EFFECTS OF X-IRRADIATION ON RESPIRATION IN FROG BRAIN

TISSUE SLICES USING THE OXYGEN ELECTRODE METHOD

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The effects of X-irradiation at dosages of 40 r, 80 r, 160 r, 240 r, 320 r, 400 r, 800 r, 4 Kr, 8 Kr, and 16 Kr on the oxygen uptake of frog brain (*Rana pipiens*) tissue slices were studied. A membrane-covered oxygen electrode method was used to measure the respiratory rate. Continuous recordings were made before, during, and following X-irradiation in all of the test experiments. X-irradiation was delivered from a G. E. beryllium window X-ray unit at 120 KVP, 5 ma with a 1/4 mm Al filter.

The mean $Q_{O_2}$ of sham-irradiated tissue slices was 6.83. At dosages ranging from 20-320 r, a significant and sustained decrease in oxygen uptake occurred. The pre-irradiated tissues exhibited a mean respiratory rate of 100 μls $O_2$-100 mg wet wgt/hr, whereas the inhibitory rates ranged 70-80 μls $O_2$/100 mg wet wgt/hr. Inhibition was noted within 30 minutes post-radiation.
At dosages ranging between 400 r and 16 Kr, a multi-phasic respiratory response was noted. It consisted of a momentary decrease, followed by an increase lasting up to 120 minutes post-radiation. A slow return to control rates was noted in some experiments at these dosages.

The data clearly indicated a metabolic effect of ionizing radiation on frog brain respiration that was dose-dependent and countered the notion that X-irradiation effects are purely physical in nature. The data may shed light on the nature of X-ray carcinogenesis as described in Warburg's respiratory concept of cell cancer.
EFFECTS OF X-IRRADIATION ON RESPIRATION IN FROG BRAIN
Tissue Slices Using the Oxygen Electrode Method

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>4</td>
</tr>
<tr>
<td>RESULTS</td>
<td>13</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>30</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>36</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>38</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Experimental Apparatus (The Oxygen Sensor)</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>Experimental Facilities</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Calibration of Oxygen Electrode</td>
<td>12</td>
</tr>
<tr>
<td>4.</td>
<td>Typical Recordings on the Speedomax H Unit</td>
<td>15</td>
</tr>
<tr>
<td>5.</td>
<td>Oxygen Uptake in Sham-irradiated Frog Brain Tissue Slices</td>
<td>16</td>
</tr>
<tr>
<td>6.</td>
<td>Effects of 40 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>18</td>
</tr>
<tr>
<td>7.</td>
<td>Effects of 80 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>19</td>
</tr>
<tr>
<td>8.</td>
<td>Effects of 160 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>20</td>
</tr>
<tr>
<td>9.</td>
<td>Effects of 240 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>21</td>
</tr>
<tr>
<td>10.</td>
<td>Effects of 320 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>22</td>
</tr>
<tr>
<td>11.</td>
<td>Effects of 400 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>24</td>
</tr>
<tr>
<td>Figure (Cont'd)</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>12. Effects of 800 r X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>13. Effects of 4 Kr X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>14. Effects of 8 Kr X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>15. Effects of 16 Kr X-irradiation on Oxygen Uptake in Frog Brain Tissue Slices</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

The effects of ionizing radiation on the nervous system of vertebrates and invertebrates have been extensively reviewed. The effects described range from biochemical (27, 31) to behavioral changes (14, 15, 18). There are two concepts concerning the nature of the radiation effects on the central nervous system. One maintains that radiation brings about a physical disruption of either cellular macromolecules or/and membranes. The second involves an effect on the metabolic integrity of the tissues, e.g. enzyme inactivation. To date, these concepts have not been resolved with complete certainty.

The present study concerned providing data to either substantiate or negate the metabolic concept in terms of changes in brain tissue respiration. Although there are many reports on biochemical changes in irradiated nervous tissue, including the brain, the literature is relatively sparse in regard to overall
tissue respiration. Moreover, according to van Cleave (27), the data are most conflicting due to differences in methods used, e.g. whole animals versus tissue slices and internal emitters versus external radiation. Mole (22) reported a decrease in basal metabolism rates in animals following 300 r to 1000 r whole-body irradiation, whereas, Florsheim et al. (9) reported no changes in oxygen uptake of minced rat brain tissues following 900-1200 r whole-body X-irradiation. Lott and Hines (19) reported a gradient in $Q_{O_2}$ from cortex to brain stem tissue slices in rats following X-irradiation. Egana (7) also reported changes in $Q_{O_2}$ in various rat brain tissues that were exposed to internal beta-irradiation. More recently, Kaack (13) reported a correlation between electrical changes and oxygen uptake in irradiated nerves. In most of the foregoing work, a manometric method was used to measure the oxygen uptake. Snezhko (26) was one of the first to report changes in respiration in intact X-irradiated rat brains using an oxygen electrode method. His technique involved the placement of the electrode into brain tissue. Such a technique, however, may have caused a coating of the membrane of the electrode and, therefore, yielded incorrect data.
A search of the literature regarding the use of Clark membrane-type oxygen electrodes revealed that not all types are suitable for a given kind of experiment. The oxygen sensor probe used in the present study was found to be suitable for making determinations before, during, and following X-irradiation of brain tissue slices. Such experimental format has a distinct advantage over studies in which tissues are either removed from irradiated animals or in which no measurements are taken during irradiation.

Summarily then, the aims of the present study were threefold: (1) to establish a dose range of effects of X-irradiation on frog brain tissue respiration, (2) to test the efficacy and use of the potentiometric method in studying oxygen uptake in isolated tissues, and (3) to provide some insight into the biochemical nature of the radiation insult to the central nervous system.
MATERIALS AND METHODS

General Format

Over 100 adult female grass frogs (Rana pipiens) ranging in weight from 100 to 200 grams (size 4-5 inches) were used in this study. The experiments were performed between October and February of the year. The experiments were divided into several series:

1. Sham-irradiated empty chamber
2. Irradiated empty chamber
3. Sham-irradiated tissue slices
4. Irradiated tissue slices.

The purpose of measuring the empty chambers was to ascertain and account for any possible exchanges of oxygen between the chamber and the electrode.

All other experiments consisted of a pre-irradiation, a radiation, and a post-radiation period. The sham-irradiated tissues were treated exactly as the irradiated tissues in re, equilibration times, handling, etc.
Tissue Preparation

Following decapitation, the brain was removed and placed inside a cold chamber on filter paper wet with fresh, aerated frog-glucose-Ringer's solution (pH 7.2). The cerebral hemispheres were separated and, using the method of McIlwain and Rodnight (21), tissue slices of less than .4 mm thickness were made, blotted, weighed, and placed in a beaker containing aerated frog-glucose-Ringer's solution for 15 minutes. The tissue slices were then loaded into the glass chamber shown in Fig. 1. The entire cutting and weighing procedure required less than two minutes. The weights of tissues used per experiment ranged between 10 and 25 milligrams. After loading, the tissues were allowed to equilibrate inside the chamber at room temperature for 30 minutes prior to recording. A 90-minute control recording period preceded the test or radiation phase of each experiment. If the tissue did not exhibit a constant oxygen uptake during this period, it was discarded.

In Fig. 1 are diagrams of the glass tissue chamber and the oxygen electrode. The purpose of the syringe needle was to establish pressure equilibrium prior to
A. **The Glass Chamber** (Pyrex)

- **Glass holder**
- **5/8" Dia.**

**Front view**  
**Side view**

B. **The Oxygen Sensor Probe** (#LP-10, Chemtronics Inc.)

- **22 Gauge needle**
- **Tissue holder**
- **Rubber stopper**
- **O-ring**
- **.0015" Polyethylene membrane**

C. **Chamber-Sensor Assembly**

- **Glass holder**
- **Tissue slices**
- **Wet filter paper**
- **Glucose-Ringers solution for frogs**

D. **Tissue Chamber**

**Fig. 1—Experimental apparatus (The Oxygen Sensor)**
each experiment and to determine the volume of the chamber. The total capacity of the chamber was about 2.5 cubic centimeters. The temperature probe (Model 403, Yellow Springs Instruments, Yellow Springs, Ohio) monitored any changes in temperature during each run. The mean temperature in the experiments was 24 ± .3 °C. If the temperature varied over .5 degrees during any single run, the experiment was discontinued.

The oxygen sensor shown in Fig. 1B and 1C (Model LP-10 Oxygen Sensor Probe) was obtained from Chemtronics Inc., San Antonio, Texas. This particular electrode is a variation of the electrode developed by Blinks and Skow (3). The current in the electrode circuit was converted into a voltage signal in the primary input resistor that was then utilized as an input signal to the amplifier. The voltage changes were measured with a Model 602 Solid State Electrometer (Keithley Instruments, Cleveland, Ohio) and displayed on a Speedomax H (Leeds and Northrup Co., Philadelphia Pa.) paper recorder.

Figure 2 shows the entire experimental facility. Note that the oxygen electrode was protected from the X-rays by a one-quarter-inch lead tube. Turning on
the line switch to the X-ray machine did not produce interfering signals to either the electrometer or the recorder. Note that the overall experimental set-up made it possible to record oxygen uptake in the tissues before, during, and following X-irradiation.

Irradiation was delivered from a Model KX-10 General Electric X-ray Unit with a beryllium window at 120 KVP, 5 ma with 1/4 mm Al filter. The target distance was 11 centimeters, resulting in a dose rate of 800 r/min to the tissues. Dosimetry was determined with the use of Bausch and Lomb silver-activated phosphate microdosimeter rods placed inside the tissue chamber and a calibration curve established by a certified radiological physicist.

Preparation and Calibration of the Oxygen Electrode

The LP-10 probe can be used in liquids or gases under a variety of situations calling for a measurement of oxygen partial pressure ($P_{O_2}$). $P_{O_2}$ measurement is a current measurement; therefore, the negative feedback amplifier has been utilized in a somewhat different way, i.e., the current was converted to a voltage signal and fed into the electrometer. Since the diffusion is much slower in liquids than in gases, and to avoid the possible
effects of materials that may have been released by the tissues, a moist, gaseous, closed system was used in this study. To insure proper moisture in the chamber, frog-glucose-Ringer's solution was added in the small recess at the bottom of the tissue chamber. For restoring linear response, the electrode was rinsed with 15% NH₄OH (16, 17). In loading, the probe was placed into a center hole in a plastic holder on a flat table. The electrolyte (0.5M KCl) mixed with a small amount of detergent, obtained from Chemtronics, was added to the probe with an eyedropper until the entire upper surface of the probe was covered. Care was taken to prevent trapping air bubbles in the probe solution. A piece of polyethylene membrane (0.0015 inch), also supplied by Chemtronics, Inc., was placed carefully over the electrode so as to avoid forming air bubbles beneath the membrane. Quickly, a rubberized O-ring was applied to the membrane, and into the O-ring groove at the tip of the probe (Fig. 1B & 1C). The excess membrane was then trimmed off with scissors and vacuum grease was applied around the margin of the membrane and O-ring. The cable of the oxygen electrode was then connected to the electrometer. The response of the electrode in pure N₂ gas was measured and
usually found to be zero. Any reading greater than zero was taken as a pre-potential of the electrode, which was then used to subtract the reading in various proportions of N₂-O₂ gas mixtures (obtained from Matheson Co., Inc., East Rutherford, N. J.). If the prepotential was greater than 1 mV., the membrane and the electrolyte solution were changed. Calibration curves were obtained by plotting potential readings against the concentration of O₂ in various mixtures as shown in Fig. 3.

The line switch to the X-ray unit was always turned on at least seven minutes prior to the application of the X-irradiation. Ten different dosages of X-irradiation ranging between 40 r and 16 Kr were used in this study. Some tissues were removed at the end of the equilibration period, placed into an aluminum planchet and dried at 100 °C in an oven to obtain dry weight as described by Lucas (20) as a factor for Q₀₂ values. The oxygen uptake was presented in terms of μls O₂/100 mg wet wgt/hr.
Fig. 3--Calibration of oxygen electrode with various $N_2-O_2$ gas mixtures at 24°C.
RESULTS

The calibration curve for the oxygen sensor probe in Fig. 3 indicates that a voltage change of one milli-volt was equivalent to 23.52 μls O₂/ml of gaseous volume. The reading of voltage changes (V) were taken from the electrometer and graphically displayed on the Speedomax H recorder. Measurements taken from an empty chamber yielded a correction factor of 0.08 mV/hr (K) that had to be applied in calculating the oxygen consumption by the tissues. When the voltage (V) was recorded over a given interval, it could then be converted to V/hr. or V'. The actual voltage change then would be V' - K.

The oxygen uptake would be:

\[(V' - K) \times 23.52 \text{ μls O}_2/\text{ml/hr.}\]

If the wet weight of the tissue (W) and the volume of the chamber (C) are known, then the overall oxygen uptake of the tissue slices could be calculated thus:

\[= \frac{1}{W} (V' - K) \times C \times 23.52 (\text{μls O}_2/\text{mg wet wgt/hr.})\]

\[= \frac{C}{W} (V' - K) \times 2352 (\text{μls O}_2/100 \text{ mg wet wgt/hr.})\]
Fig 4 contains typical graph recordings obtained during the various experimental conditions. It is clear that the oxygen electrode remained fairly stable throughout each run in re external line interference. An unexpected observation in all of the irradiation experiments was the appearance of a "ditching effect" during the time when the X-irradiation was being applied. During that time little or no oxygen uptake was indicated. Following X-irradiation, however, distinct differences in respiration rates became apparent depending on the total dosage. The offset figures along the Y-axis in Figs. 4-15 represent numbers of each experiment in that series.

Fig. 5 contains a family of curves derived from recordings of eleven experiments involving sham-irradiated brain tissue slices. The points on each curve were arbitrarily chosen so that a mean rate of oxygen consumption could be calculated for a given period; in this case, over thirty-minute intervals. These curves indicate a relatively constant rate of respiration by the tissue slices for periods up to 240 minutes. Therefore, the respiratory integrity of the brain slices appeared to be unchanged over this period of time.
Fig. 4—Typical recordings on the Speedomax H Unit showing the effects of X-irradiation on oxygen uptake in frog brain tissue slices.
Fig. 5—Oxygen uptake in sham-irradiated frog brain tissue slices.
Fig. 6 contains a family of curves derived from recordings of ten experiments in which the tissue slices received 40 r X-irradiation. The mean values for oxygen uptake depict significant and relatively sudden and sustained decreases following irradiation, with little or no sign of recovery after 120 minutes.

As shown in Fig. 7, tissue respiration following exposure to 80 r X-irradiation decreased immediately following irradiation and was sustained throughout each experiment. In a few instances, momentary increases, lasting no longer than thirty minutes, were observed.

The responses of the tissue slices to 160 r X-irradiation are depicted in Fig. 8. Again a significant, sudden, and sustained decrease in oxygen uptake was observed in all but three of the experiments. Similar responses were noted in those tissues exposed to 240 r X-irradiation shown in Fig. 9. The "ditching effect" mentioned earlier first became noticeable in those tissues exposed to 320 r X-irradiation as shown in Fig. 10. Another interesting observation was the appearance of a "plateau" following the radiation period indicating no measurable oxygen uptake. Moreover, these plateaus, even though present in all experiments, were not of the same
Fig. 6—Effects of 40 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 7--Effects of 80 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 8—Effects of 160 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 9—Effects of 240 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 10—Effects of 320 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
duration in each case. It is impossible at this time to account for these observations. Also shown in the curves in Fig. 10 were instances of increased respiration following the "plateau" phase. Only two experiments showed decreases in respiration lasting up to ninety minutes following the "plateau" phase. The "plateau" phase was even more distinct in those tissues exposed to 400 r X-irradiation (Fig. 11). The differences in the duration of the "plateau" were also most noticeable at this dosage. Moreover, an increased respiration followed the "plateau" phase in all of the experiments. Finally, there appeared a relatively high incidence of recovery to pre-irradiation oxygen uptake rates at 120 minutes post-radiation.

Fig. 12 contains a family of ten curves derived from recordings from tissues exposed to 800 r X-irradiation. The "ditching effect" and a relatively short "plateau effect" were distinct in all of the experiments. Moreover, in all cases, a significant increase in respiration, lasting up to an hour post-radiation, was noted. Following the period of enhanced respiration, a gradual return to control rates was observed. In Fig. 13, the "ditching effect" was much more prominent, due to the length of the irradiation period; however, in a few cases, there
Fig. 11—Effects of 400 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 12—Effects of 800 r X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 13—Effects of 4 Kr X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
appeared a sloping in the "ditch" which indicated at least, some respiration was occurring in the chamber. Another interesting observation was that, following a rather short "plateau" stage, a sharp but short-lived increase in respiration occurred, followed by a gradual return to control rates in all but two cases. The curves in Fig. 14 and Fig. 15 again show distinct "ditching" effects during the irradiation period followed by relatively short "plateau" phases. Following the "plateau" phase, distinct increases in oxygen uptake, lasting up to ninety minutes post-radiation, a return to control rates of respiration was apparent. In three experiments in which the tissues received 16 Kr, a decrease in respiration rates occurred at the 60-90-minute post-radiation period.

In general, the effects of X-irradiation on frog brain tissue respiration fell into at least two rather distinct categories. Dosages ranging between 40 r and 240 r brought about a significant and sustained decrease in oxygen uptake, whereas, at the 400 r to 16 Kr range, a distinct but short-lived decrease in respiration occurred followed by an increase in respiration. The breaking point appeared somewhere in the 300 r to 400 r range of X-irradiation.
Fig. 14—Effects of 8 Kr X-irradiation on O₂ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
Fig. 15—Effects of 16 Kr X-irradiation on $O_2$ uptake in frog brain tissue slices. (Dose rate: 800 r/min.)
DISCUSSION

It was most difficult to compare the data obtained in the present study with those of other authors due to the differences in techniques and experimental tissues used. The mean calculated $Q_{O_2}$ of the adult female frog brain tissues amounted to $6.83 \pm 0.02$ $\mu l$ $O_2$/$mg$ dry wgt/hr, which was relatively lower than those found in mammalian tissues. The resting oxygen uptake in the pre-irradiated tissues ($100 \mu l$ $O_2$/100 mg wet wgt/hr) was relatively similar to the rates of oxygen uptake reported for frog sciatic nerves by Kaack (13), (85 $\mu l$ $O_2$/100 mg wet wgt/hr).

The "ditching" effect observed during the irradiation period in both the empty and the tissue-filled chambers may be some kind of technical artifact of the X-ray machine. The "plateau" phase following the "ditching", however, was of different duration and was dose-dependent; therefore, one can assume that the "plateau" response was a "real" response by the tissues and not an artifact.
The general results of the present study were comparable with the overall findings of other workers measuring changes in oxidative metabolism enzymatically and using other tissues. Kaack (13) reported multiphasic respiration effects in frog sciatic nerves irradiated with a beta-emitter. In this work he reported an initial decrease followed by a biphasic increase in oxygen uptake at 15 and 50 minutes post-radiation. Snezhko (26) also reported increased respiration in intact rat brains irradiated at 900 r to 1.5 Kr. Some workers reported increased succinoxidase activity in neuroglial and neuronal cells isolated from rat cortical tissues as well as increased $^3$H-leucine incorporation in cortical cell fractions obtained from previously irradiated animals (10, 11, 23). Other workers (1, 5, 24) have studied X-irradiation effects on isolated enzyme systems in vitro and have reported changes in the activity of enzymes and in particular, sulfhydryl systems following various types of external radiation. Unfortunately, none of the foregoing reports described changes that occurred before or during irradiation in continuous sequence as was done in this study.

In attempting to explain the data reported here, one
must consider several possible levels of changes. First, histological changes of cortical slices have been extensively described by Haymaker (12). His data indicate that glial cells are more radiosensitive than neuronal cells, the latter requiring dosages in the kilo-roentgen range to show measurable deterioration. Secondly, subcellular effects of ionizing rays such as deterioration of respiratory "grana" have been mentioned by Warburg (28). Indeed, he has stated that carcinogenesis by X-rays was nothing more than a destruction of respiration by "elimination" of these respiring grana. A third possible level of radiation effect involving alterations of membrane permeability has been reported by numerous workers. Bergeder (2) stated that irradiation of muscles altered the electropotential of the surface charges, which, in turn, altered ion flux across the membrane layers. Such changes might account for the often reported changes in the electrical activity in nervous tissues. Williams and Sheard (30) have also tied electrical changes in irradiated nerves to oxygen uptake. Changes in the electrical activity are thought to result in changes in irritability of nerve components that finally produce the behavioral changes noted
in irradiated animals. A fourth possible factor to be considered involves the effects of X-irradiation on enzyme systems (1, 5, 6). Whether the effect is a direct one on the physical integrity of the enzymes per se or on the substrate has not been resolved. Chesley (4) claimed that the changes in respiration by irradiation may be due to the inactivation of catalysts already present or by a decreased production of new catalysts which would present an increasing rate of oxygen consumption. Another of the enzyme systems that has been instigated in the radiation effects is the Na-K-ATPase system. Quastel (25) and Whittam et al. (29) claimed that this system may play an important role in the oxygen uptake in brain tissue through its control over ion flux in the membranes. Finally, the effect of ionizing radiation on free-radical formation in protoplasm is well known, and therefore must be considered in accounting for the respiratory effects reported here. Egana and Velarde (8) reported on the inhibition effect of hydrogen peroxide (H₂O₂) on brain tissues. They also observed oxygen being released from H₂O₂ and explained it on the basis of increased catalase or peroxidase activity. Oxygen poisoning and radiation injury to cells
are similar in that both may involve the formation of toxic free-radicals that inhibit essential cellular reactions. One might conjecture that the plateau phase in the data reported here, indicating the absence of oxygen uptake by the tissue, may be due to the formation of inhibitory peroxides or/and the release of other oxidizing radicals.

All of the foregoing possible mechanisms of action of ionizing radiation on the nervous system at the molecular level have been integrated into the concept that the initiation of the biological effects observed in irradiated tissues result from the energy released directly within specific sensitive target molecules or transmitted indirectly to such molecules through the interaction of reaction products formed in the protoplasm by radiation.

Summarily, the data reported in this study described a multiphasic irradiation effect on brain tissue respiration that was dependent on the dosage. Low levels of X-irradiation brought about a sustained decrease in oxygen uptake, whereas higher dosages brought about an initial decrease followed by periods of enhanced oxygen consumption. Slight recovery was observed in some instances. Secondly, the data indicate that the oxygen
electrode method was suitable for measuring the effects of X-rays on tissue respiration, with one exception. The electrode did not appear to be suitable for recording changes in $P_{O_2}$ in the chamber during actual application of X-rays. The plateau observed following the "ditching effect", during radiation, was not considered to be a "rebound" from the irradiation artifact (ditching), since the longer the radiation period, the shorter the duration of the plateau phase. Finally, the results here pointed clearly to a metabolic effect on brain tissues by ionizing radiation and were not simply a physical disruption of the anatomical components of the cells. According to Warburg (28), cell cancer is basically an abnormal respiratory phenomenon. Therefore, it is hoped that the data presented here will provoke future biochemical studies on radiation carcinogenesis as outlined in Warburg's concept.
SUMMARY

The effects of X-irradiation ranging from 40 r to 16 Kr on the oxygen uptake of frog brain tissue slices were determined in this work. A membrane-covered oxygen electrode method was used to determine tissue respiration. The mean $Q_{O_2}$ of the sham-irradiated tissues was $6.83 \pm .02$. At dosages of 40 r to 320 r, a significant and sustained decrease in oxygen uptake occurred. The pre-irradiated tissues exhibited a mean rate of oxygen uptake of 100 $\mu$l s $O_2/100$ mg wet wgt/hr, whereas the inhibitory rates ranged between 70 and 80 $\mu$l s $O_2/100$ mg wet wgt/hr immediately (within 30 minutes) following X-irradiation. At dosages between 400 r and 16 Kr, a multiphasic response consisting of a momentary decrease was observed followed by an increase in respiration lasting between 90 and 120 minutes post-radiation. Recovery to control rates was noted in several experiments using the higher dosages.

The data clearly indicated a multiphasic, dose-dependent, and metabolic effect of ionizing radiation on
frog brain tissues, as opposed to a purely physical effect on the cellular integrity of membranes or macromolecules. The study also demonstrated the efficacy and use of the potentiometric determination of oxygen uptake in tissues before, during, and following irradiation.
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