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Title INVESTIGATION OF FACTORS INFLUENCING RADIATION RESISTANCE OF MICROCOCCUS RADIODURANS.

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Age dependent fluctuation of radiation resistance exhibited by Micrococcus radiodurans was investigated. The fluctuation observed in cells grown in synthetic media was less than ten percent during 44 hours of growth. The cells harvested nine hours after inoculation were the most radiation resistant. The wet mass and DNA contents of these cells were low. When the culture was "shifted" from the synthetic media to tryptone-glucose-yeast extract media, the survival dropped from 12 to 4 percent but it rebounded to 15 percent in the subsequent 60 minutes. The DNA content of these cells was low in comparison to cell mass.

Alkyliodides, 2,4-dinitrophenol, diphenylamine, malonic acid, maleic acid, ethylenediaminetetraacetate, N-ethylmaleimide and iodoacetic acid were investiaged for their radiation enhancing effects on resting M. radiodurans cells in buffer. Among them, iodoacetic acid (IAA) was the most effective.

IAA, at a non-toxic level of 100 uM, reduced the survival by 1,000 fold at all levels of radiation. This radio-toxic effect was observed only when cells were irradiated in the presence of IAA.

IAA also enhanced radiation bleaching of carotenoid pigments in the cells and produced blue-violet color upon irradiation with starch.

The starch-IAA mixture was developed as a chemical radiation dosimeter. The range of this dosimeter was from 30,000 to 500,000 rads.

INVESTIGATION OF FACTORS INFLUENCING RADIATION RESISTANCE OF MICROCOCCUS RADIODURANS

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INVESTIGATION OF FACTORS INFLUENCING RADIATION RESISTANCE OF MICROCOCCUS RADIODURANS

INTRODUCTION

The action of ionizing radiation on the biological system is characterized by the high energy and the rapidity of its deposit. The biological mechanism operates under a rigidly controlled system involving a narrow range of temperature variation and a small amount of energy turn over (33, p. 95). Ionizing radiation, therefore, exposes a cell to an extremely high energy field bringing about a profound disorder.

Radiation damage develops in steps (2, p. 1-5). The first or physical step includes exposure and energy deposit. This process takes only 10^{-16} seconds. The energy is deposited so rapidly that no biological counter action to prevent or modify this process takes place. The second, or the chemical process, involves either ionization or excitation of molecules in the cell or in the environment. This step may last about 10^{-5} seconds. The nature and the quantity of the radiation-induced chemical products of this stage depend on the type and energy content of the radiation (44, p. 79). This change may occur at the biologically essential site of the molecule. If the change was produced at the non-essential part in the molecule, it could still migrate to a vital site (29, p. 211-229).

Since the biological system contains water in large quantity, the radiolytic products of water inevitably participate in inactivating the cellular sites (44, p. 64). It is believed that the protective chemicals and oxygen exert their effects during this stage.

The biochemical stage which follows may take from seconds to hours. During this period, some of the molecular damage could be reversed without a permanent effect. The damage, however, may be fixed and develop into biochemical lesions. Once established, the damage becomes irreversible and if it is not eliminated by the metabolic processes, it could result in injury which might eventually kill the cell. The latter process is called a biological step and it may last from hours to days. The death of a portion of the cells in a multicellular system, however, does not represent the death of an organism unless the damage is so great that the lost cells can not be compensated for. This recovery depends on the regenerative capacity of an organism. The regenerative ability of an organism may be influenced by numerous factors.

Bacteria and other unicellular organisms are advantageously used as test organisms to study the radiation effects on the cell. This not only enables us to observe the radiation effects at the cellular level but also to reduce the time and cost of the experiment owing to the relative simplicity of their cultivation. The advantage gained, however, is greatly reduced when we consider that individual cells in

the culture can not be examined with a reasonable degree of accuracy and that what we observe is the population dynamics of a rather highly concentrated group of cells.

Radiation effects on a bacterium are commonly determined by noting the ability of an irradiated cell to multiply. If the cell fails to develop into a colony on a solid media, it is considered dead. Death by this criterion does not preclude all biological functions and an apparently sterile culture may still respire (8, p. 97). Therefore, inactivation rather than death is a more descriptive and preferred term.

Although it is usually assumed that the changes in the individual cell can be observed on a population, certain precautions are necessary when translating these observations at the cytological level. A bacterial culture may contain a genetic or a physiological mixture of heterogeneous cells.

Studies of bacterial populations, nevertheless, have contributed significantly to our understanding of radiation damage. The target theory was proposed by a study of microbial populations (44, p. 72-78). This theory proposes that a direct hit or a point inactivation on a biologically essential site will result in an inactivation of the cell. Assuming that no other factors influence the viability of a cell, this relationship can be stated by the following mathematical formula.

$$\ln N = \ln N_o - kD$$

Where N equals the percent of the surviving cells after irradiation and N_o is equal to 100. D is the radiation dose in designated units and k is equal to the slope of the regression. The k also represents the reciprocal of the dose which gives 37 percent survival (D_{37}). It also represents the maximum dose beyond which a multiple hit of one target could result (44, p. 74; 2, p. 63-66).

Not all irradiated microorganisms exhibit this exponential death curve. Occasionally, the exponential curve is preceded by a shoulder which may be due to multiple targets requiring more than one hit to inactivate the cell. The slope sometimes is made of more than one exponential curve. This indicates that the population is radiobiologically heterogeneous. Bacterial inactivations by radiation follow the above three major patterns of exponential, sigmoidal and composite curves (30, p. 571-581). The establishment of such inactivation curves for a given bacteria, however, does not prove that the direct hit is the only cause for the inactivation.

Micrococcus radiodurans exhibits a sigmoidal inactivation curve (22, p. 131). This curve might be produced if more than one sensitive target is in the cell. If the nucleus is considered as the target, the tendency of this cell to contain an average of four nuclei and the inclination of the cells to be arranged as tetrads increase the number of targets per sensitive volume to 16 (22, p. 69 and 81-83).

If this were the only differential characteristic of this organism responsible for its radiation resistance, the shoulder of the sigmoidal curve should extend further; but the slope of the curve should be the same as that for non-resistant bacteria. It was found, however, that the radiation inactivation curve of this organism was characterized by a gradual slope rather than by an extended shoulder (21, p. 378).

The first part of this thesis deals with an attempt to reduce the number of assumed targets (nucleus as determined by deoxyribinucleic acid content) in order to determine the extent of radioresistance which could be attributed to this aspect of cellular physiology.

The investigation of chemical agents which are primarily assumed to exert their effects on the cytoplasmic portion of a cell is reported in Part II of the thesis. The effects of the chemical agents, therefore, would be more apparent to the slope of the inactivation curve rather than to the shoulder.

Part III describes an originally developed chemical dosimeter using iodoacetic acid and starch as the indicator.

LITERATURE SURVEY

Radiation Sensitivity

The sensitivity or resistance to radiation describes the nature and the degree of cellular responses. There is no component in the cell which is immune to radiation damage. Radiation does not have a preference for any particular component in a cell. Although a given chemical bond such as the sulfhydryl linkage is constantly implicated as a radiation-sensitive site, its sensitivity derives from the inherent weakness in this biologically important bond and not from preference by radiation for this linkage.

The damages caused by common bactericidal agents are selective. Heat and antibiotics concentrate their effects on the protein and on the cell wall respectively. But the energy of radiation could be deposited in any cellular site as well as in its environment to produce a lethal effect.

The cell can only compensate for the damage produced; it can not react against radiation.

Radiation sensitivity varies from species to species and also among the different tissues of the same organism. Bergonié and Tribandeau (4, p. 983-985) have observed that the rat testicle cells showed increasing radiation sensitivities as the reproductive ability

and the mitotic period were prolonged. The least morphologically and functionally differentiated cells were also the most radiation-sensitive.

Stapleton (71, p. 357-362) has observed a similar type of sensitivity variation in bacteria. He showed an increase of X-ray sensitivity in Escherichia coli during the logarithmic growth phase. This increased sensitivity returned to the original level when the culture attained the maximum stationary phase. The most sensitive cell was also highly thermolabile during post-irradiation incubation.

Elliker and Frazier (25, p. 83-98) have shown that cells of E. coli harvested during the logarithmic phase were most thermolabile. The relationship implied between thermoliability and radiation sensitivity, therefore, appears to be a coincidence rather than a truly related phenomenon.

The cells at the earlier stage of growth (lag phase) are physically bigger and enzymatically more active (83, p. 147-186). The high resistance to radiation as well as to heat at this stage may be due to the excess regenerative capacities and increased ploidy. The nucleus in a cell has been shown to be the single most radiation-sensitive component (86, p. 558-563). A multinucleated cell, therefore, should be able to withstand a greater radiation dose.

Species variation in radiation sensitivity is the result of many factors. It is easily understood that the higher degrees of

specialization and the intercellular organization of multicellular organisms will cause them to show a greater radiation sensitivity than unicellular organisms do. Once this intercellular dependancy is broken, the individual cell requires a far greater radiation dose for inactivation. This was shown by Painter (56, p. 653-661) with tissue culture cells.

The acute radiation dose required to kill 50 percent of the original organisms in 30 days ($LD_{50/30}$) falls within the narrow range of 200 to 800 rads for mammals (46, p. 120). For microorganisms, the range extends from kilorads to megarads. The wide variation in radiosensitivity found among bacteria is not fully explained. Usually, the bacterial spores are the most radiation resistant. This phenomenon can be explained partially on the basis of metabolic rate as formulated by Bergonié-Tribandeau's law; nevertheless, the slow-growing Micobacterium phlei and Azotobacter agilis do not show marked radiation resistances (30, p. 571-581).

The radiation resistance exhibited by M. radiodurans is notable in that the organism is a non-sporulating coccus. The LD_{50} of this organism in buffer under atmospheric oxygen pressure is 500,000 rads. It does not appear to possess any unusual characteristic which may set it apart from other non-resistant bacteria.

Duryee et al. (23, p. 798-805) have observed that this organism preferred sulfur-containing amino acids to carbohydrates as

energy sources. Recently, the photosynthetic capability of this organism has been observed. It is anticipated that the understanding of these characteristics and the investigation of its radiation resistant reactions under controlled conditions may lead to an explanation of its radiation resistance mechanism.

Sensitive site. Despite the convincing demonstrations by Zircle (86, p. 558-563), Ord (55, p. 922) and others that the nucleus was the most sensitive component in the cell, nucleus inactivation could not be considered as a sole lethal effect of radiation on the cell.

The enzymatic inhibitors (40, p. 331), the chemical protective agents (58, p. 437-474) and oxygen (37, p. 466-478) all influence the cellular response to radiation.

The radiation-enhancing effect of malonic acid and maleic acid reported by Kiga et al. (40 p. 331-332) is explained on the basis of succinic dehydrogenase inhibition. The protective thiol compounds are also believed to protect cellular sulfhydryl groups from radiation inactivation (19, p. 13-21). These factors influencing the radiation response of a cell are known for their reactions with enzymes and other protein structures in the cytoplasm. If we accept the view that the nucleus is the prime radiosensitive site, the theories of protection or sensitization which assume the protein to be the sensitive target are no longer acceptable.

Bridges (10, p. 240) suggested that the protein influenced by the radiation action was nucleoprotein. Any effect produced on

nucleoprotein is equivalent to direct nucleus damage. He failed to demonstrate, however, why the nucleoprotein is peculiarly vulnerable to ratiation.

Death of a cell could be brought about by any number of damaging combinations. Apparently, the cytoplasmic constituents possess many spare parts while the nucleus has none. The observation of respiratory activity of non-viable cells may be a demonstration of this phenomenon (8, p. 97).

Target. The results obtained from irradiating dehydrated protein (27, p. 483-502) or from purified nucleic acid (13, p. 714-716) can not be extrapolated to an explanation of what might be happening in the living cell. Many workers violate or ignore this fundamental distinction a living system possesses over non-living matter. It is true that the vital theory for the life process is repudiated and any vital activity observed in vivo can be demonstrated in vitro. The fact is, however, that we do not know all these vital processes well enough to duplicate life in a test tube. As will be explained later, the cell can be inactivated by a considerably lower radiation dose than that required to inactivate protein or nucleic acid in vitro.

A simple explanation of radiation effect on a cell is given by the target theory. It assumes that a virus particle or a bacterial cell possesses a single sensitive target and chance hit by radiation will inactivate the target and subsequently the unit biological system.

Inactivation by this mechanism is influenced only by the number of targets, the target size and the radiation dose. There are no provisions for other factors which are known to influence the radiation effect on a cell. Contrary to our observations, temperature, oxygen content, environmental factors and the degree of hydration can not influence the radiation response of a cell according to the target theory (2, p. 77-78).

Alper (1, p. 573-586) proposed a modified target theory and tried to explain the oxygen effect on the basis of the target theory. She suggested that oxygen combined with the sensitive cellular target molecule prior to radiation exposure and so activated the target to radiation. Similarly, Pihl and Eldjarn (58, p. 463-465) proposed that the protective thiols combine with the sensitive sulfhydryl group, thereby shielding it from radiation damage.

It is very doubtful, however, that any target, so called because of its significance in the biological reactions, remains unaltered by such modification even though the combination was proposed to be reversible.

The paradox of these approaches is that everyone tries to isolate or identify the sensitive site or target as a cellular constituent. Although each of these constituents contributes to the vital processes of a cell, the living phenomenon is maintained by the dynamic balance of all the cellular reactions which are closely related to each other. The radiation effect upon these cellular dynamics

bears a far greater significance than that upon any given cellular component does.

Growth of Bacteria

Whenever a bacterium is inoculated into a new medium, it follows a definite pattern of growth. Immediately after inoculation, the culture undergoes a lag period. During this time, growth of the cell is manifested mainly by an increase in cell mass. Following the lag phase, the cells begin to divide exponentially and continue until the changes in the environment no longer are able to support the growth.

Balanced growth. The logarithmic phase of growth has been regarded by many workers as the most uniform and representative physiological state of a given bacterial species. The logarithmic phase, however, does not necessarily represent the balanced state (12, p. 579-584). Balanced growth is defined by Campbell (14, p. 263) as the increase of every cellular extensive property by the same factor for a given time interval. The logarithmic phase is balanced for the cell division, which is only one of many extensive properties of a growing cell. Nucleic acid content, total protein and radiosensitivity during the logarithmic phase are known to be in imbalanced states and each component increases by a different factor.

It is very difficult to obtain a completely balanced growth in

practice, for only those cells grown continuously for a considerable length could approach this state. Campbell (14, p. 271) suggested that a continuous cell division could be accomplished by a stepwise deoxyribonucleic acid (DNA) synthesis, i.e., the DNA content increases to a maximum level prior to the cell division and is reduced to a minimum immediately after the division. The rate of DNA synthesis, however, is most active when DNA content is at a minimum. If this undulating DNA synthesis operates, a continuous growth does not bring about the balanced state at any time during growth.

McFall and Stent (49, p. 580-582) have demonstrated that DNA synthesis in a cell is continuous. They exposed E. coli culture to P^{32} and stored it under freezing temperature. When aliquots were taken in given time intervals, the number of viable cells decreased in proportion to the rate of P^{32} decay. The cells were allowed to grow briefly in P^{32} so that only the cells actively engaged in DNA synthesis at the time of exposure could have incorporated P^{32}. They found that P^{32} was uniformly incorporated into all cells and argued that there was no difference in rate of DNA synthesis among cells initially exposed to P^{32}.

Synchronous growth. Synchronous growth is best defined as follows: At any given moment, every cell in the synchronous culture is doing exactly the same thing (14, p. 263). The complete synchronization of a culture is only a theoretical possibility. The degree of

synchrony is usually expressed by percent. The cells could be synchronized by subjecting a culture to basal conditions which would reduce the metabolic functions of all cells to a common minimum. Starvation, chilling and dark incubation of a photosynthetic bacteria achieve this purpose. By removing these conditions, it is expected that all the cells will initiate the identical physiological responses for a given time period.

The use of synchronous culture to study radiation effect on bacteria is advantageous in that observations made with the population approach what would be characteristic of a single cell. This procedure increases the reliability of experimental data; however, this point has either been overlooked or ignored by the majority of investigators.

Growth and radiation-sensitivity. The growth associated variation of radio-sensitivity has been inevitably linked with the cytological differentiations during growth. Stapleton (71, p. 359) suggested that the increased sensitivity during the logarithmic phase of E. coli was due to the reduced ploidy of the cell.

Bacteria contain 10 to 16 percent of their dry weight in nucleic acid, as compared to 4.3 percent in yeast (16, p. 130-133). The amount of DNA material fluctuates with the age of culture, the maximum content being obtained at the end of lag phase, followed by a steady decrease throughout the logarithmic phase. Mulay (52, p. 773)

suggested that the decline of DNA content during the logarithmic phase of E. coli was responsible for the increased radiation sensitivity. Similar findings have been reported for the mammalian cells.

Dewey and Humphrey (17, p. 503-530) have shown that the L-P 59 mouse fibroblast cell was most radiation sensitive during the active DNA synthetic cycle. They also showed that some of the damaged cells could divide but that a portion of the progeny eventually died. They suggested that the accumulation of abnormal genetic materials to these cells caused death.

Buetow and Levedahl (12, p. 579-584) analyzed the growth of Euglena gracilis and observed a steady decline of ribonucleic acid (RNA), protein and dry weight. DNA content, however, was remarkably constant during growth. The authors have shown the increasing X-ray sensitivity was best correlated with the decline of RNA, which was reduced to 37 percent of its original content following radiation exposure.

Sawada and Suzuki (65, p. 288-297), on the other hand, have shown that DNA synthesis in E. coli was more severely affected by ultraviolet (UV) radiation than RNA synthesis was. During the post-irradiation recovery stage, RNA increased sharply while DNA content remained constant.

The correlation between RNA and radiation sensitivity given

by Buetow and Levedal is erroneous. It is known that RNA synthesis precedes protein and DNA synthesis (12, p. 579). The decrease of RNA content observed by them is a mere reflection of retarded synthetic ability of extensively damaged cells. The RNA content determination may serve as an index for estimating the extent of cell damage; however, it can not be regarded as the sensitive element in a cell which determines the radiation sensitivity.

Radiation damage of DNA. Kaplan (39, p. 68-113) reported that E. coli became sensitive to UV and X-ray when 5-bromouracil replaced thymine in DNA. The degree of sensitization was proportional to the extent of replacement. The inactivation plot of such cells also showed a loss of shoulder which was characteristic of non-sensitized cells having a sigmoidal inactivation curve.

Erikson and Szybalski (26, p. 258-261) reported a similar sensitization on human tissue culture cells when halogenated uridine was incorporated.

It is believed that the sensitizing action of these analogs was brought about by inactivating the DNA to the extent that further inactivation by radiation could be achieved easily. Szybalski and Opara-Kubinska (75, p. 505-509) believed that the primary lethal effect of radiation is on DNA while all other cytological changes are the consequences of this damage.

Stuy (73, p. 56-65) observed the breakdown of DNA in

Haemophilus influenza and noted that oxygen enhanced the breakdown process. Since the role of oxygen was not explainable by the direct inactivation theory, he suggested that DNAse activation by radiation may have been the contributing factor.

The effect of oxygen on DNA synthesis was demonstrated by Pollard and Macauly (60, p. 120-125). They showed that irradiated E. coli did not take up as much P^{32} and S^{35} as the non-irradiated control and this reduced state of uptake did not recover rapidly when oxygen was present.

The effect of oxygen appears to be on DNA synthesis rather than on the preformed DNA. In any event, the damage produced to DNA could influence all other cytological functions. Pollard and Vogler (59, p. 109-119) proposed the sequence of radiation damage in the order of DNA, RNA, robosome, protein and lipid.

Radiation effects on DNA activities. The biological activity of DNA is conveniently measured by irradiating DNA in vitro and measuring its transforming ability. Stuy (74, p. 41-48) has shown that the transforming DNA from H. influenza carrying three antibiotics resistant markers gradually lost transforming ability as UV exposure was prolonged. He insisted, however, that the major cause of this phenomenon was not the direct inactivation of the markers, but the rejection by the recipient cells of the radiation-deformed DNA.

Hutchinson and Arena (38, p. 137-147) found that the

Pseudomonas DNA irradiated in air showed greater sensitivity to radiation. They also pointed out that cysteine reduced this sensitive response.

A significant point to be brought out here is that the radiation doses required to inactivate the transforming DNA in vitro ranged in millions of rads in contrast to the dose needed to inactivate a whole cell in vivo, which was in thousands of rads. This means that DNA, which is considered the most sensitive component in a cell, still requires a thousand fold more radiation than that needed to inactivate a cell before it loses its delicate biological functions.

Wilson (81, p. 230-236) showed that inactivation of DNA in vivo in Hfr cells of E. coli K 12 required nearly one million rads of gamma radiation before all recombination could be stopped. Since DNA is the sole donor component to get into an F^- cell, this study indicates that the recombining ability can remain after the donor cell has been inactivated.

The above evidences show that the direct inactivation of DNA by radiation can not be identified as the only lethal action that radiation produces on a cell.

Factors Influencing Microbial Inactivation by Radiation

Radiation. The biological effectiveness of any given type of ionizing radiation can be measured by the quantity of ions produced

in the irradiated matter (44, p. 79). By this criterion, alpha rays are more effective than beta or gamma rays. Alpha rays have a high linear energy transfer (LET) value; thus they produce a cluster of ions along their short path. An alpha particle is characterized by discrete energy determined by the source. For instance, alpha particles from U^{238} will all have 4.2 million electron volts (Mev) (79, p. 89).

Since LET is proportional to the square of charges and inversely proportional to the speed and the speed has an inverse relationship with mass, the mass of 7,296 and 2 plus charges give a high LET value for an alpha particle. Consequently, it travels only a few inches in the air and one thousandth of this range in water. The ions formed are so close together that the chance for their recombination is great. The secondary product in water is primarily hydrogen peroxide, which exerts its additional toxic effect on the cell.

The relative biological effectiveness (RBE) of alpha particles is ten times greater than that for beta and gamma rays; however, their limited ability to penetrate makes them effective only by internal emmission.

This limiation was employed rather advantageously by Zirkle (86, p. 558-563) for irradiating a selected portion of a cell, i.e., the nucleus or the cytoplasm, to demonstrate the relative sensitivities of nucleus vs. cytoplasm.

Beta particles possess a mass of one and a negative charge. The charge-to-mass ratio of beta particle is considerably greater than that for the alpha particle. The most obvious effect of this big charge is the wandering path the particle takes in matter. The biological effect of the beta particle is determined by the energy it carries, and in this respect, it is comparable to the gamma ray. The range of the beta ray is determined by its kinetic energy; for 4.2 Mev, its range is about 50 feet in air.

A gamma ray, on the other hand, consists of a packet of energy or a quantum, without charge or mass. A gamma ray, therefore, has the highest range among the three. In fact, there is no definite theoretical range for a gamma ray. The primary ions produced by beta or gamma radiation are so far apart from each other that hardly any recombination products are formed.

The biological effectiveness of any type of ionizing radiation is solely dependent on the total absorbed dose. The increased RBE for alpha can be attributed to hydrogen peroxide rather than to its direct radiation effect.

The relative effectiveness remains the same for gamma rays of different dose rates. Sinclair (70, p. 394-398) has compared 1.25 kilovolt potential (KVP) equivalent Cobalt-60, 200 KVP X-ray, and 22 million volt potential (MVP) X-ray for their effectivenesses on yeast LD_{50}, mouse $LD_{50/30}$, rat $LD_{50/30}$ and Fe^{59} uptake in rat

and chicken embryo. The result obtained indicated that the effects were reasonably comparable with respect to the total absorbed doses. The slightly higher effectiveness of 200 KVP X-ray may have been due to the skin absorption caused by back scattering.

Environmental factors. It was mentioned earlier that a biological system operates in a narrow range of temperature variations, within a constant degree of hydration and under a uniform environmental gas composition. Any drastic change of these factors, therefore, may affect the cellular physiology so severely that additional radiation effects can not be distinguished from them.

Dehydration effectively reduced the radiation sensitivity of many biological systems (50, p. 688-690). Stapleton and Edington (72, p. 39-45) showed that freezing reduced the radiosensitivity of E. coli, and Howard-Flanders and Jocky (37, p. 466-478) showed that the removal of oxygen also did. These effects can not be brought about without interfering with the normal cellular functions. Within these limits, however, no significant effect of temperature was reported (22, p. 101).

The variation of pH and the changes in mineral concentrations also affect the cellular responses to radiation. For M. radiodurans, however, no effect of oxygen, nitrogen or temperature between 0^{o} C and 20^{o} C and pH between 5 and 9 has been observed (22, p. 93, 101 and 110).

Chelation has been suggested by many workers as a possible mechanism for radiation protection. Bair and Hungate (3, p. 813) reported ethylenediaminetetra acetate (EDTA) increased radiation sensitivity of Saccharomyces cerevisiae. They noted that EDTA did not affect the viability when the cell was preincubated or EDTA was added later to the irradiated cell. Only when the cell was irradiated in the presence of EDTA, could the synergistic effect be demonstrated. Bacq et al. (78, p. 169) indicated that the chelating agent might bind the critical cellular site through heavy metal binding, thus reducing the chance for the site to be inactivated by free radicals. The most attractive hypothesis is presented by Brintzinger et al. (11, p. 468-470). They noted that the hydrogen peroxide and nucleotides reaction was catalized by heavy metals and removing the metals by chelation inhibited this degenerative process. It is a gross injustice, however, to identify the heavy metals as harmful elements. For many enzyme reactions metals are essential and by removing them, more harm than benefit will be brought to the cell.

Radiation Protective Thiols

The most extensively studied chemicals for radiation protection are the thiols. The effectiveness of these compounds have been demonstrated with mammals (48, p. 564-572) and with bacteria (43, p. 250-255). Survey of the investigations in this field, therefore,

should provide an understanding of the basic concepts followed by workers in the field of chemical protection.

Mercaptoethylamine (MEA), cysteine, and s-(2-aminoethyl) isothiouronium (AET) are the most thoroughly studied thiol compounds. The following discussion deals mainly with these chemicals.

Doherty et al. (19, p. 13-21) postulated that the action of thiols was that of accepting free radicals by sulfhydryl groups, thus sparing the essential sulfhydryl groups in the cell.

Bacq and Alexander (2, p. 469-475), on the other hand, believe that these chemicals are able to donate electrons to radiation oxidized molecules. This hypothesis, in effect, implies that the damage already produced on the cell is reversed by the protective agents.

In contrast to these views, Salerno et al. (63, p. 559-560; 64, p. 344) explain the actions of protective thiols, considering their reducing abilities. They pointed out that the degree of protection obtained with thiols was not any greater than that observed by removing oxygen.

Molecular requirements of the protective thiols. The protective thiols possess the following common features: (1) the length of the carbon chain is not more than three and usually two carbons in length, (2) the sulfhydryl group is present either in a free form or can be easily converted into the free form in a biological system,

and (3) the amino group must be present.

The above-mentioned protective mechanisms do not explain the requirement for the shorter carbon chain or the necessity of the amino group.

The requirements explained by Doherty et al. (19, p. 13-21) are very convincing. They postulated that the zwitterion formation between amino and sulfhydryl groups is essential for the stability of the free sulfhydryl groups. This zwitterion is then able to donate an electron to the cellular site in the place where the electron has been ejected by radiation. Furthermore, the zwitterion can still maintain its stability after donating an electron by resonance stabilization.

This hypothesis nicely explains why the length of the carbon chain is limited, because it becomes increasingly difficult to form a zwitterion if the length of the carbon chain increases. The amino group requirement is also explained by this hypothesis.

The mixed sulfide and disulfide hypothesis also explains this requirement. Pihl and Eldjarn (58, p. 463-465) proposed that the thiols combine with free sulfide groups of a vital molecular site and form a reversible disulfide bond. If an electron is ejected from the molecule by a direct hit, the disulfide linkage breaks down donating an electron to the hole. The disulfide linkage also donates an electron to HO. or HO_2: radicals produced in water by radiation.

By giving off electrons, the disulfide bond breaks, the thiols oxydize, leaving the essential cellular sulfhydryl group intact. This hypothesis explains the equal effectiveness of thiols to direct and indirect actions of radiation.

Practical aspect of chemical protection. In order for any chemical to have a practical protective value, the following pharmacological requirements must be satisfied (78, p. 17). The chemical must be easily administered into the animal, it must be distributed evenly in all tissue, it must not produce undesirable side effects or result in the accumulation of toxic effects, and finally, the margin of protection has to be reasonably great.

Unfortunately, the chemical protectors so far reported do not satisfy any of the above requirements. Most of thiols and other chemicals are toxic to the cell and the toxicity is sometimes closely related to the protective capability (78, p. 18). The margin of safety claimed by any of the protectors is not sufficient and it is highly doubtful whether any of these chemicals has any significant value.

Maisin and Doherty (48, p. 565) proposed a rule to the effect that the protective chemicals should at least increase $LD_{50/30}$ by 30 percent or increase the minimal lethal dose (MLD) to 150 percent. This is a small improvement in radiation tolerance. If $LD_{50/30}$ of a given animal was 500 rads, the minimum increase by above criteria

would be 650 rads. Not all the so-called protective agents even measure up to this minimum (58, p. 444-445, 446, 447, and 448) and consequently the term protection must always be qualified.

There is even a flat denial that the prospect for chemical protection is completely negative (76, p. 77). This may be so; for the protective compounds already reported, however, such denial may be regarded, at best, as too hasty. The problem of radiation protection needs a fresh approach. Unless we are able to bring out new chemical agents and approach the problem from an entirely different concept, the future of chemical protection is bleak indeed.

Radiation Sensitizing Compounds

The radiation sensitizing compound is defined by Bridges and Horne (8, p. 105) as follows. "A substance which enhances the effect of radiation without itself being toxic; or if it is toxic, the resulting effect should be more than the sum of the radiation effect and the agent's effect separately."

The sensitizing compounds may be classified according to the proposed mechanisms: (1) the radiomimetic agents, (2) the metabolic analogs, (3) the excess normal metabolites and (4) the sulfhydryl combining agents.

The above classification is by the author and because the information on the subject is limited, it must be regarded as

premature. Nevertheless, it will provide a basis for organization of the diverse and disorderly information under a unified heading.

Nitrogen mustard (55, p. 921-922) and halogenated pyrimidines (75, p. 508-509) injure the nucleus. Sensitization observed with this group of compounds is considered to be the result of the synergestic action of the chemical agents and radiation on the nucleus. The action of each element, however, is not simultaneous. The nucleus is already damaged somewhat by the chemicals prior to the radiation exposure.

Synkavite, a vitamin K analog, is another compound which produces a radiomimetic effect. Hanel et al. (31) reported that the radiation sensitivity of mice increased when irradiated in the presence of this agent. They also showed an excessive nuclear aberration in mouse liver when it was irradiated in the presence of synkavite. Similar sensitizing effects have been observed with synkavite in mammalian systems by many workers (2, p. 477-479); however, this agent was not effective in bacteria (42, p. 351-353; 36, p. 518-540).

Another synthetic vitamin K analog, vitamin K_5 is reported to sensitize E. coli, M. radiodurans and Pseudomonas fragi by Shehata (68, p. 78-85). Silverman et al. (69, p. 432-440) further investigated vitamin K_5 related compounds 4-amino-1-naphthol and 1-amino-2-naphthol. They found these compounds exhibited similar

sensitizing effects on E. coli and Staphlococcus feacalis.

An excessive amount of vitamins when administered to mice produced increasing incidences of anophthalmia to the fetus after the pregnant mice had been exposed to X-ray (85, p. 1219-1221). This is an example of a normal metabolite functioning as a sensitizer.

Radiation protection and sensitization are not opposing phenomena. This is exemplified by the fact that when an excessive amount of protective compound was given, sensitization resulted (78, p. 29).

The sulfhydryl enzyme inhibitors, malonic and maleic acids, were reported to enhance radiosensitivity of Sacch. ellipsoides (40, p. 331-332). This effect, however, was nullified by the addition of fumaric or aspartic acid.

Bridges (9, p. 467-472; 10, p. 232-242) has shown that N-ethylmaleimide, iodoacetic acid and p-chloromercuric benzonate sensitized E. coli and Pseudomonas species. The sensitization, however, was brought about by some of these agents only when oxygen was removed. The exact mechanism for the oxygen effect is not known. Bridges postulated that the reaction involving the sensitizer might have been competing with oxygen for the same sensitive site.

The sensitizing action of a sulfhydryl binding agent is postulated also by Bridges. He proposed that radiation produces more reactive sulfhydryl groups in the cell and they react with the binding

agents. He observed that a highly reactive sulfhydryl binding agent was also the most efficient sensitizer.

PART I. THE MODIFICATION OF RADIATION RESISTANCE BY CONTROLLING GROWTH CONDITIONS

Duggan et al. (21, p. 382) observed that the radiation resistance of M. radiodurans was a thousand times greater when the culture was 48 hours old than it had been at eight hours. There was no explanation and no further attempt has been made to analyze the nature of this variation.

It is possible that certain cellular constituents, which are responsible for the radiation resistance, might have undergone changes during growth. This part of the thesis will deal with investigations designed to study this observation.

Stapleton (72, p. 358) showed that the increase of radiation sensitivity of E. coli during logarithmic phase was due to the reduction of the average numbers of nuclei. Besides the classical demonstration of Zirkle (86, p. 558-563), who showed that the nucleus was the most sensitive component in a cell, Mortimer (51, p. 312-326) and Wood (84) have shown that the numbers of nuclei per Saccharomyces cerevisiae cell influenced the effectiveness of radiation.

Instead of counting the numbers of nuclei under the microscope, quantitative measurement of deoxyribonucleic acid (DNA) was made from the cells harvested at various growth stages, in order to observe the changes of this component during growth.

An investigation had already been made to correlate the radiation resistance with the pigment content of the cell (45, p. 26). It was found, however, that the pigment content did not parallel the radiation resistance.

Several growth-associated parameters, such as turbidity, viable count and percent survival, were determined along with the DNA content of each aliquot taken from a growing culture, and an attempt was made to correlate these values with radiation resistance.

Another approach was that of forcing the culture to change its physiological makeup while growing by subjecting the cells to different environments. The cells were grown in a culture medium of one composition and then abruptly changed to an entirely different medium (47, p. 45-52). When growing cells have been subjected to a rich medium and then are suddenly forced to grow in a poor medium, the process is called "shift down." The reverse process is called "shift up" (66, p. 592-606).

Only the "shift up" type of experiment was carried out because of its simplicity. This approach also eliminated the steps of centrifugation or filtration of the culture, which reduce the viability of the cell and introduce errors.

MATERIALS AND METHODS

Normal Growth

The culture. A 12 liter Pyrex bottle was fitted with an air sparger and stop cocks connected to tubes to regulate the air flow. An illustration is shown in Figure 1. The culture was continuously sparged with sterile air supplied by a small pump. When stop cock A was closed, the air pressure increased in the bottle causing the culture to overflow from the three-way stop cock B via tube b. Upon completion of sampling, the stop cock A was opened to release the pressure and the three way stop cock B was turned to let the sterile air from tube d replace the culture fluid remaining in tube c. A small portion of fluid remaining in area E was flushed out by repeating the sampling procedure and stopping before the culture had reached stop cock B. All contracts with outside air were made through the sterile cotton-filled tubes in order to avoid contamination during growth. A 200 ml aliquot of culture was withdrawn at a given time intervals and immediately centrifuged and washed twice with 0.05 M phosphate buffer. Following this procedure, the washed cells were resuspended in buffer and divided into volumes appropriate for DNA, turbidity and viable count. A portion of the cells in buffer was saved for future irradiation. Viability determination and

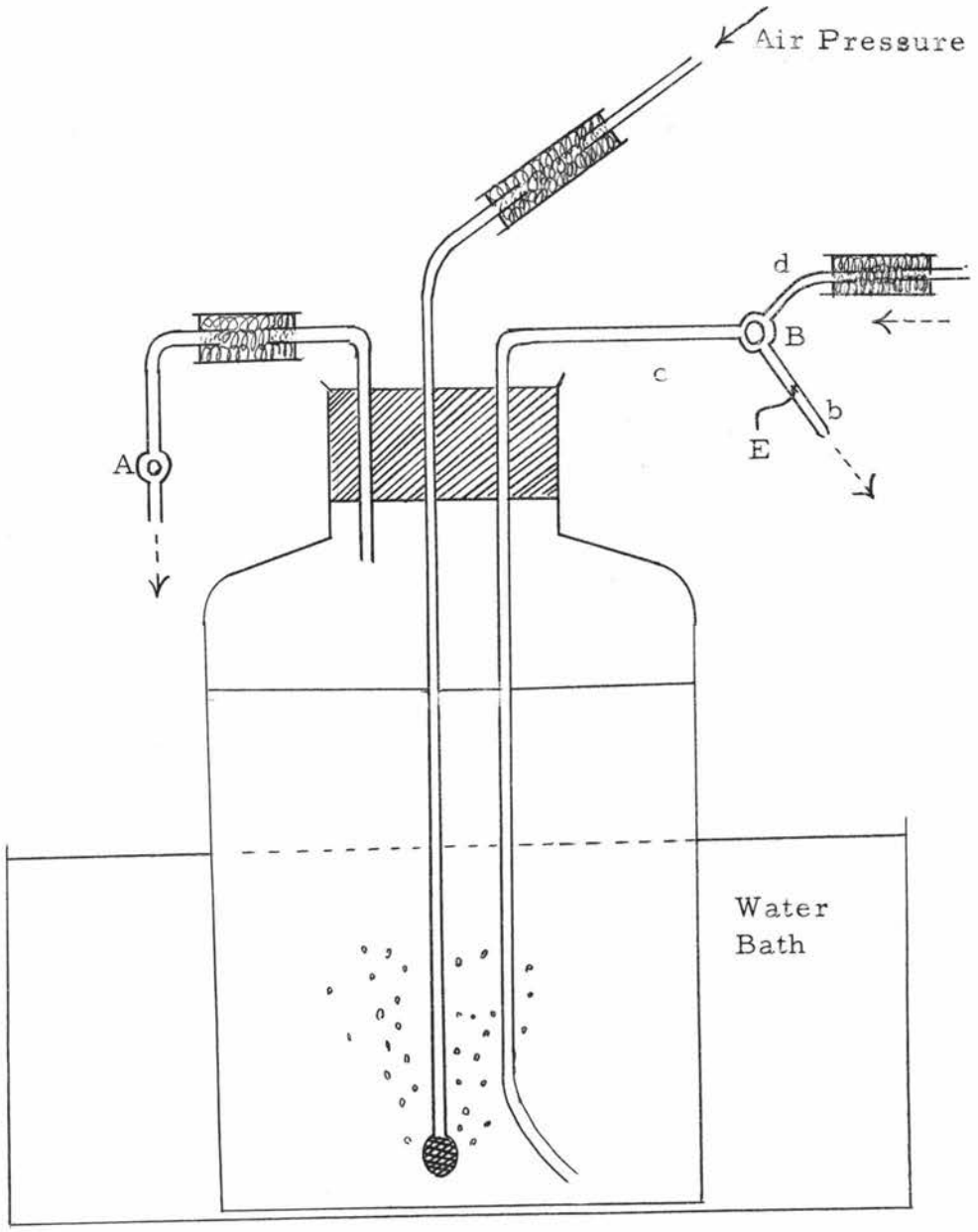

Figure 1. The culture tank.

irradiation were completed within 24 hours from the time the cells were harvested. No change of viability was observed during this time if the buffer suspension was kept at 4° C. The viability of the buffer-suspended cell did not change after storage at 4° C for four days.

DNA content determination. A 100 ml portion of the cells prepared as above was centrifuged and the resulting packed cells partially dried in a vacuum desiccator for 30 minutes to free them from excess water. They were then resuspended in 5 ml of physiological saline (0.85 percent NaCl) plus 1.0 ml of 70 percent perchloric acid. This was then heated at 70° C for 30 minutes.

The determination for DNA was made from the above supernatant according to the method described by Dische (18, p. 285-305). This measured the amount of omega-hydroxylevlialdehyde produced by the acid hydrolysis of deoxyribose. This developed a blue color in the presence of diphenylamine reagent(1). The intensity of blue color was determined by the optical density (O.D.) at 595 mu. This process was carried out by adding 1.0 ml of diphenylamine reagent to 1.0 ml of supernatant and heating it at 100° C for ten minutes. The color formed was stable at room temperature. When the

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(1) Doubly recrystalized in 70 percent ethanol. One gram of this was dissolved in 100 ml of gracial acetic acid and 2.75 ml of concentrated H_2SO_4 was added.

intensity of color was too great, appropriate dilutions were made with gracial acetic acid.

The DNA standard was prepared by dissolving commercial herring sperm preparation in warm 2 N NaCl solution.

Turbidity. Total protein in the sample was measured by the O. D. of a cell suspension in 0. 05 M phosphate buffer measured at 680 mu.

The "Shift Up"

A culture tank containing five liters of synthetic medium (SM) (62, p. 292) was inoculated with cells grown at 30° C on a shaker in 100 ml volumes. The size of inoculum varied between 2 to 20 percent depending on the experiment. When the growth reached the midpoint of the logarithmic phase, an equal volume of double strength (by concentration) tryptone-glucose-yeast extract medium (TGYM) was poured into the tank. The TGYM was prewarmed to 30° C before addition, in order to maintain a constant temperature during growth. Aliquots were removed from the tank prior to and after the shift time. The aliquots were treated in a manner similar to that described previously.

RESULTS AND DISCUSSIONS

The Growth

The cells grown in TGYM or in SM showed similar levels of radiation resistance. The growth characteristics, however, were markedly influenced by the media. The TGYM-grown cells reached the maximum growth phase faster as well as giving the higher yield. This comparison is made in Table 1.

The rate of growth was also influenced by the size of inoculum. The smaller the inoculum, the faster the growth rate. The maximum cell yield, however, was not influenced by the inoculum size and the maximum yield obtainable from SM was always less than that from TGYM.

DNA Content

The difference in growth rate was reflected in DNA content per viable cell. Table 3 shows the DNA contents of cells grown in TGYM and SM. In either medium, the maximum DNA content per cell was obtained during the lag phase and it declined gradually as the culture progressed through the logarithmic phase. The rate of decline was faster and more pronounced in TGYM.

The decrease in content of DNA is, to some extent, due to

Table 1. The Growth of M. radiodurans in TGYM and SM at 30° C.

Age of Culture (hours)	No. of Viable Cells Per Ml.	
	TGYM	SM
0	3.0×10^6	3.0×10^6
4	4.5×10^6	3.3×10^6
8	1.0×10^7	3.3×10^6
12	1.2×10^8	4.0×10^6
25	7.0×10^{10}	5.0×10^6
36	5.5×10^{10}	6.0×10^6
49	5.6×10^{10}	7.0×10^7
56	- -	4.0×10^7
61	- -	5.0×10^7
73	7.0×10^{10}	5.0×10^8
84	- -	1.4×10^9
97	7.0×10^{10}	2.0×10^9

Table 2. DNA Standard.

DNA(ug/ml)	O.D. at 595 mu
0	0.000
25	0.028
50	0.041
75	0.062
100	0.088
150	0.134
200	0.167
250	0.211(1)
300	0.245
350	0.308
400	0.330
500	0.374

(1) The O.D. from 250 ug/ml or more of DNA were measured from respective dilutions and multiplied to give the full values.

Table 3. DNA Content During Growth.

Age(hrs.)	O.D.^{595}	DNA(ug/ml)[1]	DNA(ug/cell)[2]
In TGYM			
4	-[3]	-	-
8	-	-	-
12	0.324	489.0	4.07×10^{-6}
25	1.128	1,671.0	2.39×10^{-8}
36	1.098	1,627.0	2.96×10^{-8}
49	1.098	1,627.0	2.91×10^{-8}
In SM			
4	-	-	-
8	0.080	110.0	3.33×10^{-5}
12	0.094	130.0	3.25×10^{-5}
25	0.093	130.0	2.60×10^{-5}
36	0.078	105.0	1.75×10^{-5}
49	0.534	800.0	1.60×10^{-6}
97	0.412	600.0	2.00×10^{-7}

(1) Determined from DNA standard (Table 2).

(2) DNA (ug/ml), divided by viable count (Table 1).

(3) Insufficient cells.

the reduction of ploidy, however, the minimum DNA content per cell may or may not represent the haploidal state. A recent electron microscopic study by Dr. R. G. E. Murray of the Faculty of Medicine, University of Western Ontario (personal communications) indicates that the cell divides in such a manner that each cell of the tetrad undergoes nuclear division prior to the cell division; therefore, a single cell always possesses four nuclei and frequently as many as 16 nuclei. According to this observation, M. radiodurans does not exist as haploid at any time.

The dry weight of 5 ml of 97-hours-old cells from SM was approximately 27 ug. The DNA content of this sample was 600 ug/ml (Table 3). The calculated percent DNA per dry weight was 11. This content lies within the 10 to 16 percent which was given as average DNA content of bacteria by Davidson (16, p. 130-133).

Table 3 shows that the amount of DNA per cell could be reduced to one-twentieth of that of the maximum during growth. This phenomenon could be explained by assuming that the numbers of nuclei or the sensitive unit per cell might take any number between 1 and 16. As indicated earlier, this possibility may not exist and the variation must be regarded as chiefly due to the cell size fluctuation during growth.

The role of polyploidy in increasing radiation resistance is not adequately explained by the target theory. Mortimer

(51, p. 312-326) irradiated polyploidal cells ranging from two to six targets and noted that only the diploidal cell was more resistant than the haploid. Contrary to the expectation, the radiation sensitivity increased as the ploidy multiplied. Wood (84) explained this on the basis of the increased target volume which accompanies the ploidal increase. This explanation, in effect, deviates from the basic assumption that each target is independent.

Growth and Radiation Resistance

The percent survival in Table 1 and Table 3 was not determined. The quantity of cells obtainable from these experiments was too small for determining all of the desired parameters. This limitation was most acutely manifested at the early stage of growth. The cells at this stage undergo active physiological changes and the measurements of DNA, turbidity, viable count and the survival count must be made. In order to do this, an extremely large quantity of cells has to be inoculated, sacrificing the subsequent growth rate. Such experiments were carried out using 20 percent inoculum. The results are shown in Table 4.

The number of viable cells increased to approximately twice that of the inoculum in 44 hours in SM. Although SM did not support growth as well as TGYM, the reproducibility of the experiment was better with SM. The results shown in Table 4 are the average of

Table 4. The Cellular Components in Relation to Radiation Resistance During Growth[1].

Age of Culture (hours)	Viable Count	Survivor[2]	Percent Survival	O. D. ^{680}	O. D. ^{680}[3] / V. C.	DNA (ug/ml)	DNA / cell[4]
0	2.00×10^8	-	-	0.390	1.95	330	1.65
4	1.77×10^8	1.51×10^7	8.53	0.392	2.21	315	1.77
6	2.08×10^8	2.43×10^7	11.68	0.488	2.34	370	1.77
9	2.37×10^8	3.60×10^7	15.18	0.520	2.19	416	1.75
20	3.58×10^8	4.20×10^7	11.73	0.600	1.67	416	1.16
24	3.20×10^8	4.40×10^7	13.75	0.632	1.97	430	1.34
30	3.60×10^8	3.00×10^7	10.71	0.684	1.79	450	1.84
44	3.10×10^8	3.00×10^7	9.67	0.680	2.19	432	1.39

(1) Grown in SM.
(2) After receiving 750,000 rads.
(3) O. D. at 680 mu was multiplied by 10^9 and divided by viable count to give relative mass per cell.
(4) DNA (ug/ml) was multiplied by 10^6 then divided by viable count to give relative DNA content per viable cell.

Table 5. The Changes of Cellular Constituents During "Shift Up."

Time from Shift Point (minutes)	Viable Count	Survivor[1]	Percent Survival	O. D. $_{680}$	O. D. $_{680}$[2] / V.C.	DNA (ug/ml)	DNA/cell[3]
-30	3.0×10^8	3.0×10^7	10	0.480	1.27	360	1.20
0	3.2×10^8	3.8×10^7	12	0.470	1.16	385	1.22
15	3.0×10^8	1.2×10^7	4	0.466	1.22	385	1.28
30	2.8×10^8	1.7×10^7	6	0.476	1.34	410	1.19
60	4.0×10^8	6.0×10^7	15	0.480	1.20	440	1.10

(1) After receiving 750,000 rads.
(2), (3) See Table 4 for explanation.

three separate runs.

Approximately ten percent of initial cells survived 750,000 rads. The fluctuation of survivals was between 8.53 and 15.18 percents. This represents only $\pm$6.5 percent variation of survivals during 44 hours of growth. The magnitude of the survival variation does not compare with the original observation of 1,000 fold reported by Duggan (21, p. 382).

The difficulty may have arisen because of the slow rate of growth in SM or because the cells in this experiment were irradiated in buffer while Duggan irradiated cells in a growth medium (TGYM).

Another observation made from these experiments was the correlation between relative DNA content and survival. It appears that the maximum survival is characteristic of cells having the least amount of DNA. These cells also had the least amount of cell mass (Table 4).

It is very difficult to explain this phenomenon on the basis of target theory or by the indirect radiation inactivation theory. The cells having more mass should be more resistant to radiation. They should have regenerative reservoirs and a greater multiplicity of sensitive targets. The observation contradicts all these expectations. The observation, therefore, could only be interpreted as meaning that the cells presenting the smallest targets are better able to survive radiation.

Changes in Radiation Resistance During "Shift Up"

Immediately after "shift up," the percent survival dropped from 12 to 4 percent followed by an increase of 15 percent in the subsequent 60 minutes (Table 5).

The turbidity and DNA increased and decreased simultaneously during normal growth; however, after "shift up," the decrease of DNA content was more apparent than the turbidity decrease.

The decrease of survival from 12 to 4 percent is significant, because all the measurements made after "shift up" had been multiplied by the factor of two to account for the doubled volume of medium. This treatment might have given slightly higher values for the determinations made at 60 minutes, because a fast growing culture can double its cell content in a relatively short time.

The results obtained from the "shift up" experiment also point to the fact that the cell containing the least amount of DNA shows the highest resistance to gamma radiation.

Limitations of the Experiments

The deductions made above must be weighed against the following limitations inherent in the experimental design.

First of these was the amount of cells needed to perform a reliable determination. A ten liter culture was used to achieve this

goal; however, a still larger volume of culture would have been more desirable.

A vigorous growth of cells is desirable, not only for large quantities of cells, but desirable also to amplify the magnitude of the variations during growth. The growth rate was sacrificed in order to obtain sufficient numbers of cells at the early stage of growth.

Secondly, the single culture tank was used in order to eliminate the errors inherent in the batch method. This modification improved the reproducibility of viable count. Nevertheless, a further refinement of this technique would be desirable if more precise data are to be obtained.

Finally, the "shift up" did not bring out the desired sharp contrasts in DNA and turbidity before and after the shift. The gradual transitions of DNA and cell mass contents may have been due to the media. Usually, both very rich and very poor media are used for "shift" experiments. The transfer from SM to TGYM may not have been drastic enough to induce an abrupt change on the cell.

Summary

The fluctuation in radiation resistance was not significantly great for the cells obtained at different ages of culture. Not more than a ten percent variation in survival was observed among the cells harvested over a growth period of 44 hours.

The age-associated factor, therefore, can not be the major contributing factor in the radiation resistance of M. radiodurans.

PART II. THE MODIFICATION OF RADIATION RESISTANCE WITH CHEMICAL AGENTS

The modification of growth conditions did not change the radiation inactivation pattern of M. radiodurans appreciably. Therefore, it was felt necessary to attempt a further modification on the cell by means of several known chemical sensitizing agents.

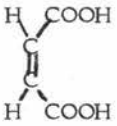
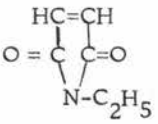
A wide variety of chemical agents have been reported to enhance the radiation sensitivity of bacteria (8, p. 96-115; 24, p. 231-296). Radiation resistance of M. radiodurans was also reported to be influenced by vitamin K_5 (68, p. 78-85).

MATERIALS AND METHODS

Chemical Agents

The descriptions of the chemicals are summarized in Table 6. All chemicals were dissolved in 0.067 M phosphate buffer except the alkyliodides and diphenylamine (DPA). These compounds were first dissolved in 95 percent ethanol and diluted with phosphate buffer. The final ethanolic concentration which came in contact with the cell was less than 0.01 percent. This amount of residual ethanol did not affect the cell in any detectable way. The alkyliodides could be dissolved in buffer; however, DPA could not be dissolved completely by this method.

Table 6. Chemicals Used for Sensitization Experiments.

Chemical	Structure	Abbreviation Used	Molecular Weight	Solubility[1] in H_2O	Source
Methyliodide	CH_3I	MI	141.95	$1.8/15^{\circ}$	Eastman Organic (E.O.)
Ethyliodide	C_2H_5I	EI	155.98	$0.4/20^{\circ}$	Matheson, Coleman & Ball
n-Propyliodide	C_3H_7I	PI	169.99	v. sl.	E.O.
Malonic Acid		MO	104.07	$138/16^{\circ}$	E.O.
Maleic Acid		ME	116.07	$79/25^{\circ}$	E.O.
N-Ethylmaleimide		NEM	125	v.s.	E.O.
Iodoacetic Acid	ICH_2COOH	IAA	185	v.s.	E.O.
2,4 Dinitrophenol	$C_6H_3(NO_2)_2(OH)\cdot 2HCl$	DNP	197.08	s.	E.O.
(Ethylenediamine) tetraacetic Acid Disodium salt	$HOOC-CH_2\ (NaOCCH_2)_2NCH_2CH_2N(CH_2C-ONa)CH_2COOH\cdot 2H_2O$	EDTA	372.25	v.s.	E.O.
Diphenylamine	$(C_6H_5)_2NH$	DPA	169.22	i.	E.O.

(1) Solubility according to Lange's Handbook of Chemistry. $1.8/15^{\circ}$ for example indicates 1.8 part by weight is soluble in 100 ml of H_2O at 15° C. v.s. = very soluble, v.sl. = very slightly soluble and i. = insoluble.

The Cell

The cells were cultured in SM at 30° C on a shaker. After 48 hours, the culture reached the terminal stage of logarithmic growth. It was then centrifuged, washed twice with phosphate buffer, and resuspended to give the proper concentrations of cells. This stage of growth produced the most consistant patterns of survival after irradiation.

Total Pigment Determination

The complete method for determining the carotenoid concentration has been reported elsewhere (45, p. 13). For the quantitative determination of the pigment, the irradiated cells were centrifuged and partially dried under vacuum for 30 minutes to free them from excess water. The cells were then resuspended in 20 ml of 10:10:3 mixture by volume of petroleum ether, acetone and absolute methanol and shaken vigorously for from five to ten minutes. This mixture was stored at 4° C for 24 hours to achieve the maximum pigment extraction. The solvent extract was then filtered through Whatman No. 1 filter paper to free it from the cellular residue. The O.D. of this solvent extract was measured at 475 mu, against a solvent blank to determine the pigment content of the original cell mass. All procedures involving the solvent extract had to be carried out in a

cold room.

Toxicity of Chemical

The cells and chemicals in different concentrations were mixed in 0.067 M phosphate buffer. The viable counts were made from this mixture at definite intervals to determine the time required for the chemicals to act. The viable count was made after the original mixture had been diluted from 10^5- to 10^6-fold in order to reduce the bacteria to countable numbers and to reduce the carry-over of chemicals on the plate to a negligible level. The colony count was made after incubating at 30° C for 48 to 52 hours.

Irradiation

The cells with different chemical agents in buffer were exposed to gamma radiation from a Cobalt-60 source having a dose rate of 300,000 roentgens per hour.

Viable counts were made as soon as all the vials had received the given radiation doses. The whole process required a total of four hours of irradiation and handling at room temperature. The controls were subjected to the identical conditions except for the radiation exposure. The plates were handled in a manner similar to that described under toxicity studies.

RESULTS AND DISCUSSIONS

The Toxic Levels of Chemical Agents

The alkyliodides, malonic acid (MO), maleic acid (ME) and 2, 4-dinitrophenol (DNP) were not toxic to the cells suspended in buffer at or below 1, 000 uM. No change in the viability of cells incubated at 4^{o} C for four days with the above chemical agents have been observed.

The ethylenediaminetetra acetic acid disodium salt (EDTA) and diphenylamine (DPA) exhibited slight inhibitions at 1, 000 uM; however, these were used for direct comparison with other agents.

On the other hand, iodoacetic acid (IAA) and N-ethylmaleimide (NEM) exhibited toxic effects at or higher than 350 and 150 uM respectively. There was a definite time of exposure required for both IAA and NEM to reach the maximum toxic levels. The toxicity increased progressively during 30 minutes of cell-chemical contact; however, after this period, no further increase of toxic effects was observed.

The progressive increase of toxicity with the length of exposure indicates that NEM and IAA are gradually incorporated into the cell. It is not clear whether this increase is due to an active accumulation mechanism by the cell or to simple adsorption. The fact

that different saturation points could be achieved by different initial concentrations of chemicals favors the latter possibility.

The toxicities of IAA and NEM are plotted in Figure 2. The curves are drawn from the values obtained with 3.0×10^7 cells per ml. As the cell concentration increased, higher chemical concentrations were necessary to produce comparable effects. The vertical lines in the graph show the variations observed with 1.0×10^8 and 3.0×10^6 cells per ml.

The Sensitization

The term sensitization is not very descriptive. Although the definition given by Bridges (8, p. 105) is broad enough to include all chemical agents which can bring about a synergistic effect with radiation, the term suggests that the agents can exert their effects independently without radiation. The term sensitization is used in its broadest interpretation throughout this thesis.

The sulfhydryl-combining agents. Kiga et al. (40, p. 331-332) have shown that malonic acid (MO) and maleic acid (ME) could enhance the lethal effect of radiation on Saccharomyces ellipsoides. Another sulfhydryl-combining agent, NEM has been successfully used to sensitize E. coli. Bridges (9, p. 467-472) noted that the sensitizing effect was evident only when the oxygen was removed. This

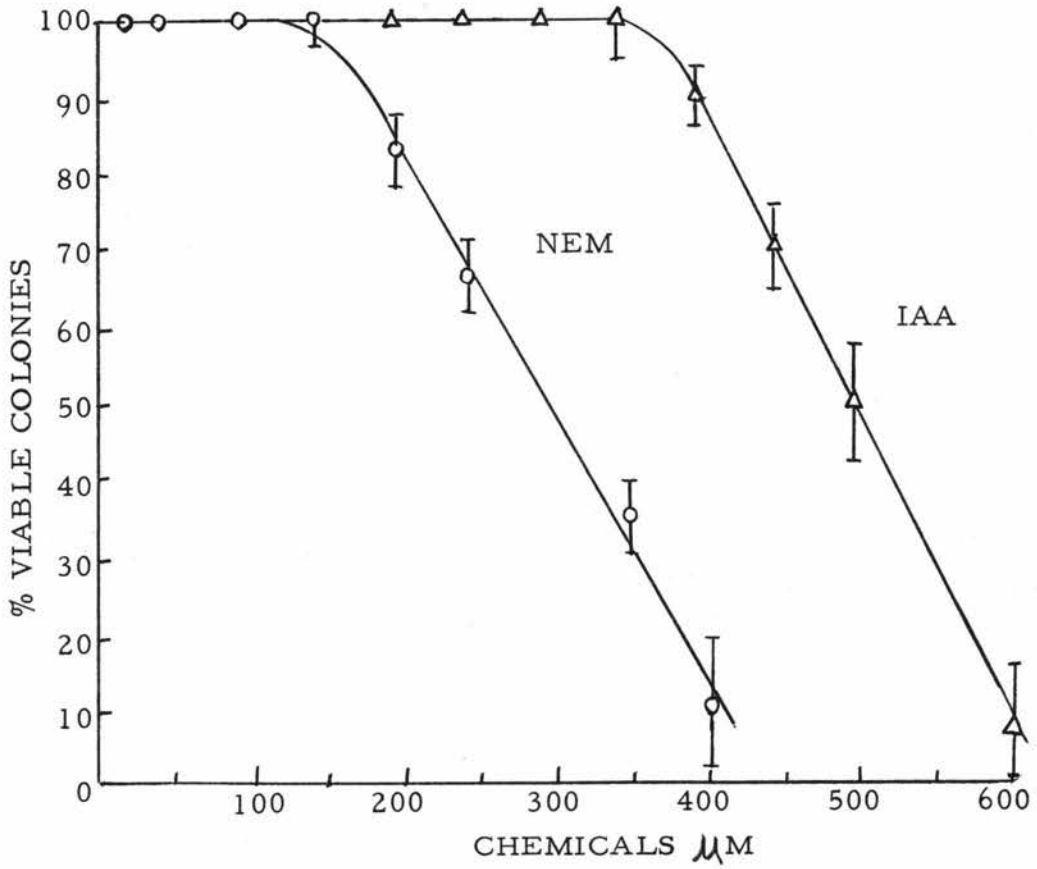


Figure 2. The toxic effects of NEM and IAA on the cells suspended in 0.067 M phosphate buffer. See text for explanations.

suggested that the sensitizer was in some manner competing for the same cellular site with oxygen. His results, however, could not be duplicated using M. radiodurans as a test organism (Table 9). NEM did not sensitize M. radiodurans either in the presence or absence of atmospheric oxygen (Table 7, 8 and 9). The effects of MO or ME were also negligible (Table 7 and 8).

The effect of IAA, was marked. Less than one percent of the original cells survived 700,000 rads (Table 8). When one considers the facts that the concentration of IAA in buffer was far below the toxic level and that the 100 uM used was one tenth of the chemical concentrations employed for other agents, the significance of this sensitizing effect becomes apparent.

IAA is considered to be a sulfhydryl-combining agent of questionable specificity (7, p. 527-529). Since NEM, which has higher specificity to the sulfhydryl group, failed to sensitize this organism, the effect of IAA could not be considered as due to its action to the cellular sulfhydryl group alone.

Alkyliodides. The above observation of the effect of IAA suggested that iodine might have helped produce this phenomenon. It is probable that iodine might have been liberated from IAA by radiation.

There was no reported data concerning the effects of radiation on IAA. Radiolysis of alkyliodides, however, have been investigated by many workers. Schuler and Petry (67, p. 3954-3958) have

Table 7. Viable Counts Made After Cells Have Been Exposed to Radiation with Following Chemicals.

Chemicals	Concentration (uM)	Viable cells/ml after exposure to 0	250 k rads	500 k rads	700 k rads
Buffer	0	2.60×10^6	2.18×10^6	1.82×10^6	1.38×10^6
NEM	100	2.55×10^6	1.90×10^6	1.53×10^6	1.05×10^6
IAA	100	2.62×10^6	1.37×10^6	1.30×10^5	2.10×10^2
MI	1,000	2.60×10^6	2.06×10^6	1.74×10^6	1.20×10^6
EI	1,000	2.61×10^6	2.16×10^6	1.93×10^6	1.43×10^6
PI	1,000	2.61×10^6	1.98×10^6	1.21×10^6	8.60×10^5
MO	1,000	2.64×10^6	2.08×10^6	1.35×10^6	9.08×10^5
ME	1,000	2.58×10^6	1.83×10^6	1.07×10^6	6.74×10^5
DNP	1,000	2.60×10^6	2.10×10^6	1.56×10^5	5.30×10^1
EDTA	1,000	2.16×10^6	1.31×10^6	4.93×10^5	8.00×10^4
DPA	1,000	1.85×10^6	1.48×10^6	1.06×10^6	2.85×10^5

Table 8. Percent Survivals After Irradiation in Presence of the Following Chemicals[1].

Chemicals	Concentration (uM)	Percent survival after exposure to			
		0	250 k rads	500 k rads	700 k rads
Buffer	0	100	84	70	53
NEM	100	100	73	59	40
IAA	100	100	53	5	0.008
MI	1,000	100	79	63	46
EI	1,000	100	83	74	55
PI	1,000	100	76	46	33
MO	1,000	100	80	52	35
ME	1,000	100	70	41	26
DNP	1,000	100	81	6	0.002
EDTA	1,000	83	50	19	0.3
DPA	1,000	71	57	41	11

(1) Calculated from Table 7 data.

shown a progressive release of iodine from methyliodide, ethyliodide, propyliodide and isopropyliodide when these compounds were irradiated with 120 KVP X-ray for several minutes. Although the rate of iodine release was different for each alkyliodide, the release was dose-dependent. Hornig and Willard (34, p. 2429-2434) further studied the effects of temperature and oxygen on this phenomenon.

Since the release of iodine from alkyliodides during irradiation has been demonstrated, they were examined for possible sensitizing effects.

The results obtained with alkyliodides are also summarized in Table 7 and Table 8. The alkyliodides were not as effective as IAA, perhaps because they evaporated during irradiation, for they were highly volatile.

Miscellaneous Chemical Agents

The 2,4-dinitrophenol (DNP) is a well known uncoupling agent. Some workers indicated that the conditions which do not favor the rapid growth of irradiated cells would improve the chance for recovery (72, p. 361). Since DNP would limit the available energy, it was expected that it would affect the recovery of irradiated cells.

Controversy exists concerning the effectiveness of diphenylamine (DPA) as a carotenogenesis inhibitor (28, p. 531-538); however, it was observed that the pigmentation of M. radiodurans was

reduced by the presence of this agent in the growth media. The effect of this chemical in the resting cell suspension may not be identical to that observed with the growing cell; nevertheless, this compound was included in order to observe any possible effects it might have.

The heavy metal binding agent, ethylenediaminetetra acetate (EDTA) was shown to enhance the effect of radiation on yeast (3, p. 813). This compound was examined as a representative of chelating agents which might have a sensitizing effect on this organism.

The DNP and DPA presented similar effects (Tables 7 and 8). The effects on the cells irradiated at lower radiation levels were not pronounced. However, at higher radiation levels, the effect was to reduce the surviving numbers considerably. The DNP was far more effective than DPA in this respect; nevertheless, no direct comparison can be made owing to the poor solubility of DPA.

The EDTA appears to have a different inactivation mechanism from DNP or DPA. Fewer cells survived when irradiated in the presence of this agent; however, the rate of inactivation was comparable to that in buffer (Tables 7 and 8).

The Effect of IAA

The radiation sensitizing effect of IAA upon M. radiodurans

was unique: it was the most effective agent in the reduction of survival and changed the cells visibly by decolorizing the pigment. This effect is duscussed in detail below.

The toxicities of NEM and IAA in connection with radiation have been postulated to be due to the specific combining reactions with vital sulfhydryl groups. Bridges (9, p. 467-472) observed that NEM was effective as a radiation sensitizer only in the absence of oxygen. No radiosensitizing effect of NEM has been observed with M. radiodurans, either in the presence or absence of oxygen, while the effect of IAA was pronounced. This sensitizing effect of IAA was not influenced by oxygen.

Both NEM and IAA were toxic to M. radiodurans at higher concentrations. Based on Bridges' hypothesis (10, p. 238-241), this toxicity may be due to the blocking of certain sulfhydryl groups essential to the vital processes. The concentrations of IAA used for the irradiation experiment, however, were below the toxic level. The action of IAA under radiation exposure, therefore, appears to be distinct from the normal sulfhydryl-binding action.

Radiation is considered to produce more reactive sulfhydryl groups in the cell (10, p. 240). Although this might have accounted for some of the sensitizing effect of IAA, the markedly different radio-sensitizing effects of NEM and other sulfhydryl-combining agents from IAA indicate that IAA also reacts with other vital groups

Table 9, The Effects of NEM and IAA in Reducing the Survival of Cells Exposed to Radiation.

Radiation Dose (rads)	No. Viable Cells/ml.			Percent Survival		
	Buffer	NEM[1]	IAA[1]	Buffer	NEM	IAA
0	3.00×10^7	2.90×10^7	3.00×10^7	100	100	100
100,000	2.24×10^7	2.14×10^7	1.18×10^7	74.7	71.0	71.0
200,000	1.48×10^7	1.48×10^7	6.65×10^6	49.3	49.3	22.2
300,000	1.35×10^7	1.40×10^7	9.50×10^5	45.0	47.0	3.2
500,000	1.04×10^7	1.27×10^7	4.00×10^4	34.7	42.3	0.13
700,000	7.70×10^6	1.00×10^7	2.30×10^3	25.7	33.3	0.8×10^{-2}
1,000,000	1.28×10^6	3.80×10^6	6.50×10^1	4.3	12.7	0.22×10^{-4}

(1) The concentrations of NEM and IAA were 100 uM. Also compare with Tables 7 and 8 and note that survival is influenced by the initial cell concentration.

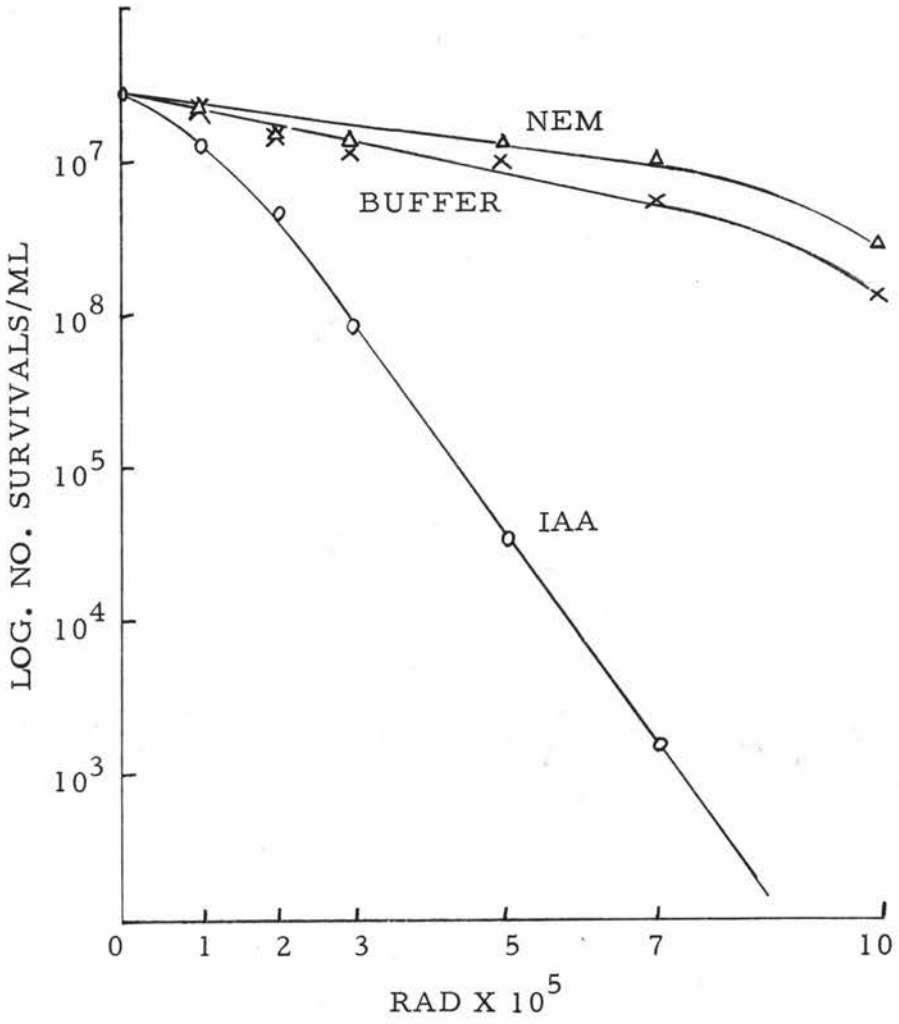


Figure 3. The effects of NEM and IAA on the irradiation survival of M. radiodurans. The concentrations of chemicals were 100 uM. (From Table 9)

in the cell.

The presence of IAA in the irradiation mixture was necessary to bring out this effect. When cells were incubated with the chemical but centrifuged prior to irradiation and suspended in phosphate buffer, the survival was not influenced. The toxicity test has indicated that IAA must have been accumulated in the cell. The loss of the sensitizing effect when IAA was removed might indicate that the amount of this chemical incorporated inside of the cell was not sufficient and IAA outside the cell must furnish an additional sensitizing agent. Addition of equal molar L-cysteine inhibited this effect of IAA, apparently because of the removal of free IAA in buffer by reaction with cysteine.

Radiolysis of IAA

It was postulated that the pronounced radio-sensitizing effect of IAA might have been due to the free radicals or ions produced by irradiating IAA.

The radiolysis of IAA was demonstrated by irradiating IAA and a starch mixture in buffer. The mixture was colorless before irradiation. Upon irradiation, however, the typical iodine starch complex of blue-violet color was formed. The color intensity was proportional to the radiation dose. Ultraviolet light produced the same effect.

Nature of IAA Radiolytic Product

The blue-violet color of irradiated starch and IAA appears to be similar or even identical to that of the iodine-starch color complex. The maximum O. D. of 1, 000 uM IAA and 0. 05 percent starch obtainable by irradiation was 1. 190 at 665 mu while the color intensity obtained with 1, 000 uM by atomic weight of iodine was 0. 550. This indicates that the radiolysis of IAA produces approximately twice as many color forming units as one could expect from the iodine molecule. The color forming units produced from IAA having this characteristic would be iodide radicals or ions. This also indicates that iodide ions or radicals are as capable of forming a complex with starch as iodine molecules.

The color forming units produced from IAA were also very short lived. The color was produced only when a mixture of IAA and starch was irradiated. No color was obtained when separately irradiated IAA and starch were mixed. The irradiated IAA produced color with non-irradiated starch only when the radiation dose was higher. The color developed by this method was not dose dependent. (Table 10).

The radiation sensitizing effect of IAA also appears to be due to this short lived radiolytic product. There is a very close parallelism between color formation of an IAA-starch mixture and sensitization brought about by irradiating a cell-IAA mixture.

The sensitization could be brought about only when the cell was irradiated in the presence of IAA. Addition of irradiated or non-irradiated IAA to the irradiated cells did not produce any effect. If the radiation-activated sulfhydryl groups in the cell were solely responsible for the sensitization with this agent, the irradiated cell should have been equally sensitive to non-irradiated IAA. The above evidences strongly support the hypothesis that the effect of IAA is due to the highly reactive but short lived primary radiolytic products of IAA.

Pigment Decolorization

It has been repeatedly observed that the irradiated cells lost the pink pigmentation of the non-irradiated cell. This pigment-bleaching effect was markedly enhanced when cells were irradiated in the presence of IAA. The degree of bleaching with and without IAA is compared in Figure 4. The pigment content was determined by the procedure described in the discussion of methods.

It has been already postulated that the radiolytic product of IAA was responsible for the sensitizing effect. The pigment decolorization enhanced by this compound further indicates that the action of the sensitizing agent is not confined to sulfhydryl groups.

It is known that IAA reacts with phenolic hydroxyl and amino groups besides sulfhydryl (7, p. 531).

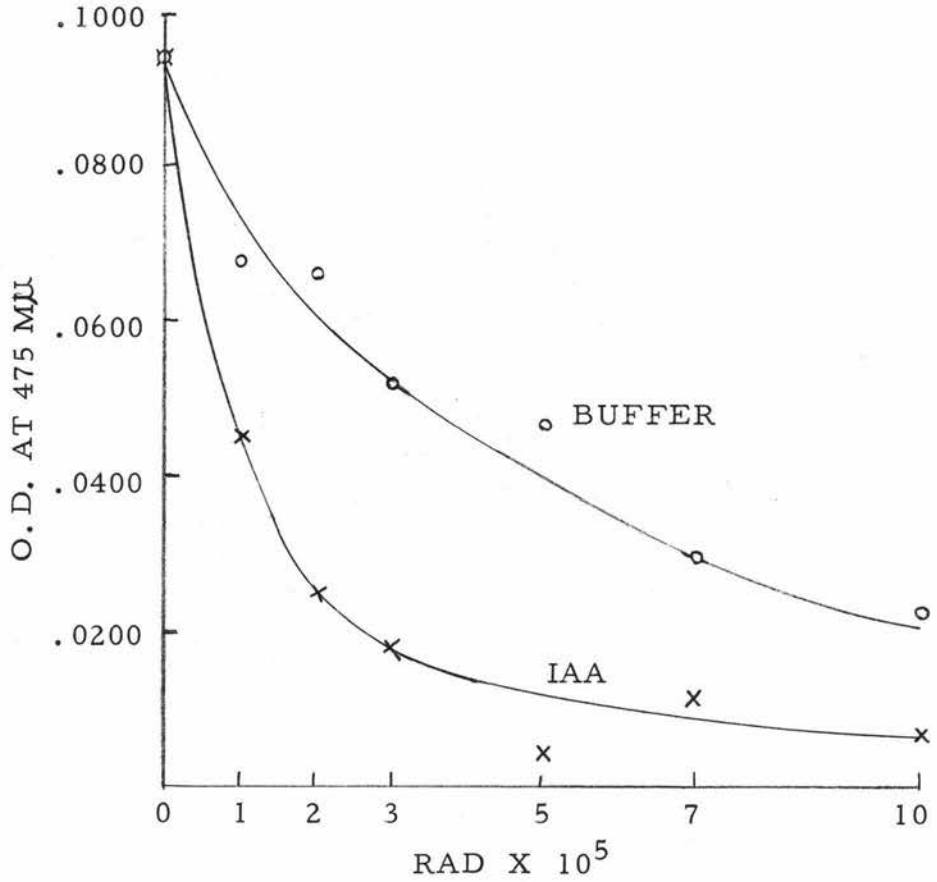


Figure 4. The effect of IAA with radiation on the total pigment content of M. radiodurans.

The bleaching of carotenoid pigments is an example of such non-specific action of IAA, particularly when radiation has promoted its reactivity. The inability of NEM or other sulfhydryl-binding agents to sensitize suggests that the non-specific reaction of the radiolytic product of IAA with various cellular constituents is significant in sensitization.

Limitations of the Experiments

The chemical agents were evaluated solely for their abilities to influence the viability of the resting cell. The cells were exposed to the chemicals in buffer prior to and during irradiation. No attempt was made to examine the effects of chemicals on the growth. This rigid distinction was made in order to simplify the experiments and also to eliminate any additional variables. It is desirable, however, to study the effects on growth and further evaluate the radiosensitivity of cells so treated.

The effects of chemicals on the recovering cells have not been examined. In order to do this, the cells should be irradiated in buffer and be permitted to grow in the presence of chemical agents.

The possible modifications of the experiments suggested above, however, would not measure the direct action of chemicals under the influence of radiation. It is quite probable that the above chemicals tested may have different effects when they are permitted to

influence the growth.

Summary

The alkyliodides (MI, EI and PI), sulfhydryl-combining agents (MO, ME and NEM), an uncoupling agent (DNP), a carotenogenesis inhibitor (DPA) and a chelating agent (EDTA) did not influence the survival of M. radiodurans to any appreciable extent. The effect of IAA was significantly greater than that of any of these, and the radiation resistance of this organism was reduced to the level of non-resistant bacteria when the cells were irradiated in its presence.

This effect of IAA appears to be produced by the radiolytic products of IAA, i.e., the iodide radicals and ions. These products are short lived and the presence of cells with IAA during irradiation is required to bring out this effect. The radiolytic products may not only react with sulfhydryl groups but also with other vital cellular components. One of the cellular components, carotenoid pigment, is shown to be influenced by the proposed agents.

PART III. COLOR COMPLEX FORMATION OF IRRADIATED STARCH-IAA MIXTURE AND ITS USE AS A RADIATION DOSIMETER

A starch and IAA mixture was irradiated to demonstrate the radiolysis of IAA. During this investigation, it was observed that the color intensity of the starch-iodine complex increased proportionally with the radiation dose and this suggested a possible application of this system as a radiation dosimeter.

The Nature of Starch-Iodine Color

The blue-violet color formed when starch is mixed with an iodine solution is a commonly known phenomenon. Despite its simplicity, the mechanism of this phenomenon is not clearly known.

The iodine color reaction is uniquely produced by 1,4 glucosidic polymers. Starch and glycogen are the only naturally occurring polymers of this nature (80, p. 641-708). The glucose units in starch are linked in such a manner that they form an alpha helix on each turn containing six glucopyranose units (20, p. 174-176). According to Downes (20, p. 176), one iodine molecule fits into one turn of alpha helix and the iodine eventually forms a long chain inside the helix. In this manner, one mole of iodine is incorporated into six moles of glucose units.

Hallő and Szejtli (35, p. 380-386) proposed that the resonating

iodine atom chain rather than the iodine molecule occupies the inner groove of the helix and they calculated that the minimum of 10 to 20 atom lengths of iodine was necessary to produce color.

Noller (53, p. 396-398) pointed out that the reaction between iodine and starch was not a true chemical reaction but was due to the formation of a chemical inclusion body. The inclusion body is not as stable as the chemical reaction products.

By degradating 1, 4 glucosidic linkages in a stepwide manner, Thoma and French (77, p. 4144-4147) showed that a minimum of eight glucose units was needed to form a perceptible color with iodine and at least 12 to 15 glucose units in chains were needed to obtain a typical starch-iodine color.

Starch

The chief constituents of grain and tubular starches can be divided into two fractions. The A fraction or amylose is composed of 300 average glucose units arranged in a straight chain having a molecular weight of less than 50, 000 (20, p. 174). This is the component in starch mainly responsible for the deep blue color formation with iodine. It takes up 18 to 20 percent by weight of iodine (53, p. 397). Amylose is insoluble in cold water. When heated, it gels and an irreversible precipitation (retrogradation) takes place upon cooling.

The B fraction or amylopectin has a higher molecular weight. It could be as high as 500,000 with approximately 1,300 to 3,000 glucose units found per molecule. This is a highly branched polymer with alpha-1,6 glucosidic linkage. This fraction takes up a small amount of iodine, i.e., 0.5 to 0.8 percent by weight of iodine being incorporated (53, p. 397). The color of the iodine-amylopectin complex is red to purple. It contributes the violet portion of starch iodine color. The highly branched structure makes amylopectin readily soluble in hot water and once formed, the solution neither gels nor undergoes retrogradation.

The most naturally occurring starches, corn, wheat, arrow root and potato contain about 20 to 25 percent of amylose and 72 to 80 percent of amylopectin (80, p. 641-708). The corn starch used for this experiment contained 27 percent of amylose as determined by the differential precipitation method described by Wilson et al. (82, p. 1380-1383).

Each starch has different physico-chemical properties owing to its occurrance as a granule. Corn and rice starch granules are smaller in comparison to the potato starch granule. The starch granule has a definite orientation and shape according to the source; however, the size is variable.

When the starch granule is heated in water, it undergoes a sudden and irreversible swelling within a definite temperature range.

This temperature is characteristic of each starch granule. The swelling temperature for corn starch is between 64 and 71° C (80, p. 641-708). At this point, the viscocity of starch suspension increases suddenly and the volume of the granule increases several hundred times.

Amylose and amylopectin both play significant roles in determining the physico-chemical property of a given starch suspension. Amylopectin acts as a stabilizing colloid for the less soluble amylose and gives the starch an increased solubility.

Partial degradation of starch increases solubility without sacrificing the color-forming ability. Soluble starch is a partially degradated starch produced either by mild acid or by beta-amylase treatment.

Starch is used for many purposes and many specialized starch products are on the market. Cold-swelling, thin boiling, oxidized starches and British gum, yellow and white dextrins are a few of the examples (15, p. 20-27).

Conditions Influencing the Color Complex Formation

The stability of the iodine-starch complex is influenced by heat, sunlight, hydrogen ion concentration and the proportional concentrations of iodine and starch. The increased temperature is said to break down the length of the iodine chain inside the groove of the

alpha helix. The temperature effect is reversible on returning to the cooler temperature (54, p. 125).

Sunlight, ultraviolet light and X-ray also promote degradation of the iodine-starch complex. In the above cases, however, the color can not be restored (5, p. 205-206; 6, p. 291-293). The optimum pH for the complex formation is reported to be around 5.0. Deviation from this gives a less intense color (32, p. 76).

The iodine-starch complex is formed by I^- and I_3^- ions equally well (80, p. 641-708).

MATERIALS AND METHODS

Stock Reagents

Starch. The stock starch suspension was prepared by dissolving 1.0 gram of a commercial corn starch (Argo) in 20 ml of boiling 0.067 M phosphate buffer. It was then stirred for five minutes to a fine paste and, upon cooling, additional cold buffer was added to obtain one percent suspension.

IAA solution. The 10 mM stock solution was prepared by dissolving IAA (Table 6) in 100 ml of cold buffer. Both stocks were stored at 4° C.

Irradiation Mixture

An equal volume each of the 2,000 uM of IAA and one percent starch prepared from the above stocks were mixed thoroughly in buffer. The 10 ml of the above mixture was then pipetted into glass vials to be irradiated. The final mixture contained 1,000 uM of IAA and 0.05 percent starch in 0.067 M phosphate buffer of pH 7.0. The irradiation was carried out in a Cobalt-60 gamma radiation source (Dose rate: 300,000 rads/hour).

The Color Intensity Determination

The maximum absorption of the color complex was observed at 665 mu. The O.D. readings at this wave length determined the absorbed doses of gamma radiation.

RESULTS

This system measures gamma radiation doses from 10,000 to 1,000,000 rads. The accurate range lies between 30,000 to 500,000 rads. The linear relationship observed between radiation and color intensity is shown in Figure 5. This linear relationship no longer holds at the higher radiation levels and the curve shown in Figure 6 does not represent a zero order function. It is noted, however, that the radiation dose could be measured accurately from

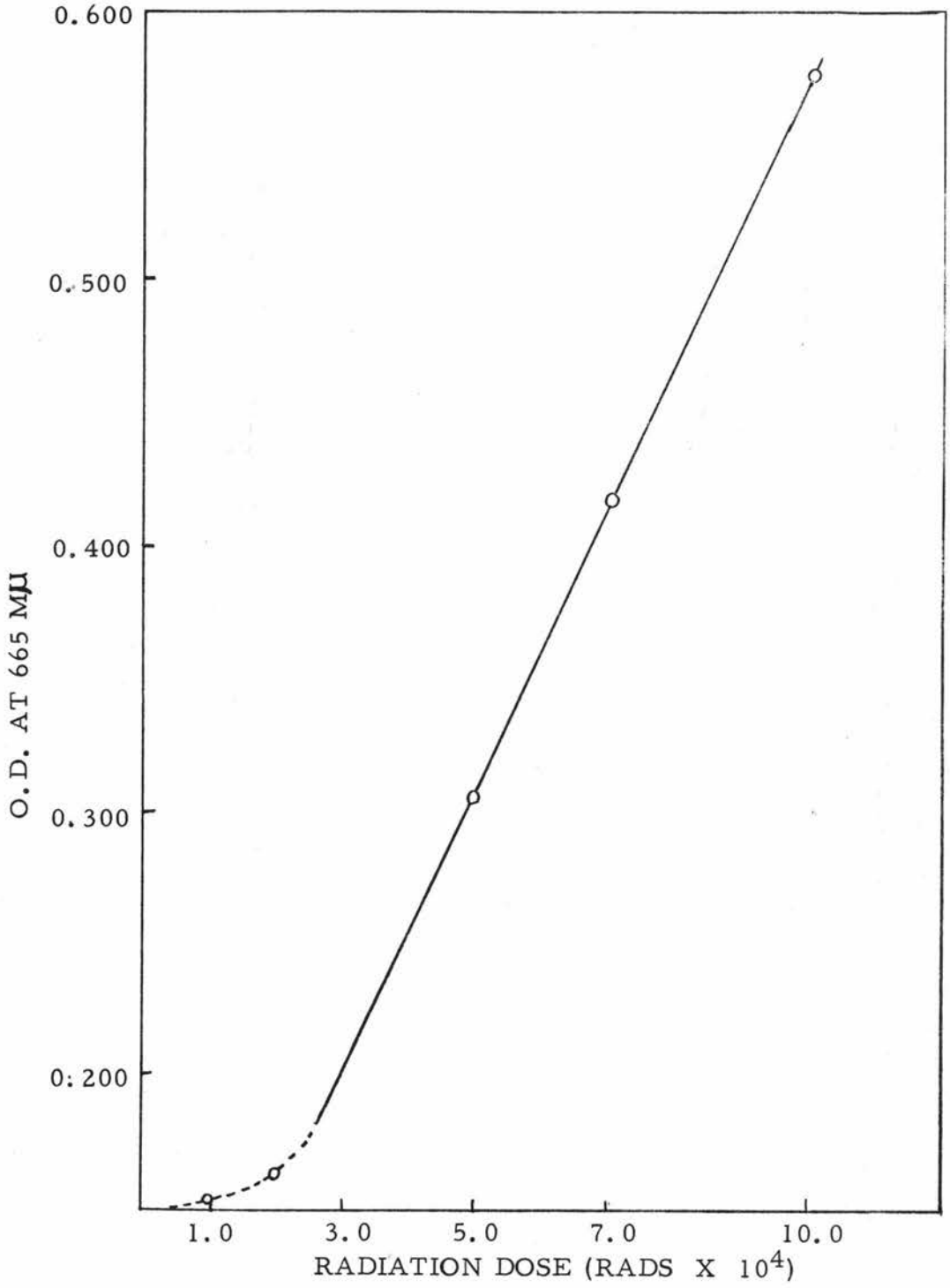


Figure 5. O.D. increase of IAA-starch mixture upon radiation exposure.

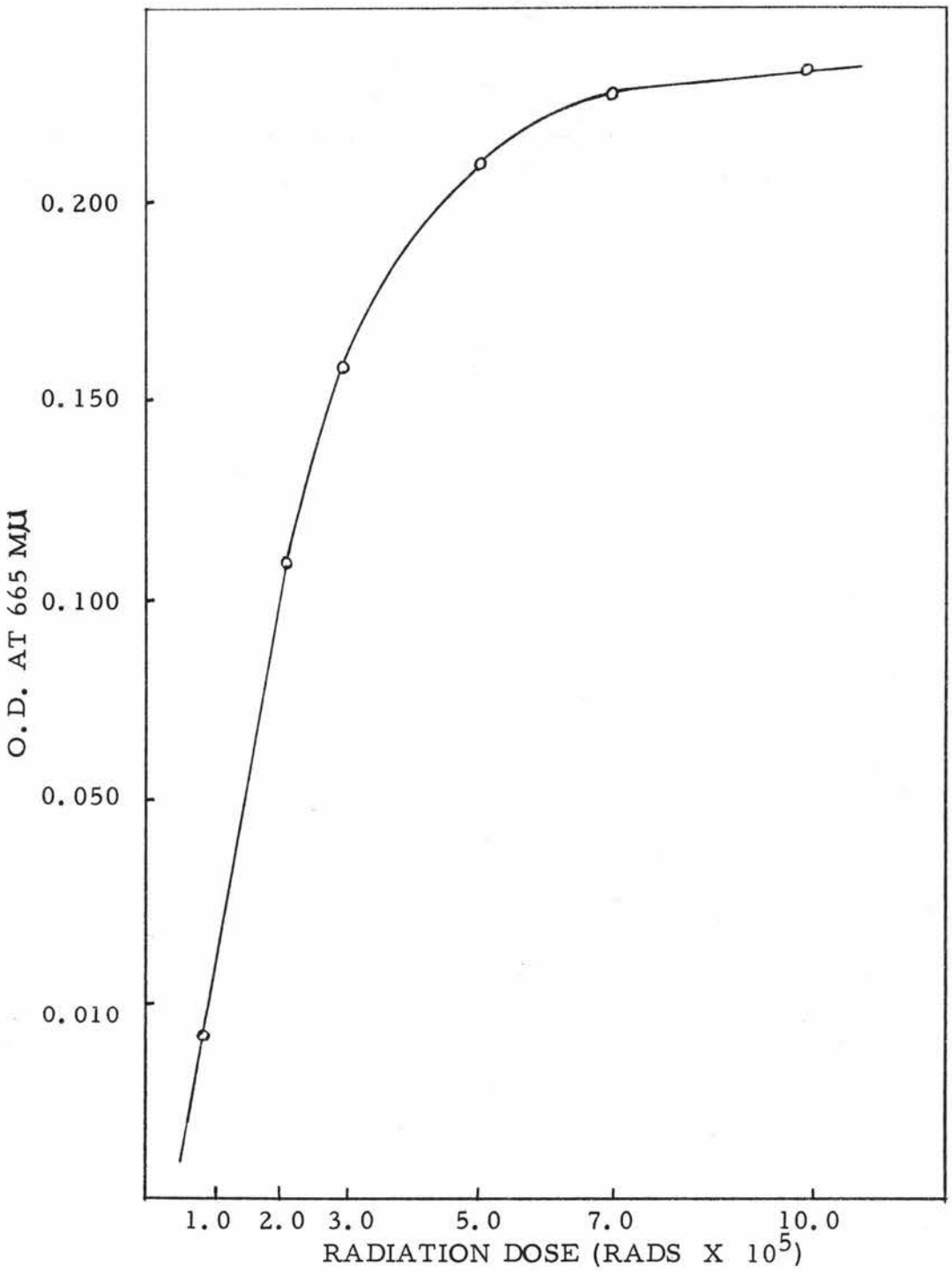


Figure 6. The O. D. increase of IAA-starch mixture upon irradiation (readings were made from 1/5 phosphate buffer dilutions).

30,000 to 500,000 rads with the use of standards such as those represented by Figures 5 and 6.

DISCUSSIONS

The color developed from IAA appears to be similar to or identical with that observed from starch and iodine. However, the color-forming units from IAA appear to be either iodine ions or radicals, or both, rather than iodine molecules (see Part II).

The color-forming units produced from IAA were also very short lived and therefore must be trapped immediately with the starch present in the mixture. The irradiated IAA and non-irradiated starch produced color of lesser intensity at higher radiation levels; however, the color intensity did not correspond to the radiation dose (Table 10). No color was produced from separately irradiated IAA and starch.

By comparing these two phenomena, one can postulate that the radiation has destroyed a part of the color-forming starch units. This may have been brought about by the radio-hydrolysis of starch. This phenomenon may account for the gradual decline of color intensities at the increased radiation levels. Some of this effect, however may be due to the exhaustion of IAA.

Despite the simplicity of preparation and handling of the starch-IAA mixture, the O.D. readings were reproducible within the

Table 10. O. D. at 665 mu of Separately Irradiated Starch Plus Irradiated IAA, and Irradiated IAA with Non-Irradiated Starch.

Radiation Dose (rads)	O. D. at 665 mu	
	Irradiated IAA + Irradiated Starch	Irradiated IAA + Starch
100,000[1]	0.000	0.000
200,000	0.005	0.003
300,000	0.003	0.091[2]

(1) No color developed at lower radiation levels.

(2) No further increase of O. D. was observed at higher radiation levels.

Table 11. O. D. 665 mu[1] of Various IAA and Starch Concentrations After Receiving 300,000 Rads.

Starch Concentration (%)	O. D. at 665 mu of IAA concentrations (uM)				
	5,000	4,000	3,000	2,000	1,000
0.5	0.000	0.000	0.000	0.000	0.000
0.3	0.000	0.021	0.100	0.000	0.070
0.1	0.137	0.128	0.134	0.150	0.156
0.05	0.113	0.133	0.145	0.154	0.162

(1) The values are from 1/5 dilutions.

range of $\pm$0.010, for several independent determinations.

The mixture containing 1,000 uM IAA and 0.05 percent starch suspension appears to be the optimal proportioning of both reactants. Various concentrations of IAA and starch have been tested in order to determine the optimal proportions. This result is shown in Table 11.

The starch prepared by the described method did not stay in suspension and repeated agitation was necessary to read the O.D.

A commercial soluble starch (Fisher, ACS certified) did not produce color when irradiated with IAA. The molecular weight of this starch is considerably lower than the untreated corn starch and radiation might have degradated this starch so that it no longer was able to form color with the radiolytic product of IAA.

Several attempts have been made to improve the solubility of starch without sacrificing the sensitivity. Heat treatment or autoclaving have been tried. Also partial degradation of starch with HCl has been attempted (Table 12).

The autoclaving of starch for ten minutes did not seem to affect the sensitivity of starch while improving the solubility considerably. The color produced with this starch, however, did not show the close correlation with the radiation doses.

The IAA-starch mixture was stable before or after radiation at room temperature. No decrease of color intensity has been observed from the irradiated vials left on the laboratory desk top for a

Table 12. The Integrity of Starch Molecule[1] and the Color Forming Ability with IAA.

O. D. ^{630}[2]	O. D. ^{665} after exposure to radiation (rads)					
	With I_2[3]	50, 000	70, 000	100, 000	300, 000	700, 000
0. 003	0. 010	0. 000	0. 000	0. 000	0. 000	0. 000
0. 010	0. 380	0. 156	0. 200	0. 234	0. 303	0. 367
0. 018	0. 280	0. 190	0. 233	0. 280	0. 300	0. 367
0. 043	0. 370	0. 250	0. 275	0. 330	0. 345	0. 418
0. 045[4]	0. 430	0. 210	0. 288	0. 363	0. 990	1. 450
- [5]	0. 550	0. 332	0. 443	0. 584	0. 800	1. 055

(1) The starch was hydrolysed by 0. 1 N HCl with increased period of autoclaving.
(2) The degree of starch integrity was measured by the opacity.
(3) 1, 000 uM iodine by atomic weight.
(4) The starch autoclaved for ten minutes in buffer.
(5) The original starch preparation.

week.

Limitations of the Dosimeter

The radiation dose range covered by this system is limited between 30,000 and 500,000 rads. This system may have a limited use, however, in radiation food pasteurization.

By virtue of its simplicity and accuracy, the measurement of radiation doses can be made very easily. This will eliminate the guess-work involved in measuring the depth doses of bulky food materials.

The range must be improved before this system can gain general use. The use of other iodinated compounds or the modification of the starch may solve this problem.

The poor solubility of starch and the apparent correlation between the integrity of the starch molecule and the sensitivity of this system pose another problem. A moderate heat treatment of starch may eliminate this difficulty.

Summary

A simple and accurate radiation dosimeter was devised with starch and IAA.

(1) The range of this dosimeter is from 30,000 to 500,000 rads.

(2) The accuracy of this system is with $\pm$0.010 O.D. readings.

(3) The IAA-starch mixture is stable before radiation and the resulting color is also stable at room temperature.

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