measurements of collagen synthetic rates may be a more reliable index of lung damage than pulmonary DNA synthesis.

## MATERIALS AND METHODS

*Materials.* Female, CD-1 derived mice, 8–12 weeks of age, bred and maintained in the Animal Resources Center at the University of Texas at Austin, were used for these studies. BHT, ninhydrin, proline, and 4-hydroxyproline were obtained from the Sigma Chemical Co., St. Louis, MO. Dulbecco's Modified Eagles Medium was obtained from GIBCO, Grand Island, NY, and *p*-dimethylaminobenzaldehyde from MCB, Cincinnati, OH. L-[5-$^3$H]Proline (32 Ci/mmole) was obtained from Schwarz/Mann, Orangeburg, NY, and [methyl-$^{14}$C]thymidine (56 Ci/mole) and D,L-hydroxy[2-$^{14}$C]proline (21 Ci/mole) were from Amersham, Arlington Heights, IL. All other chemicals used were of reagent grade.

*Methods.* BHT was dissolved in corn oil and administered to mice intraperitoneally. BHT concentrations were adjusted so that all injection volumes were 0.1 ml/10 g body weight. Control mice received an equivalent volume of corn oil.

Acid-insoluble hydroxyproline synthesis, an index of collagen synthesis, was measured *in vitro* in minced lung tissue using slight modifications of the method described by Bradley *et al.* [17]. Mice were killed by cervical dislocation, and their lungs were perfused free of blood with isotonic saline through the pulmonary artery. Lungs from five mice were pooled for each experiment and minced into pieces approximately 2 mm$^3$. Portions (200 mg) of the minced lung tissue were incubated in 25 ml flasks at 37° in 1.8 ml of Dulbecco's Modified Eagles Medium containing 100 μg/ml ascorbate under an atmosphere of 95% $O_2$:5% $CO_2$. β-Aminopropionitrile was omitted from the incubation mixture to maximize collagen crosslinking and thereby minimize degradation. After 1 hr the medium was removed and replaced with 0.5 ml of identical medium. [5-$^3$H]Proline, 10 μCi, was then added to each flask, and the incubation was continued as before. A flask was removed after 1, 2, 3 and 4 hr for the determination of hydroxyproline synthesis.

Minced lung tissue was next washed with cold phosphate-buffered saline (pH 7.4) [18] and homogenized in 3 ml $H_2O$ with a Tekmar Tissumizer, Cincinnati, OH. Proteins were precipitated by the addition of 1 ml of 20% trichloroacetic acid (TCA). After centrifugation at 1000 *g* for 5 min, the supernatant fraction was removed and analyzed for total radioactivity and free proline [18]. These data were used to calculate the specific activity of free proline in the minced lung tissue.

The TCA-insoluble pellet was washed two times with 5 ml $H_2O$, lyophilized, weighed, and hydrolyzed for 18 hr at 107° in 4 ml of 6 N HCl. The hydrolysate was neutralized with NaOH, treated with decolorizing carbon (Norit A), filtered, and brought to a final volume of 7 ml with water. A 0.1 ml aliquot of this solution was counted in Ready-Solv MP, Beckman, Fullerton, CA, for the determination of total TCA-precipitable radioactivity. A 2.0 ml aliquot was used to measure the quantity of TCA-insoluble [$^3$H]hydroxyproline [19]. The percentage of total protein synthesis committed to the synthesis of acid-insoluble collagen was calculated from the total TCA-insoluble radioactivity and the amount of TCA-insoluble [$^3$H]hydroxyproline using the factor 2.06 to correct for the differential incorporation of proline in noncollagen protein and into the hydroxyproline of lung collagen [20]. Under these incubation conditions, the synthesis of [$^3$H]hydroxyproline was found to be a linear function of time for the 4-hr incubation period.

The total quantity of collagen present in lung tissue was estimated by measuring total pulmonary hydroxyproline. Mice were killed by cervical dislocation, and the lungs were excised and lyophilized intact. The entire lung was then hydrolyzed as before. The hydrolysate was neutralized with KOH, and 0.01 μCi [$^{14}$C]hydroxyproline was added to each sample. The samples were then treated with decolorizing carbon, filtered, and brought to a final volume of 8 ml. A 0.1 ml aliquot was counted in Ready-Solv MP to determine the recovery of hydroxyproline and another 0.1 ml aliquot was assayed for total hydroxyproline content [21].

Pulmonary DNA synthesis was estimated 3 days after the administration of BHT by measuring thymidine incorporation into total lung DNA. Mice were injected i.p. with 0.5 μCi [$^{14}$C]thymidine. Ninety minutes later the animals were killed, and the specific activity of pulmonary DNA was determined by a modification of the method previously described [22]. Each mouse lung was removed, homogenized in 3 ml of $H_2O$, and mixed with 2 ml of 0.5 N $HClO_4$. After centrifugation the pellet was washed two times with 5 ml of cold 0.5 N $HClO_4$. The pellet was then resuspended in 4 ml of 1.5 N $HClO_4$ and digested at 70° for 20 min. This mixture was centrifuged, and 2 ml of the resultant supernatant fraction was counted for radioactivity in Ready-Solv MP. A 0.5 ml aliquot of the supernatant fraction was assayed for total DNA using Richard's modification of the diphenylamine reaction [23].

The rate of acid-insoluble hydroxyproline synthesis was calculated by linear regression analysis using the quantity synthesized at 1, 2, 3 and 4 hr (plus a 0 hr value of 0 pmole/mg dry wt). Experiments examining the rate of collagen synthesis were carried out over a 4-month period. Control mice and each experimental treatment group were analyzed three to four separate times in a random order during this time. The control values remained constant as shown by the small standard errors. All data points were used for regression analysis except when the correlation coefficient for an individual experiment was less than 0.95. In those experiments, the aberrant data point was discarded, and the slope was recalculated using the remaining data points. The slopes of the regression lines (rates of hydroxyproline synthesis) were then compared using a test analogous to Student's *t*-test [24]. The percentage of total protein synthesis devoted to collagen was calculated after 1, 2, 3 and 4 hr of incubation in each experiment and the data from identical treatment groups were combined. These data were then analyzed by one-way analysis of variance and multiple comparisons were done with Scheffé's test [25]. Data for pul-

monary DNA synthesis were analyzed by Student's *t*-test [24]. A P value of less than 0.05 was considered significant for all experiments.

## RESULTS

The rate of acid-insoluble hydroxyproline synthesis in lung tissue at various days after the administration of 400 mg/kg BHT is shown in Fig. 1. Two days after BHT, the rate of synthesis was significantly greater than that from untreated mice. This rate continued to increase thereafter and reached a maximum 7 days after BHT. There was then a decline until the rate of hydroxyproline production reached a level slightly, but significantly, greater than untreated mice 14 days after BHT.

A specific BHT-induced increase in collagen synthesis could be seen when the synthesis of acid-insoluble [$^3$H]hydroxyproline was expressed as the percentage of total protein synthesis devoted to the formation of acid-insoluble collagen (Fig. 2). A maximum of approximately 1.5% of total protein synthesis was committed to collagen 7 days after BHT. Significant, although smaller, increases compared to untreated mice were seen 2 and 3 days after BHT. Collagen synthesis on days 1 and 14 was not significantly different from untreated mice which had about 0.6% of total protein synthesis devoted to collagen.

There was a direct relationship between the dose of BHT administered and both the rate of net hydroxyproline synthesis and the percentage of protein synthesis devoted to collagen when measured 7 days after BHT (Fig. 3). There was a significant increase in both the rate and relative amount of collagen synthesized at doses of BHT 200 mg/kg or greater. Lower doses of BHT did not produce any significant increases in collagen synthesis. Linear regression analysis of the dose of BHT from 200 to 400 mg/kg versus the rate of collagen synthesis produced a correlation coefficient of 0.999 while the same analysis versus the percentage of collagen synthesis gave a correlation coefficient of 0.958.

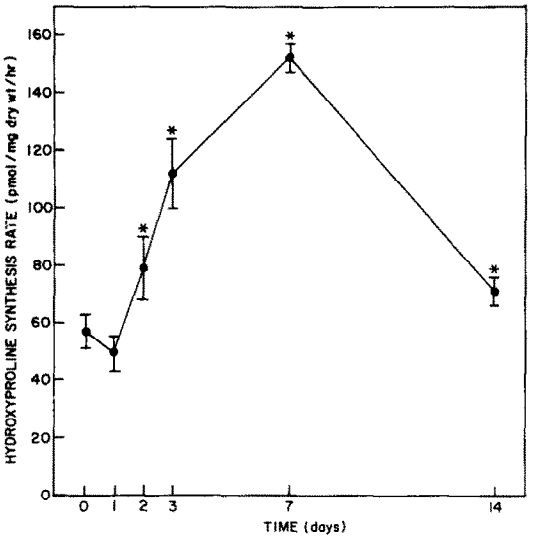


Fig. 1. Rate of pulmonary hydroxyproline synthesis at various times after the administration of 400 mg/kg BHT. The data are expressed as the mean ± S.E. of the slopes obtained from three to four separate experiments at each time point. Significant differences were determined using all individual data points from each separate experiment (N = 16–20). Key: (*) significantly greater than untreated mice (day 0) (P less than 0.05).

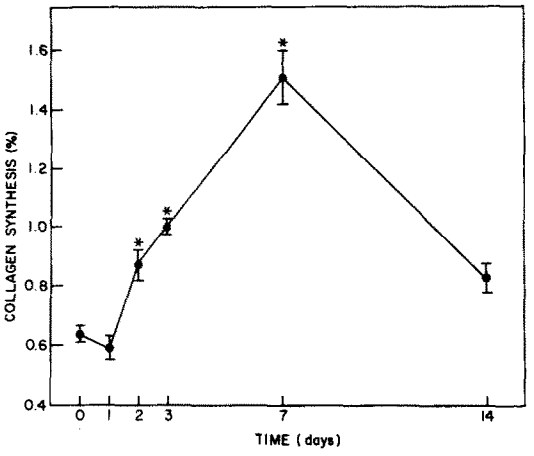


Fig. 2. Percentages of total protein synthesis committed to the synthesis of acid-insoluble collagen in lung tissue. Mice were injected i.p. with 400 mg/kg BHT dissolved in corn oil. Each point represents the mean ± S.E. of all values obtained after 1, 2, 3 and 4 hr of incubation at each individual day assayed (N = 12–16). Key: (*) significantly greater than untreated mice (day 0) (P less than 0.05).

All CD-1 mice given 200 mg/kg BHT or less survived while at 300 mg/kg there was 20% mortality and at 400 mg/kg 50% mortality. Pulmonary DNA synthesis was significantly elevated 3 days after the administration of 200 mg/kg BHT (Table 1). Lower doses failed to stimulate while higher doses produced an even larger stimulation. Maximum thymidine incorporation was evident after 400 mg/kg BHT. The time course of changes in pulmonary thymidine incorporation after 400 mg/kg BHT is shown in Table 1. There was no significant increase in DNA synthesis 1 day after BHT. Significant increases were seen on days 2, 3 and 7 with maximum levels evident on day 3.

The administration of 300 or 400 mg/kg BHT to CD-1 mice produced a significant increase in total lung hydroxyproline within 2 weeks (Fig. 4). Lower doses of BHT failed to result in a measurable accumulation of excess hydroxyproline. Similar increases were obtained when pulmonary hydroxyproline levels were measured 3 weeks after BHT (data not shown).

## DISCUSSION

Lung damage has been indirectly quantitated by measuring pulmonary DNA synthesis associated with the proliferative response of lung cells after an acute insult [9, 10]. Previous studies have shown that in male Swiss–Webster [3] and Balb/c (J. P. Kehrer and H. R. Witschi, unpublished data) mice a BHT dose of 100 mg/kg or less produced no increase in pulmonary DNA synthesis while a dose of 200 mg/

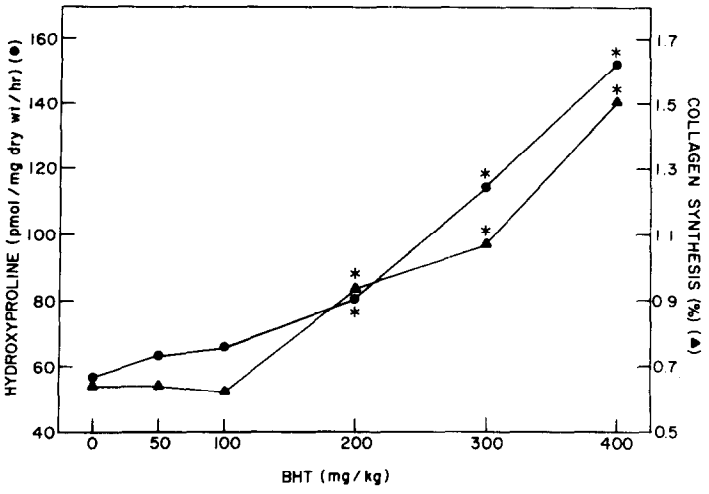


Fig. 3. Effect of various BHT doses on collagen production. Mice were injected i.p. with BHT dissolved in corn oil or an equivalent volume of corn oil alone. Hydroxyproline production and the percentage of collagen synthesis were measured 7 days after BHT. Hydroxyproline production rates were obtained from the slope of the regression lines (N = 4–20). The percentage of collagen synthesis is expressed as the mean of values obtained after 1, 2, 3 and 4 hr of incubation (N = 4–16). Key: (*) significantly greater than oil-treated controls (P less than 0.05).

kg only resulted in a slight increase. Doses greater than this rapidly increased DNA synthesis with maximum levels seen 3–4 days after 400 mg/kg BHT [26, 27]. The present study has demonstrated a similar dose and time response after BHT in female CD-1 derived mice. This thymidine incorporation data suggested that CD-1 and Balb/c mice developed similar levels of lung damage at similar doses of BHT. BHT at a dose of 400 mg/kg was lethal to only about 10% of the Balb/c mice (unpublished data), however, while the CD-1 mice exhibited a much higher level of mortality. The reason for this difference is not clear. Morphologically the lesion in CD-1 [28, 29] mice is the same as that in Balb/c mice [4, 5]. Smith and Brody [28] have reported a poor correlation between BHT doses and the extent of lung injury in CD mice. It is possible that, while the average response of a group of mice was similar between strains, the greater individual variation in CD-1 mice resulted in greater mortality. It is also possible that CD-1 mice are more susceptible than Balb/c mice to a lethal BHT effect at equivalent levels of lung damage. The assumption has been made for this study that the average lung damage induced in each strain was the same, even though

Table 1. Effect of BHT treatment on pulmonary thymidine incorporation

| BHT (mg/kg) | Time* (day) | Thymidine incorporation† (dpm/mg DNA) | N |
|---|---|---|---|
| 0 | | 770 ± 104 | 13 |
| 50 | 3 | 754 ± 103 | 9 |
| 100 | 3 | 923 ± 148 | 13 |
| 200 | 3 | 1521 ± 352‡ | 7 |
| 300 | 3 | 2519 ± 706‡ | 6 |
| 400 | 1 | 881 ± 133 | 5 |
| 400 | 2 | 4352 ± 319‡ | 5 |
| 400 | 3 | 7712 ± 823‡ | 18 |
| 400 | 7 | 2796 ± 744‡ | 8 |

* Day after BHT when thymidine incorporation was measured.

† There were no significant differences between groups given indentical treatments at different times so the data were combined. Data are expressed as means ± S.E.

‡ Significantly different from oil-treated controls (P less than 0.05).

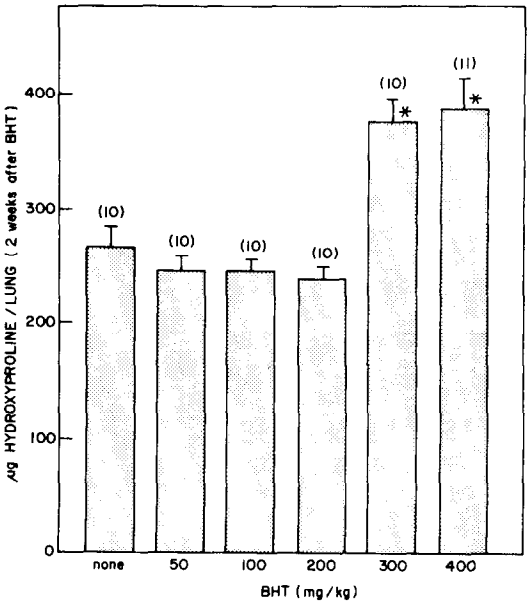


Fig. 4. Total lung hydroxyproline after various doses of BHT. Mice were injected i.p. with BHT dissolved in corn oil or an equivalent volume of corn oil alone. Two weeks later the mice were killed, and the lung tissue was analyzed for total hydroxyproline as described in the text. Data are expressed as mean μg/lung ± S.E. Values in parentheses = N. Key: (*) significantly greater than oil-treated controls (P less than 0.05).

mortality differed. Comparisons may be made, therefore, between the *in vitro* data obtained in this study with CD-1 mice and the *in vivo* data previously obtained in Balb/c mice.

Greater than 97% of the amino acid hydroxyproline present in lung tissue is located in collagen [30]. Hydroxyproline itself is not incorporated into collagen, however. Rather proline is incorporated and subsequently hydroxylated by the enzyme prolyl hydroxylase [31]. Thus, measurements of the conversion of radioactive proline to hydroxyproline have been widely used to estimate collagen synthesis. It is now recognized that collagen-producing cells are capable of degrading significant amounts of collagen within the cell prior to secretion [32]. The rate at which collagen was produced *in vitro* in normal and BHT-damaged lung tissue was measured using acid-insoluble proteins. This technique excludes the small fragments of newly synthesized, but degraded collagen from the analysis. The values reported, therefore, represent net rather than total collagen synthesis.

The rate at which collagen was produced in lung tissue after 400 mg/kg BHT was found to be significantly greater than that of untreated mice for up to 14 days. This rate averaged about 100 pmoles·(mg dry wt)$^{-1}$·hr$^{-1}$ during the 2 weeks after BHT. If one assumed that all of this hydroxyproline was deposited in the lung, an average mouse lung of 30 mg dry weight would contain 132 μg more hydroxyproline after 2 weeks. This calculation agrees quite well with the actual value of 118 μg (Fig. 3). However, untreated mice produce 55 pmoles·(mg dry wt)$^{-1}$·hr$^{-1}$ with no net deposition. Correcting the above calculation for the basal production rate leaves an increase of only about 60 μg hydroxyproline, much less than the actual increase. Previous data have also shown that net collagen accumulation ceases by 7 days after 400 mg/kg BHT [16] although elevated rates of collagen synthesis are evident after day 7. These data suggest that processes in addition to increased collagen synthesis are involved in determining how much collagen is ultimately deposited in acutely damaged lung tissue.

The lung damage produced by BHT is accompanied by a general stimulation of total protein synthesis [33]. It was therefore possible that the observed increase in collagen synthesis merely reflected this generalized stimulation. Expressing hydroxyproline synthesis as a percentage of total protein synthesis committed to collagen revealed a specific stimulation of net collagen synthesis above the general stimulation of protein synthesis. BHT at a dose of 400 mg/kg increased collagen synthesis to a maximum of 1.5% of total protein synthesis by day 7. This level then declined until day 14 when specific collagen synthesis was not significantly different from control values of 0.6%. Since the rate of collagen synthesis remained elevated 14 days after BHT, it appears as though the BHT-induced increase in total protein synthesis declines at a slower pace than does the increase in collagen synthesis.

The percentage of total protein synthesis committed to collagen *in vitro* can be compared to similar data previously obtained *in vivo* in normal and BHT-treated mice [16]. Normal mice in both studies exhibited 0.6% of total protein synthesis committed to collagen. This level increased to a maximum of 1.5% *in vitro* and 1.7 to 2.0% *in vivo* at 7 days after 400 mg/kg BHT. These similarities between the *in vivo* and *in vitro* values provide the first strong evidence that *in vitro* assays of collagen synthesis are an accurate reflection of the *in vivo* situation. The percentage of collagen synthesis measured in untreated mice both *in vivo* and *in vitro* also compared favourably with *in vitro* studies in other species which reported 1.0% for hamsters [13] and 0.6% for rats [14]. The time course of changes in the percentage of collagen synthesis after BHT reported in the present *in vitro* study (Fig. 2) was not clearly evident in the previous *in vivo* study, however [16]. This difference could be due to the different days examined or to the greater statistical precision achieved *in vitro* by the use of a greater number of individual data points at each day studied (four points *in vivo* vs twelve to sixteen points *in vitro*).

Doses of BHT as low as 200 mg/kg were found to increase both the relative synthesis of collagen and the rate at which collagen was produced by lung tissue *in vitro*. These studies were done 7 days after BHT since this was the time of maximum synthetic rates after 400 mg/kg BHT (Fig. 1). The increase in rate and percentage of collagen synthesis measured 7 days after BHT 200 mg/kg was not accompanied by a significant accumulation of total lung collagen by day 14. Although the increase in the rate of hydroxyproline production was statistically significant, the maximum increase above control levels was only 25 pmoles·(mg dry wt)$^{-1}$·hr$^{-1}$. Assuming a smaller average increase in collagen production, measurably more hydroxyproline would not be deposited in lung tissue within 2 weeks. It is apparent that, while the *in vitro* rate of collagen synthesis is a sensitive index of lung damage, the relationship to *in vivo* collagen deposition is unclear.

The rate of collagen synthesis exhibited a linear dose–response relationship with BHT doses of 200 mg/kg or greater ($r = 0.999$). This was in contrast to thymidine incorporation which had a correlation coefficient of only 0.930. The linear relationship between BHT and collagen production was used to extrapolate to a toxic threshold dose for BHT of 160 mg/kg. A similar linear response was seen in rats between the rate of pulmonary collagen synthesis and the level of ozone to which they were exposed [11], suggesting the broad utility of this technique for determining toxic levels of various pneumotoxins.

This study has shown that the percentage of protein synthesis committed to collagen *in vitro* is the same as that *in vivo* in both normal and BHT-damaged lung tissue, providing the first clear indication that *in vitro* assays of collagen synthesis reflect relative *in vivo* changes. It is not clear, however, whether the absolute values of *in vitro* collagen synthesis are the same as the *in vivo* values. One might expect lower values *in vitro* because of the abnormal state of the tissue. The data presented here suggest that the elevated rate of collagen production measured *in vitro* is not sufficient to explain the accumulation of collagen in lung tissue damaged by BHT. Although it is possible that increased synthetic rates are primarily responsible for the deposition of excess

collagen seen after BHT, other changes in collagen metabolism are probably involved. *In vivo* studies have shown a decreased degradation of newly synthesized collagen in lung tissue damaged by BHT which may contribute to collagen deposition [16]. It is also possible that changes in established collagen are occurring in damaged lung tissue and that these changes play a role in collagen homeostasis. Finally, this study suggests that *in vitro* rates of collagen synthesis provide a sensitive index of pulmonary damage which exhibits a linear dose–response relationship and which may be used to detect lung damage following non-fibrotic lung insults.

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