Edited by

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A. HOWARD

Current Topics in RADIATION RESEARCH

VOLUME I

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FOREWORD

It is the inspiration and experience of individuals which leads to progress in science. In radiation research a general advance relies also on a variety of disciplines and their interrelation. We felt that a real contribution to radiation research could be made by inviting individual workers to summarize their subjects from their own point of view, free from the requirements of handbook or review articles. We believed that this would help authors to cut across the traditional frontiers of their parent disciplines, and would encourage a measure of spontaneity.

We are grateful to the contributors for the thought and care which went into their chapters, and to our publishers for their willing co-operation and speed in producing the present volume.

Manchester, August 1964. The Editors

CONTENTS

I

NEW LIGHT ON RADIATION BIOLOGY FROM ELECTRON SPIN RESONANCE STUDIES

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CONTENTS

1. Introduction

At first glance one might feel tempted to put a question mark rather than a stop at the end of the title of the present contribution. A quotation from a fairly recent review on "Electron Paramagnetic Resonance in Biology" [ANDROES and CALVIN 1962] reflects a widely held view: "In the EPR[1] observations of the effects of ionizing radiation on essentially complete biological systems, there are few regularities . . . The ionization studies might be said to be suffering from too much detail — every system gives different un-interpretable results." It is, in fact, not easy to derive the regularities from the already vast literature in this specialized field nor to interpret the spectra. Nevertheless, there are quite a few regularities to be found and several recent publications offer very valuable interpretations of ESR spectra. We feel, therefore, that a certain justification exists for the claim that ESR studies have thrown some new light on radiation biology, particularly so when we take into consideration the general rate of progress of radiation biology. It is probably fair to say that during the last few years our understanding of the basic mechanisms involved shows little progress in view of the large amount of work done and published. Three reasons cause the slowness of progress: (i) The enormous diversity and complexity of effects produced by radiation in living material. The search for "*the* mechanism of *the* biological action of radiation" had to be given up a decade or two ago and it seems, in fact, somewhat doubtful if it will ever be possible to work out general laws or principles governing such actions. There certainly seems to be some truth in the statement quoted above. (ii) A pronounced lack of ideas concerning new ways of attacking the problems involved. (iii) A lack of new experimental methods and of promising analytical tools.

It is in connection with (iii) that the ESR technique brought improvement. Available for less than two decades and used to analyze radiobiological effects for less than one, ESR methods have certainly not solved all our problems. But they have led to the demonstration of some quite general and regular phenomena to be described below. Admittedly these regularities, once established, may seem somewhat trivial to those expecting more spectacular results. To those working

[1] Electron spin resonance (= ESR) or electron paramagnetic resonance (= EPR) are synonymous and stand for a method of spectrometry using electromagnetic waves in the centimeter range.

in the field they were a little unexpected and very much so were the interpretations which it is possible to work out (quite at variance with the above quotation) for some of the spectra.

Two more remarks seem appropriate before entering the discussion of our subject. It is not our intention to list the relevant publications numbering about 150 in the form of a written-out card index. We aim rather at bringing out general principles and exemplifying these by experimental results. Consequently, our contribution takes the form of a critical analysis. Completeness is attempted in the list of references but not in the text where it will not be possible to mention all the detailed findings. We sincerely hope the investigators concerned will forgive our way of dealing with the material.

Finally we should like to emphasize that in the present contribution we take "radiation biology" to comprise the biological actions of ionizing radiations only. The various effects caused by ultraviolet, visible and infra-red light are now usually classed as "photobiology". Hence the very numerous and important ESR studies in the field of photobiology and particularly of photosynthesis will not be dealt with here.

2. Some reasons for applying ESR methods to radiobiology

There is no need to give another description of the ESR technique here. For an elementary introduction ZIMMER [1961] might be consulted. Excellent surveys on the physical principles underlying the application of ESR to biology are given by ANDROES and CALVIN [1962] and by BOAG [1963]. Several monographs are available describing every aspect of the physical theory and of experimental methods [GORDY, SMITH and TRAMBARULO 1953; INGRAM 1955, 1958; BLYUMENFEL'D, VOEVODSKII and SEMENOV 1962; SCHOFFA 1964].

For the purpose of the present discussion we can limit ourselves to stating that ESR as usually applied to radiobiology is a kind of absorption spectrometry using "light" of a wavelength in the centimeter region. This kind of absorption spectrometry has a marked advantage over the more usual types of spectrometry using ultra-violet, visible, or infra-red: the ESR spectrometer looks through many materials without specific absorption and sees only certain kinds of electrons such as occur in free radicals, in certain ions, in electrically conducting metals, and in rare states of electronic excitation. Of these, only free

radicals need concern us here. The others will either not be produced by radiation in biological material, or else will give rise to spectra that can easily be distinguished from the spectra of free radicals [cf. ZIMMER, 1959, 1961].

The ESR method is therefore used in radiobiology mainly as a convenient tool for determining the production, concentration and decay of free radicals in living material. Its convenience rests on its being virtually undisturbed by surrounding material, on its high sensitivity of detection and on its non-destructiveness: wavelength and power of radiation used are such as to exclude the production of any damage in the specimen under investigation.

Moreover, an analysis of number, intensity, and form of the lines in an ESR spectrum can, in principle, be used to identify the type of free radical present in a specimen. By extending the observations over some interval of time, changes in the spectrum, if they occur, may also be used to elucidate reactions which the free radicals originally formed by radiation may undergo with each other or with radicals present in the surrounding material.

For anybody familiar with the development of radiation biology during the past 40 or 50 years the reasons for applying ESR to this field of research will be evident from the foregoing remarks. If one is at all interested in the first steps of the chain of reactions started by the absorption of radiation and eventually leading to the biological effects under study, ESR has many advantages over the earlier physical and chemical methods. In fact, ESR gives a much better chance than earlier techniques to study some aspects of the physicochemistry of irradiated biologically important molecules in their natural environment as well as *in vitro*. Though ESR spectrometry is far less hampered by the influence of surrounding material than other kinds of spectrometry, it still suffers from one severe limitation: using the methods presently available it can only with difficulty be applied to specimens in aqueous suspension or containing a large amount of water[1] (COOK and MALLARD,

[1] After completion of the manuscript a paper by R. F. COOK and L. G. STOODLEY appeared: An electron-spin-resonance spectrometer for use while irradiating wet biological systems. (Internat. J. Radiat. Biol. **7**, 155). It describes a low frequency ESR spectrometer operating at 300 Mc/sec instead of the usual 10 000 Mc/sec and making it possible to obtain spectra from specimens containing considerable amounts of water. The application of this technique, however, appears to be somewhat limited by its inherently lower sensitivity which can be compensated for only by using much larger samples.

1963]. Another limitation is the time needed to take a spectrum which is inversely connected to sensitivity of detection. This means that fast radical reactions cannot be studied at the low concentrations of particular interest in radiobiology. Nevertheless the application of ESR to radiobiological research seemed promising from the very beginning and has indeed been rewarding, as we hope to show.

To facilitate reading the spectra shown in the following sections we should like to draw attention to two peculiarities of experimental procedure. For purely technical reasons (i) most of the commonly used ESR spectrometers record the first derivative of the spectrum instead of the spectrum itself, and (ii) the abscissae of the ESR spectra nearly always give magnetic units instead of units of wavelength as used in optical spectrometry. Because of (i) one integration is required in order to obtain the spectrum from the record produced by the spectrometer. A second integration furnishes the number of absorbing centres (free radicals), which is proportional to the area under the spectrum just as in optical spectrometry. In accordance with (ii) line widths and distances between lines are generally stated in magnetic units, not in units of wavelength nor as wave numbers per centimeter.

3. ESR studies on irradiated "complete" biological objects (excluding experiments on phage)

ESR absorption in a "complete" biological object after X-ray treatment was first demonstrated in experiments by ZIMMER, EHRENBERG and EHRENBERG [1957]. These early studies on barley seeds together with the earlier investigations on biochemicals [COMBRISSON and UEBERSFELD 1954; GORDY, ARD and SHIELDS 1955] already made it possible to draw attention to several regularities which could be shown by subsequent work to hold in a rather general way for other biological materials, and for other kinds of ionizing radiation as well.

In our opinion these regularities are up to now the most important result of the very numerous ESR studies on "complete" biological systems. Moreover, it is convenient to use these regularities as a starting point for an attempt at surveying the relevant literature. Naturally, we shall limit ourselves here to discussing investigations and results directly connected with problems of radiobiology. Findings more closely related to other fields of research (such as the physics and chemistry of the solid state) will have to be left aside however interesting they

may be. At this stage, we are concerned with the following 6 regularities:

3.1. IONIZING RADIATION PRODUCES CENTRES OF ESR ABSORPTION IN SUBSTANCES OF BIOLOGICAL IMPORTANCE (BIOCHEMICALS). THE CENTRES OF ESR ABSORPTION PRODUCED MUST, BECAUSE OF THEIR PROPERTIES, BE REGARDED AS FREE RADICALS IN THE MAJORITY OF CASES

This point is well brought out in a large number of papers[1]. There seems to be no point in listing the biochemicals tested in these investigations: they include many classes of compounds such as amino acids, proteins, nucleic acids and their constituents, fats, lipids, sugars, vitamins, enzymes and hormones. Very rarely one finds in the literature a statement that *no* ESR absorption was detectable in a certain material after irradiation. We tested some of these substances (for other reasons) and could usually show ESR absorption to occur though the energy (radiation dose) required to form an absorbing centre may occasionally be much higher than in the average case. We shall return later to the question of free radical yield per unit of absorbed radiation energy. In

[1] (BLYUMENFEL'D and KALMANSON, 1957, 1958, 1961; BOAG and MÜLLER 1959a, 1959b; BOGLE, BURGESS, FORBES and SAVIGE, 1962, BOX and FREUND, 1959; BOX, FREUND and LILGA, 1961; COMBRISSON and UEBERSFELD, 1954; DORLET, VAN DE VORST and BERTINCHAMPS, 1962; DREW and GORDY, 1963; DUCHESNE, 1962a, 1962b, EHRENBERG, EHRENBERG and ZIMMER, 1957; FRÄNZ and RANDOLPH, 1963; GORDY, 1958, 1959; GORDY, ARD and SHIELDS, 1955a, 1955b; GORDY and REXROAD, 1961; GORDY and SHIELDS, 1958, 1960, 1961; HENRIKSEN, 1961, 1962a, 1962b, 1962c, 1962d; HENRIKSEN and PIHL, 1960; IMAI, 1961; KALMANSON and BLYUMENFEL'D, 1958; KATAYAMA and GORDY, 1961; KIRBY-SMITH and RANDOLPH, 1960; KOCH and FRÄNZ, 1963; KOCH, FRÄNZ and MARKAU, 1962; KÖHNLEIN, 1962, KÖHNLEIN and MÜLLER, 1962; KURITA, 1962; KURITA and GORDY, 1962a, 1962b; LÖFROTH, EHRENBERG and EHRENBERG, 1961; MCCORMICK and GORDY, 1958; MIYAGAWA and GORDY, 1960; MÜLLER, 1962a, 1962b, 1963a, 1963b; MÜLLER, KÖHNLEIN and ZIMMER, 1963; NIELSEN and RASMUSSEN, 1962; POHLIT, RAJEWSKY and REDHARDT, 1961; PRYDZ and HENRIKSEN, 1961; PULATOVA, 1962; PULATOVA, ROGULENKOVA and KAYUSHIN, 1961; RAJEWSKY and REDHARDT, 1962a, 1962b, 1963a, 1963b; RANDOLPH, 1961; RANDOLPH and PARRISH, 1958; REXROAD and GORDY, 1959; ROTBLAT and SIMMONS, 1963a, 1963b, 1963c; SCHIRMER and SOMMERMEYER, 1962; SHEN, BLYUMENFEL'D, KALMANSON and PASYNSKII, 1959; SHIELDS and GORDY, 1958, 1959; SIMMONS, 1962; SOMMERMEYER and SCHIRMER, 1963; USATYI and LASURKIN, 1962; VAN DE VORST, 1963; VAN DE VORST, VAN DER KAA, DEPIREUX, DUCHESNE and BERTINCHAMPS, 1960; VAN DE VORST and WILLIAMS-DORLET, 1963; ZIMMER, 1959; ZIMMER, KÖHNLEIN, HOTZ and MÜLLER, 1963).

view of the many ESR spectra which we shall need for discussing more specific problems we see no reason for giving any examples here.

3.2. IONIZING RADIATION PRODUCES CENTRES OF ESR ABSORPTION IN "COMPLETE" BIOLOGICAL SYSTEMS. BECAUSE OF THEIR PROPERTIES THERE IS LITTLE DOUBT THAT IN THE MAJORITY OF CASES THESE CENTRES ARE FREE RADICALS

Fewer investigations support point 3.2, but the evidence is still ample[1]. Obviously, two main reasons are responsible for the comparatively small number of experiments related to point 3.2: the difficulty (mentioned above) of conducting ESR studies with systems containing much water, and the wide-spread view that further work of this kind holds little promise. We readily agree with this view as far as "ordinary" biological objects are concerned such as seeds and spores. With these, in fact, there is little hope of ever finding out in which of the morphological sites or biochemical constituents the radiation-produced ESR absorbing centres reside that lead to the biological effect under inves-

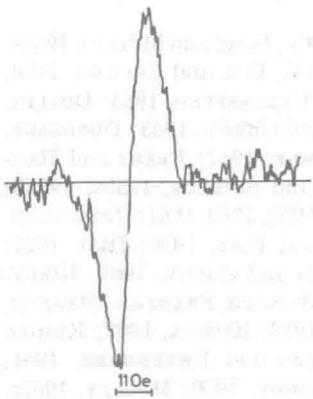

Fig. 1. Recorded curve of the first derivative of ESR absorption in barley embryos after irradiation with X-rays in air of known water content [ZIMMER, EHRENBERG and EHRENBERG 1957].

[1] (BHASKARAN, 1964; BHASKARAN and KÖHNLEIN, 1964; CONGER, 1961; CONGER and RANDOLPH, 1959; COOK and WHIFFEN, 1962; EHRENBERG, 1961; EHRENBERG and EHRENBERG, 1958; EHRENBERG, EHRENBERG and LÖFROTH, 1962; EHRENBERG and ZIMMER, 1956; FAIRBANKS, 1957; INGRAM, 1955, 1958; KLINGMÜLLER, 1961a, 1961c; KLINGMÜLLER and SAXENA, 1959; KOLOMIITSEVA, 1963; KOLOMIITSEVA, KAYUSHIN and KUZIN, 1963; MÜLLER, 1963a, 1963b; MÜLLER and ZIMMER, 1959, 1961; POWERS, 1961a, 1961b; POWERS, EHRET and SMALLER, 1961; POWERS, WEBB and EHRET, 1960; RANDOLPH and HABER, 1961; SINGH, VENKATERAMAN, NOTANI and BORA, 1963; SMALLER and AVERY, 1959; ZIMMER, 1959, 1961, 1962a, 1962b; ZIMMER, EHRENBERG and EHRENBERG, 1957).

tigation. However, the situation is different in at least one class of biological entity: bacteriophage. With this material the investigation can be carried far beyond a statement of the production of free radicals by ionizing radiation within the entity, as will be shown in subsequent sections.

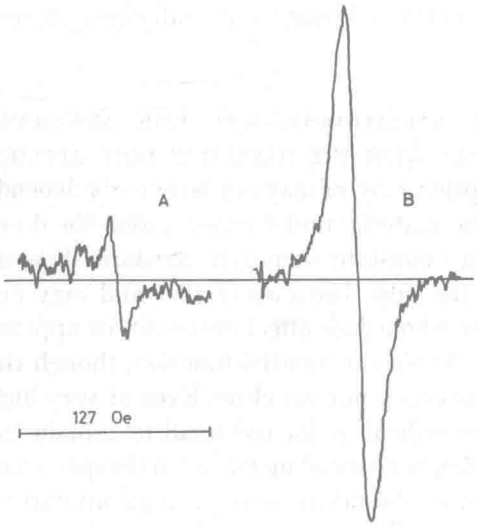

Fig. 2. First derivative of ESR spectrum in Seeds of *Agrostis stolonifera* equilibrated with air of 3.1% water content. A: before irradiation, B: after irradiation, measured at half sensitivity of spectrometer [EHRENBERG and EHRENBERG 1958].

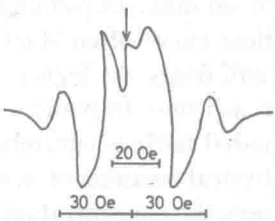

Fig. 3. Second derivative of ESR spectrum in spores of *B. megatherium* irradiated anaerobically at 313°K to a dose of 4 Mr and measured anaerobically at 77°K [POWERS, WEBB and EHRET 1960].

Figures 1 to 3 illustrate the kind of ESR spectra obtained from three biological materials frequently used in the studies cited above: (i) barley embryos (i.e. barley seeds after removal of much of the starch by dissection), (ii) seed of the grass *Agrostis stolonifera* and (iii) spores of *B. megatherium*. The spectra usually show little detail (hardly any indication of hyper-fine structure) unless the experiment is done under special conditions (fig. 3). Normally the spectra of the numerous kinds of constituent molecules superimpose to give only one fairly wide absorption line, though many of the constituents if taken alone show very pronounced hyper-fine structure. The spectra obtained in studies on other seeds such as wheat, broad bean etc. are very similar to those

given in figs. 1 and 2. Another point is well brought out in fig. 2: the existence before irradiation of an ESR spectrum very similar to, though of far smaller intensity than the one found after irradiation. The same observation is made in all living biological material. It is due to the metabolic processes always going on, many of them by means of free radical mechanisms. This "natural background" which must always be tested for, has of course to be subtracted from the overall effect observed after irradiation.

3.3. THE CONCENTRATION OF RADIATION-INDUCED ESR ABSORBING CENTRES GENERALLY INCREASES WITH THE RADIATION DOSE APPLIED

The increase of ESR absorption may or may not be linearly dependent on dose. Depending on the material under investigation the dose-effect curve often starts with a constant or nearly constant slope at small doses. At higher doses the slope becomes smaller and may approach zero. In many cases the whole dose-effect curve can be approximated fairly accurately by a simple exponential function, though the physical meaning of such a function is not yet clear. Even at very high doses the concentration of free radicals is far too small to explain the decrease of slope by a depletion of unchanged molecules in the specimen: i.e. the dose-effect curves must not be taken to mean single hit curves. In some cases, when spectra of different shape are observed at high doses, the dose-effect curve is even more difficult to interpret and may have no meaning at all. Such changes in shape are usually due to secondary reactions of the radiation-produced radicals giving rise to radicals of another type.

Again there is quite a number of studies supporting point (3) in biochemicals as well as in "complete" biological systems[1]. As an example of the kind of dose-effect curve obtained, fig. 4 shows the dependence on X-ray dose of the concentration of free radicals in barley embryos. The curve does not start from the origin because of the con-

[1] (BOAG and MÜLLER, 1959a; CONGER and RANDOLPH, 1959; EHRENBERG and FHRENBERG, 1958; HENRIKSEN, 1951, 1962a, 1963c; HENRIKSEN and PIHL, 1960; HENRIKSEN, SANNER and PIHL, 1963a; KIRBY-SMITH and RANDOLPH, 1961; KÖHNLEIN, 1962, 1963; MÜLLER, 1962a, 1962b, 1963a, 1963b; MÜLLER and ZIMMER, 1959, 1961; NIELSEN and RASMUSSEN, 1962; PRYDZ and HENRIKSEN, 1961; RAJEWSKY and REDHART, 1962b; RANDOLPH and HABER, 1961; ROTBLAT and SIMMONS, 1963a, 1963b, 1963c; SCHIRMER and SOMMERMEYER, 1962; ZIMMER, 1959, 1961, 1962a; ZIMMER, EHRENBERG and EHRENBERG, 1957; ZIMMER, KÖHNLEIN, HOTZ and MÜLLER, 1963).

centration of free radicals already present in the sample before irradiation. In addition, fig. 4 gives some indication of another very general phenomenon: dependence of detectable radical concentration on additional parameters during irradiation (air or nitrogen in the present case). Several other dose-effect curves will be given in later sections.

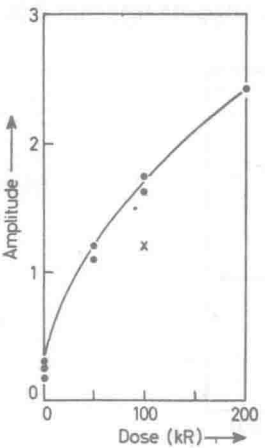

Fig. 4. Amplitude of the first derivative of ESR spectra in barley embryos *versus* dose of X-rays. Irradiation and measurement at room temperature in air • and in nitrogen × of equal and known water contents [ZIMMER, EHRENBERG and EHRENBERG 1957].

3.4. THE DETECTABLE CONCENTRATION OF RADIATION-PRODUCED ESR ABSORBING CENTRES DEPENDS ON TEMPERATURE AND WATER CONTENT OF THE SPECIMEN AS WELL AS ON THE GAS SURROUNDING IT[1]

An example is already given in fig. 4.

3.5. IN MOST CASES, THE DETECTABLE CONCENTRATION OF RADIATION-PRODUCED ESR ABSORBING CENTRES DECREASES COMPARATIVELY SLOWLY AFTER IRRADIATION

Because of this finding a view widely held in radiobiology had to be given up: absorption of radiation by biological materials does not

[1] (BHASKARAN, 1964; BHASKARAN and KÖHNLEIN, 1964; CONGER, 1962; CONGER and RANDOLPH, 1959; DUCHESNE, 1962a; EHRENBERG and EHRENBERG, 1958; EHRENBERG, EHRENBERG and LÖFROTH, 1962; EHRET, SMALLER, POWERS and WEBB, 1960; EIDUS and KAYUSHIN, 1960; HENRIKSEN, 1962c, 1963a, 1963b; HUNT, TILL and WILLIAMS, 1962; KIRBY-SMITH and RANDOLPH, 1961; KLINGMÜLLER, 1961a, 1961b, 1961c; MÜLLER, 1962a, 1962b; MÜLLER and ZIMMER, 1959, 1961; PATTEN and GORDY, 1960; POWERS, 1961a, 1961b; POWERS, EHRET and SMALLER, 1961; POWERS, WEBB and EHRET, 1960; RANDOLPH and HABER, 1961; SPARRMAN, EHRENBERG and EHRENBERG, 1959; ZIMMER, 1959, 1961, 1962a, 1962b; ZIMMER, EHRENBERG and EHRENBERG, 1957).