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A plethysmographic method for measuring function in locally irradiated mouse lung

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ABSTRACT

A plethysmograph has been developed to measure pulmonary function in mice after single doses of X rays to both lungs. The apparatus consists of a whole-body airtight chamber fitted with a Lavalier microphone. The microphone acts as a sensitive electrical capacitance manometer converting pressure changes in the chamber into an electrical signal which is electronically processed and recorded on a pen recorder. Two parameters of lung function were simultaneously monitored, breathing rate and amplitude.

Lung function has been tested in male CBA mice aged two to six months and in animals which have received graded X-ray doses to both lungs. No diurnal rhythm or age-related increase has been observed up to six months in control mice. The two lung-function parameters exhibited a dose-dependent response in irradiated lungs tested 16 weeks after irradiation; the response was reproducible in successive experiments. Respiration rate was increased above a threshold dose of 11 Gy (1100 rad), while amplitude decreased, also with a threshold at 11 Gy. These changes were observed before histological evidence of fibrosis became apparent and before pulmonary insufficiency led to deaths in the higher dose groups.

The measurement of lung function by plethysmography is an alternative to lethality for assessing radiation damage in the lungs of small animals. The technique is non-destructive, responding to lower doses than LD_{50}, and allows quantitative assessment of sequential changes in the lungs in each mouse over long post-irradiation times.

New quantitative endpoints for the assessment of normal tissue injury after irradiation are being developed by an increasing number of investigators (Chauser *et al.*, 1976; Glatstein *et al.*, 1975; Hayashi and Suit, 1971; Hirst *et al.*, 1977; Hopewell, 1975; Stewart, 1977; Van der Kogel *et al.*, 1977; Vatistas and Hornsey, 1966; White and Hornsey, 1977; Withers, 1967; Withers and Elkind, 1969). Because radiation damage to normal tissues is the dose-limiting factor in radiotherapy it is important that endpoints should be established which enable injury, particularly late injury, to be quantitated. Many of the techniques presently available are invasive or involve sacrifice of the animal. Functional assays which permit sequential assessment of injury in the same groups of animals over a period of time are available for only a few organs in small laboratory animals.

The present study reports a new non-invasive technique of measuring changes in pulmonary function after irradiation of both lungs of mice.

MATERIALS AND METHODS

Experimental animals and radiation procedures

Lung function was tested in mice in which both lungs were irradiated and in sham-irradiated controls. Male CBA/Ht mice, 10–12 weeks old, were given single doses of X rays ranging from 8 to 20 Gy. Radiation was delivered with a 250 kV X-ray unit, operated at 240 kV with an HVT of 1.3 mm Cu at a target skin distance of 20 cm. The dose rate was 3.65 Gy per min. Four mice were irradiated simultaneously on a specially constructed jig. The total dose was delivered within four to ten minutes in four equal increments with the jig rotated 90 deg after each increment to allow for a constant 10% dose-difference between the four irradiation positions. Both lungs were irradiated through two anterior 20×15 mm apertures in a 3.0 mm thick lead shield which protected the remainder of the body. Structures in the thoracic mid-line were also protected by a 1.0 mm wide strip in the lead shield separating the two lung fields. Perspex pillars fixed to the base of the jig kept the mouse in a supine non-rotated position and held the rib cage in position with respect to the radiation fields. This positioning was checked in some animals by radiographs which showed that less than 5% of the total volume of lung was protected by the mid-line shield. Dose distribution in the lung was checked using thermoluminescent dosimeters in dead mice.

The mice were anaesthetized before irradiation with sodium pentobarbital (60 mg/kg) given intraperitoneally. Megimide (15 mg) was given to each mouse after irradiation to speed recovery from the anaesthetic.

Pulmonary function was measured at monthly intervals up to 26 weeks in the control group and at 16 weeks in the irradiated groups. A specially constructed total body plethysmograph, described below, was used to assess pulmonary function.

Equipment and electronics

The changes in thoracic gas volume during respiration can be measured by using a whole-body

plethysmograph and a sensitive electrical capacitance manometer. The design of the apparatus used in the present experiments was based on the larger apparatus commonly used for measuring lung function in human subjects.

The plethysmograph was a clear Perspex airtight cylinder with a volume of 125 cm^3, both ends of which could be opened (Fig. 1). One end was fitted with a wire mesh screen and, immediately outside it, a hinged door. This allowed the cylinder to be opened without releasing the mouse. The mouse was placed in the chamber at the opposite end which was then closed by a removable door. The chamber was airtight when both ends were closed.

The pressure transducer was a modified Lavalier microphone (Mytex TMX 1250) which acted as the electrical capacitance manometer. The microphone was housed in the removable door of the chamber and responded to pressure changes in the whole chamber. One side of the microphone diaphragm was exposed and in direct contact with the gas in the chamber. The other side of the diaphragm was maintained in pressure equilibrium with the atmosphere through a narrow tube which had a high acoustic impedance except at very low frequencies. The diaphragm and a fixed backplate formed a capacitor. Capacitance changes were translated into voltage changes by having a permanent electrical charge impressed on the diaphragm (electret element; Fraim and Murphy, 1970). These voltage changes were amplified by a high impedance buffer amplifier close to the capsule. The overall frequency response was determined by the input impedance of the amplifier ($> 10^{10}\,\Omega$) and by the dimensions of the tube for pressure equalization. The low frequency cut-off (-3 dB) of the modified microphone unit was 1.2 Hz.

Initial experiments with the transducer indicated that further filtering was required to remove interfering signals that were not associated with respiration. It was also determined that the breathing rate of the mice was in the range 4–10 Hz. This is a higher value than reported in the literature but it has been checked by further experiments. The signal was therefore electrically filtered to exclude frequency components below about 3.3 Hz (arising due to movement, temperature changes, etc.) and above about 11 Hz (due to sounds of scratching, sniffing, etc.). From measurements on unfiltered signals it was found that a rate of 24 dB per octave would be necessary for the high-pass filter while a 12 dB per octave low-pass filter would be sufficient. To obtain the optimum characteristics the final response was trimmed to provide minimum overshoot consistent with the required cut-off slopes as this was considered more important than the precise cut-off frequency. The resulting filtered signal could be monitored on an oscilloscope or a chart recorder at this stage. The action of the filters on the signal from the transducer is shown in Fig. 2.

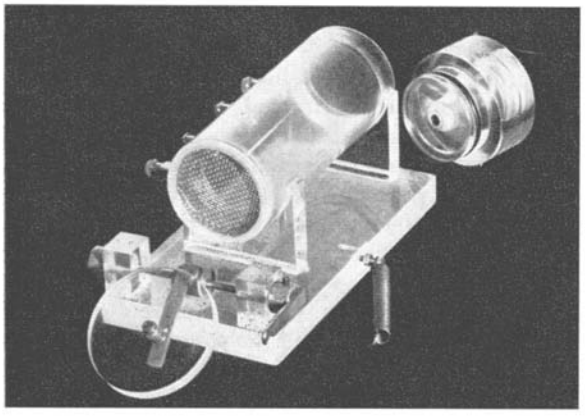

FIG. 1.

The plethysmograph shown with both ends open. The pressure transducer is located in the centre of the removable door at the far end. Wire mesh screen and a hinged door are at the opposite end allowing the chamber to be opened without releasing the mouse.

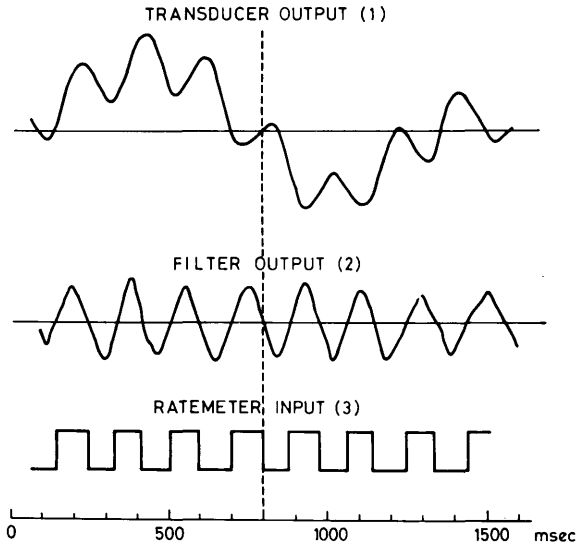

FIG. 2.

Typical waveforms obtained from the plethysmograph. The waveform numbers refer to various points in the circuit indicated in Fig. 3. The top trace (1) is a typical output from the transducer showing a slow component due to movement of the animal. In the filtered output (2) only the fast component (~5 Hz) is present. This signal was converted to a pulse waveform by the zero-crossing detector (3) suitable for frequency measurement.

A diagram of the modified transducer and electronic processing equipment is shown in Fig. 3. This system provides a simultaneous record of the rate and amplitude of pressure changes in the chamber which were due to the respiration of the mouse. The rate signal was calibrated with an oscillator in 1 Hz increments from 1 to 10 Hz, enabling the reading on the chart paper to be converted to Hz which corresponded to breaths per second. The amplitude signal was not calibrated and its magnitude was recorded directly in chart units.

Testing procedure

All mice were allowed to settle in the chamber for ½ to 1 min before the ends were closed and measurements recorded. However, after the mice had been in the chamber several times, the settling period was less than ½ min. At no time did the mice appear stressed in the chamber and they voluntarily entered the chamber with no assistance after several experiences in the chamber.

Continuous measurements were made for two min after closing the chamber. If sufficient data had not been obtained in this time air was allowed to enter the chamber and the measurements were repeated. It was often necessary to repeat the measurements before the mice became accustomed to the chamber; after this time the data were obtained in the first testing period. The average of the resting data for both parameters was read from the trace between 1 and 2 min of testing. A minimum period of 15 sec was regarded as a reliable trace and usually much more than this was obtained in the testing period.

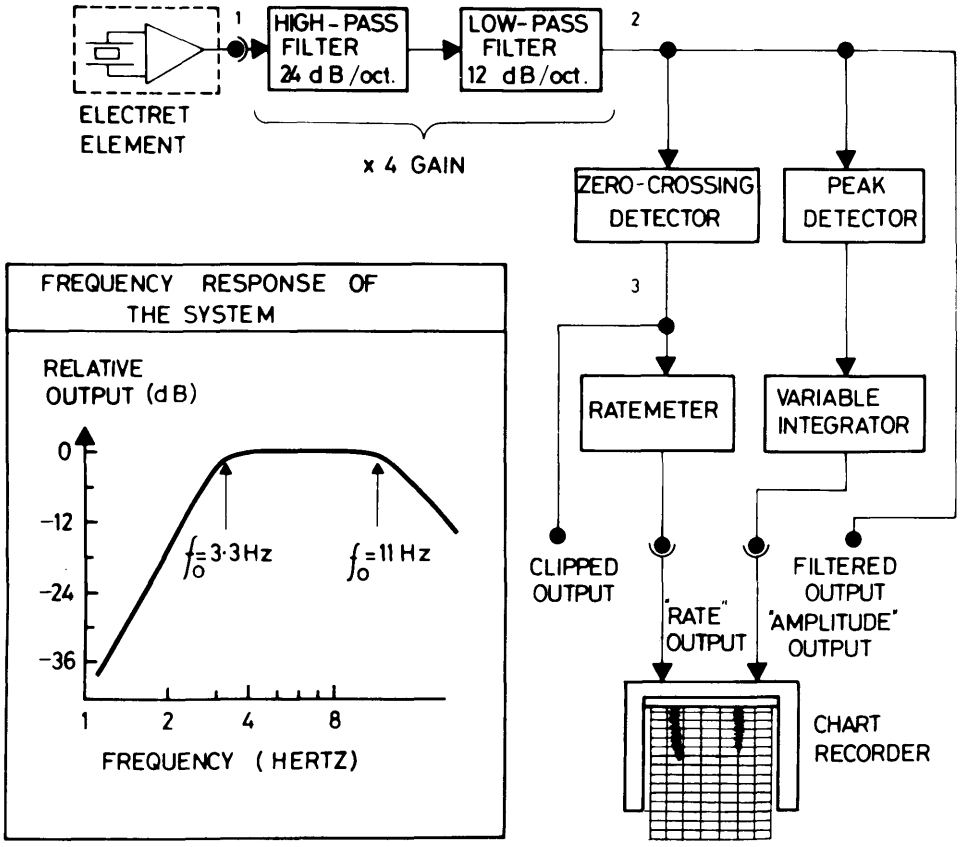

FIG. 3.

Block diagram of processing electronics. The filters were of the Sallen and Key configuration, Butterworth response (Tobey *et al.*, 1971); their frequency response is shown in the bottom left. The peak detector was a precision half-wave rectifier circuit followed by a variable time-constant integrator (20 ms–1 s). The rate-meter was based on a diode-transistor pump circuit (Anderson, 1972) with the output time constant also variable (100 ms–1 s). Typical output levels were ~100 mV on the amplitude output and 70 mV Hz^{-1} on the rate output. The clipped output could be fed to an external ratemeter. The numbers 1, 2 and 3 refer to the waveforms in Fig. 2.

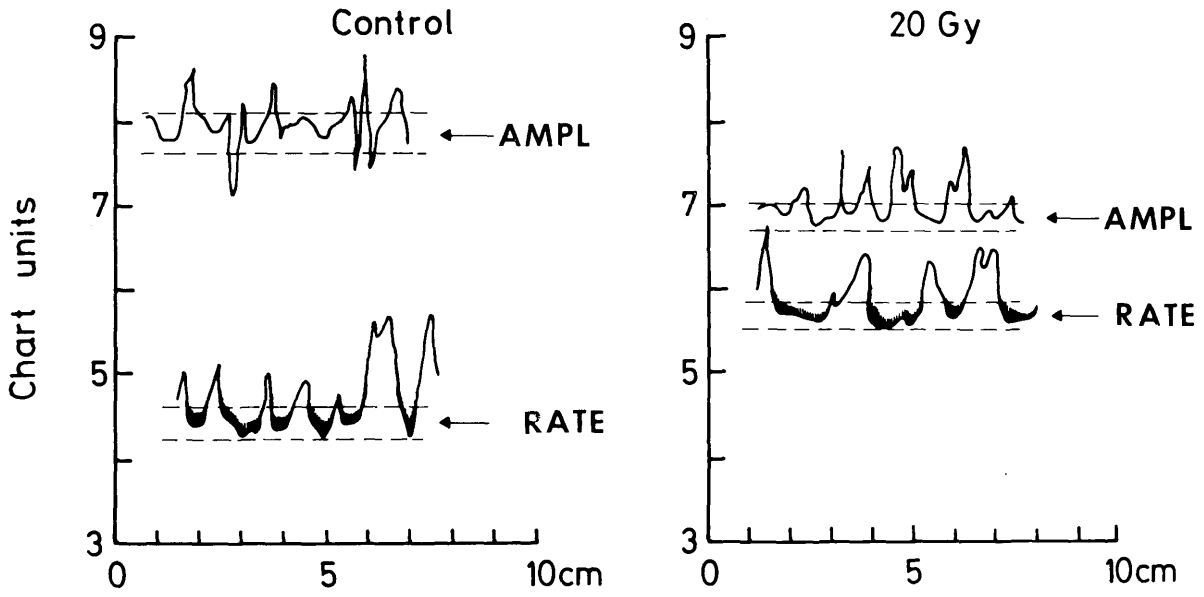

FIG. 4.

Pen recorder tracings from a sham irradiated mouse and a mouse given 20 Gy to both lungs. Testing time was 16 weeks after irradiation. The upper tracings represent the amplitude of pressure changes in the chamber, the lower the rate of pressure changes. The area between the dotted lines on each trace indicates the range of resting values from which the mean was calculated. The peaks represent animal movement which could not be electronically removed. Amplitude data were recorded in arbitrary chart units as indicated; the rate data were converted to Hz which corresponded to breaths per second. All rate data were expressed in breaths per minute. In this example, the rate of breathing was 330 (4.3 chart units) and 480 (5.8 chart units) breaths per minute for the control and irradiated mice respectively. The traces represent a time of 1.5 min; chart speed was 5 cm per min (bottom axis).

RESULTS

An example of the raw data from the chart recorder is shown in Fig. 4 for a sham-irradiated mouse and for a mouse tested at 16 weeks after 20 Gy. The lower trace represents the rate and the upper the amplitude. Some animal motion produced unwanted signals which were in the frequency range of interest. Because it was not feasible to eliminate these signals, fluctuations are apparent in the traces of both signals. The operator marked the trace to indicate when a mouse was at rest, so that spurious fluctuations were not incorporated into the data analysis. In this example the rates of breathing were 330 and 480 breaths per min for the control and irradiated mice respectively, *i.e.* 5.5 and 8.0 Hz. The amplitudes were 7.9 and 6.9 arbitrary units respectively. It is clear that significant changes occur in both of these functional parameters after irradiation.

The breathing rate of unanaesthetized sham-irradiated CBA mice varied from 321 to 350 breaths per min ($\pm 4\%$) between 9 am and 5 pm on five consecutive days. A larger variation was seen from three to nine months in this same group, ranging from 300 to 350 breaths per min ($\pm 8\%$). These variations did not appear to reflect a trend due either to a diurnal or an age related change. At 16 weeks after irradiation the breathing rate progressively increased after doses to both lungs greater than 11 Gy as shown in Fig. 5. Although the breathing rate of mice given only 11 Gy to both lungs exhibited a slight increase, this increase was not significant compared to the controls. A dose of 8 Gy produced no change in rate. The results from two separate experiments are plotted in Fig. 5. There were no significant differences in breathing rate between the two experiments in any dose group.

The amplitude exhibited the opposite dose-dependent pattern to the rate, decreasing with increasing dose, *i.e.* breathing became more shallow as it became faster (Fig. 6). Dose-effect curves were again obtained with a threshold at 11 Gy. Dose-response curves from the second experiment exhibited the same pattern as the first although there was more scatter in the data. In addition, there were significant differences between the two experiments at two points, control and 8 Gy. During measurement of the 8 Gy group at 16 weeks it was found that clamping the end door more tightly caused a 10% increase in the amplitude reading. Subsequently, a stronger spring and new O-ring seal around the door

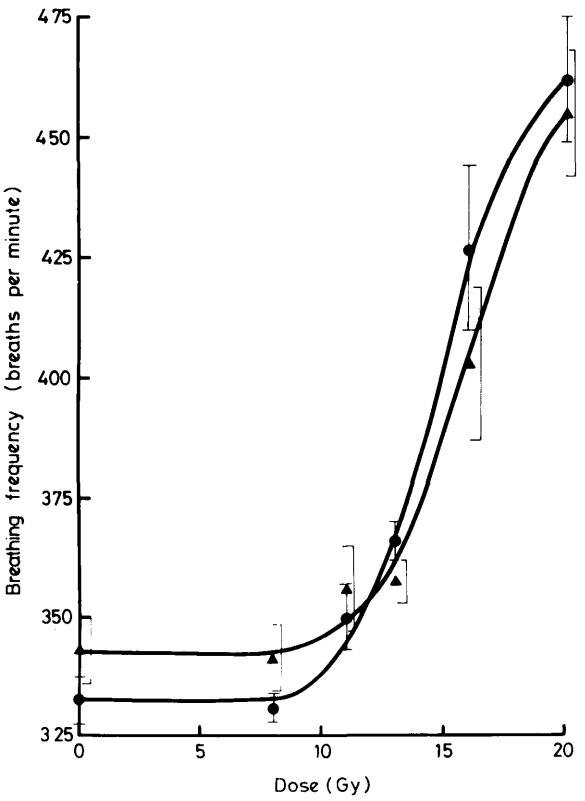

FIG. 5.

Breathing frequency as a function of radiation dose at 16 weeks after irradiation, showing a progressive increase in rate with dose with a threshold at 11 Gy. The results from two experiments are plotted; there was no significant difference in breathing rate at any dose between the two experiments. Each dose point represents the mean of 10 mice in one experiment (●) and 7 in the other (▲). Error bars are 1 SEM.

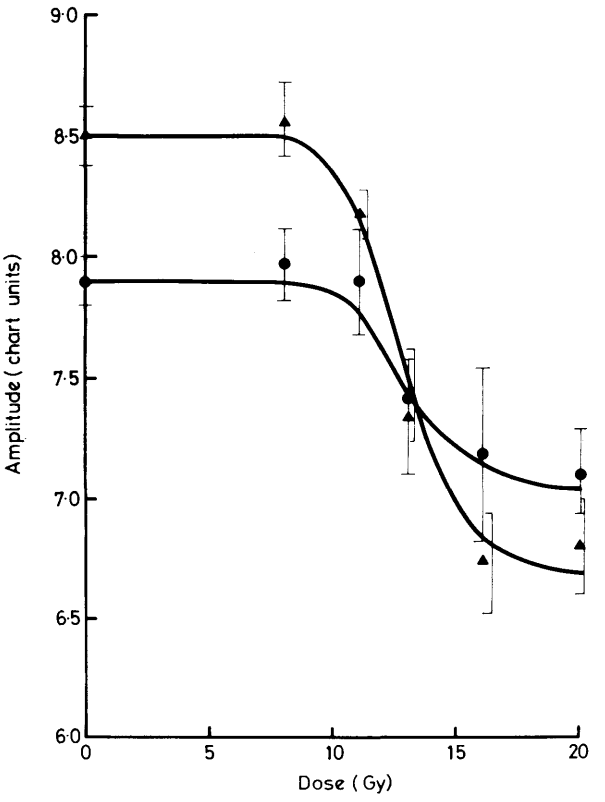

FIG. 6.

Amplitude of breathing at 16 weeks after graded X-ray doses to both lungs, showing a reduction with increasing dose. Data are from the same mice for which breathing rate is plotted in Fig. 5; the symbols correspond to the two experiments plotted in Fig. 5. Error bars represent 1 SEM. Significant differences are evident in the sham and 8 Gy group between the two experiments; a minor air leak found in the chamber probably contributed to these differences.

were introduced. In addition, the amplitude signal was routinely checked by testing the same control mouse as a "standard" before testing the experimental groups; *i.e.* several times per day. If his amplitude had changed significantly, the system was checked for air leaks or electronics problems and rectified before further use. This occurred at intervals of a few months when the O-ring seal became deformed.

DISCUSSION

Total body plethysmography is often used to measure pulmonary function in human subjects (Bedell *et al.*, 1956; Comroe *et al.*, 1959; Du Bois, 1959; Du Bois *et al.*, 1956a; Schmidt and Cohn, 1961). Two parameters of lung function in mice were simultaneously monitored in this study, respiration rate and pressure amplitude. The ratemeter output was accurately converted to breaths per min by calibrating the equipment with an oscillator. The mean breathing frequency of 328 breaths per min in control mice is higher than previously reported values for mice, 160 and 190 breaths per min with ranges of 60–230 and 97–123 respectively (Spector, 1956; Crosfill and Widdicombe, 1961). However, breathing frequency is strain dependent and often sex dependent, factors which were not reported in the previous studies (Travis *et al.*, in preparation). Another factor which might be expected to increase breathing rate is stress. Subjectively the mice appeared unstressed in the chamber. Further experiments have also shown that breathing rate is decreased rather than increased as a result of swimming stress. These findings suggest that the higher rates measured in this study were not due to stress. Because of the discrepancy between

our value for breathing frequency and those previously reported, the value reported in this study has been confirmed by two other methods, one visually by high speed filming and one using a recording stethoscope. The latter simultaneously measured heart rate, which was approximately 2–3 times higher than the breathing rate.

The amplitude signal, which was not calibrated, was subject to more technical problems than the rate signal. It was affected by small movements of the mouse and, although the signal was filtered to eliminate large movements, it was not feasible to eliminate all movement in the frequency range of interest. Therefore, mean values were more difficult to obtain for breathing amplitude than for rate. The amplitude signal was also affected by temperature and humidity changes after the mouse had been in the closed chamber for more than two min. An increase in either of these factors would cause an increase in pressure, therefore temperature and humidity were controlled by opening the chamber every two min during testing of an animal. This introduction of fresh air also eliminated the potential problem of O_2 depletion and CO_2 accumulation in the closed chamber, changes which in themselves would affect breathing frequency and depth. Because the rate and amplitude of breathing were continuously recorded when a mouse was in the closed chamber, sudden changes in either signal were immediately visible on the chart paper. Few significant changes were observed in either trace during the two min of testing in any group of mice, irradiated or control, suggesting that the amount of O_2 depleted and CO_2 accumulated in this time had no significant effect. In a few traces a gradual small increase (of 5 to 10%) in rate was seen over the two min period, which may have been due to the increase in CO_2 content. The value then used was the mean of the rate before and after the increase.

Appendix 1 shows that the effect of CO_2 accumulation would be expected to be possibly just significant.

These observations are consistent with calculation of gas composition changes in the chamber based on the known rate of exchange of O_2 and CO_2 in normal human subjects. Applying these figures to mice having a respiration rate of 328 breaths per min, 1.13% of the available O_2 is converted to CO_2 in 1 min, 2.2% in 2 min and 3.2% in 3 min (Appendix 1). The amount of O_2 depleted and CO_2 accumulated in 2 min would not cause changes in either functional parameter, as was indeed observed experimentally.

Although lung gas volume and airway resistance in patients can be calculated from plethysmographic measurements (Du Bois *et al.*, 1956a; Du Bois *et al.*, 1956b; Bedell *et al.*, 1956), the amplitude results in the present study are more difficult to interpret. The amplitude of the pressure signal is related to four lung parameters: airway resistance, ventilation, lung volume and the shape of the breathing cycle. A reduction in any one or combination of these four would cause a reduction in the amplitude of the pressure change.

It is unlikely that airway resistance is decreased. Rather, airway resistance would be *increased* if fibrosis were occurring, a histological change which has not been observed at 16 weeks in any of the dose groups. Although a change in airway resistance cannot be excluded, this parameter is the least likely contributor to the reduced amplitude observed.

A more probable factor influencing amplitude is the shape of the breathing cycle. Normally the breathing cycle consists of a slow intake of air during inspiration followed by a rapid exhalation of air during expiration. One method of compensating for respiratory insufficiency is to optimize the breathing cycle to equal periods of steady flow during inspiration and expiration, thereby effectively reducing the amplitude of the pressure change in the plethysmograph.

The most probable lung parameters reducing breathing amplitude in the present study are a reduction in lung volume and ventilation. Decreased ventilation has been observed in dog lungs at 16 weeks after irradiation, the time when amplitude was reduced in the present study (Teates, 1965). In both studies, this functional change occurred before histological evidence of fibrosis and during severe diffuse pulmonary oedema. The latter pathological change could cause a decrease in both lung volume and ventilation.

In the present study, the amplitude signal represents a complex of pulmonary functional parameters in the mouse but undoubtedly reflects the depth of breathing. Although none of the four functional parameters affecting amplitude can be isolated in the system used, it is reasonable to assume that the magnitude of change in any of these parameters would be constant within a group of mice receiving a specific dose. In addition, the relative contribution of each of these factors is not important for a dose-response curve, provided that each does depend upon the dose.

Both rate and depth of breathing yield clear dose-response relationships at 16 weeks. A threshold dose of 11 Gy was observed for both effects. The dose response curves show a mirror image relationship of

the two parameters, respiration rate increasing while the depth of breathing proportionally decreased. At this post-irradiation time, the shapes of the two curves are not significantly different and may reflect the same pathological change. Rapid shallow breathing indicates a reduction in lung compliance. This breathing pattern effectively reduces the increased work of breathing resulting from stiffening of the lungs. Although decreased compliance is usually associated with fibrosis, rapid, shallow breathing was observed before the onset of fibrosis and during severe pulmonary oedema (Travis *et al.*, 1978). A reduction in lung compliance has been reported during the oedematous phase of the radiation response in rat and dog lungs (Shrivastava *et al.*, 1974). The doses used were similar to those used in the present study. It is likely that the rapid, shallow breathing pattern observed may be a compensatory response to decreased lung compliance due to oedema, the latter causing a concomitant reduction in lung volume, one of the factors which affect amplitude. Further investigations are being conducted to measure pulmonary compliance.

If the relationship between breathing rate and depth observed at 16 weeks is true at other times after irradiation, it may be feasible to assess routinely only one of these parameters. Because the amplitude signal cannot be calibrated absolutely in the present system and because there are more fluctuations and scatter in the amplitude than in the breathing rate, the latter is the parameter of choice.

The present tests indicate changes in rate and depth of breathing at earlier times and after lower doses than those used when death is the endpoint (Field and Hornsey, 1974; Hornsey *et al.*, 1975; Phillips and Margolis, 1972; Steel *et al.*, 1978). CBA mice exposed to 13 Gy to the whole thorax, a dose within the reported range of LD_{50} values for pulmonary lethality in other strains, have all survived 36 months (Travis *et al.*, unpublished). The rate and depth of breathing had, however, changed significantly from control values by 16 weeks after 13 Gy. All mice given 16 Gy or higher doses were killed between 15 and 20 weeks after irradiation because of respiratory distress, but significant changes in rate and depth of breathing were recorded for at least six weeks before killing.

These functional changes therefore provide a useful new method for assessing pulmonary response of mice to radiation. The technique described not only responds at lower doses than LD_{50} and is non-non-invasive, but is also a quantitative method.

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APPENDIX 1

The following equations were used to calculate O_2 depletion and CO_2 production in the chamber after various periods of respiration.

Equation 1. O_2 depletion

$$n_z(O_2) = n_0(O_2)\left[1-\left(\frac{V_T}{V_c}[1-f]\right)\right]^Z$$

Where:

Z = number of breaths
$n_z(O_2)$ = amount of O_2 or CO_2 in chamber after Z breaths
n_0 = amount of O_2 or CO_2 in chamber at $Z=0$
V_T = tidal volume (volume of air exhaled) by a mouse (0.08 cm³, eq. 2 and 3)
V_c = volume of chamber less the volume of the mouse (95 cm³)
f = ratio of exhaled to inhaled O_2 (16%/20%)

Equation 2. Calculation of V_T

$$V_T = \frac{\text{Minute volume (cm}^3\text{ air/min—Eq. 3)}}{\text{Breathing frequency (mean observed value)}}$$

$$= \frac{26.9\text{ cm}^3/\text{min}}{328\text{ breaths/min}}$$

$$V_T = 0.08\text{ cm}^3/\text{breath}$$

Equation 3. Calculation of minute volume (Guyton, 1947).

$$\text{Minute volume} = 2.10 \times \text{wt (gm)}^{0.75}$$

Minute volume = 26.9 cm³ for a 30 gm mouse

Substituting in Eq. 1 and calculating vol. % of O_2 and CO_2 in chamber

Z	t (minutes)	n_z/n_0	Vol. %O_2	Vol. %CO_2
0	0	1	20.99	0.03
1	0.003	0.9998		
330	1	0.9461	19.86	1.13
660	2	0.8950	18.79	2.20
990	3	0.8468	17.77	3.22
1650	5	0.7579	15.91	5.08

Breathing rate would be expected to increase when 3 to 5% of CO_2 had been accumulated. Even if the above times should be shortened by a factor of two, because the normal breathing rates of our mice were faster than those in the literature, no increase in rate due to CO_2 would be expected until near the end of our test period. In practice the traces were flat between 1 and 2 min when the values were read off the chart.

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