AN ABSTRACT OF THE THESIS OF

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(Date thesis is presented) July 16, 1963

Title THE ROLE OF METHIONINE IN THE METABOLISM OF

MICROCOCCUS RADIODURANS

Abstract approved

(Major Professor)

Studies on methionine, methionine analogue and methionine precursor utilization by Micrococcus radiodurans demonstrate: 1) methionine is required in synthetic medium, with D-, L-, and D,L-forms equally well utilized; an excess is detrimental to growth; 2) of methionine sulfonium compounds, S-adenosylmethionine, but not S-methyl-L-methionine can replace it; 3) vitamin B₁₂ can replace methionine, the sulfur requirement probably satisfied by inorganic sulfate, or enhance growth in the presence of methionine precursors; 4) purine and pyrimidine bases do not replace methionine or vitamin B₁₂; 5) anaerobic growth can be supported by the addition of hemoglobin to synthetic medium containing vitamin B₁₂ and 6) a sulfurous gas other than H₂S is produced from methionine metabolism.

Anaerobic photosynthetic growth was demonstrated which may result in reclassification of the organism.
Photosynthetically-grown cells are less pigmented but as radiation resistant as heterotrophically grown cells.

Proposed pathways of methionine degradation suggest the mercaptide to be either $\beta$-mercaptoethylamine (I) or 3-mercaptopropylamine (II) with chemical analyses compatible with (II) and possibly with (I).

Radiation sensitive organisms grown in the presence of this gas exhibit slightly increased radiation resistance.
THE ROLE OF METHIONINE IN THE METABOLISM OF
MICROCOCCUS RADIODURANS

by

ANNE O'CONNELL DEENEY

A THESIS
submitted to
OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY
August, 1963
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Date thesis is presented July 16, 1963

Typed by Ruth Baines
DEDICATION

In memory of my husband,
Hugh Jerome Deeney, Jr.
June 26, 1923
July 6, 1960
Without his inspiration, I would never have embarked on, or persisted in, this endeavor.

*******

I also wish to thank the faculty of the Department of Microbiology, my family, and my friends, for their help and encouragement during my personal trials and during the course of this research.
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INTRODUCTION

The "atomic age" has brought with it many questions and many problems. Some of the questions asked include: 1) in what ways can atomic radiation be utilized in non-military affairs; 2) how much radiation will be required for these methods; 3) will the amount of radiation required be the same for $\alpha$, $\beta$, $\gamma$, and $x$-rays; and 4) what is the comparison with the effects of ultraviolet rays?

As an answer to the first question, Meisel (108, p. 159-161) proposes for the Russian 5-year plan:

1) Study of the effect of ionizing radiation on biosynthetic processes of microorganisms, including the yield of ergosterol, RNA and nucleotides, vitamins.

2) In fermentation plants, the application of radiation in oxidation-reduction and other enol chemical processes.

3) The genetic effects of radiation on microorganisms, genetics, and biochemical mutations.

4) Radiation sterilization of antibiotics, hormones, vitamins, tissue transplants, and some foods.

5) Development of radiomimetic compounds.

6) Use of tagged compounds to follow their use in biological processes.

Among problems encountered are: 1) undesirable side-effects of radiation; 2) a suitable radiation source; 3) limits and hazards of accidental radiation exposure; and 4) existence of compounds available to protect the organism in case of the necessity of undesirable radiation exposure, or therapeutic compounds utilizable after radiation exposure.
The discovery in 1954 (3, p. 575) by Anderson et al. of a remarkably radiation resistant microorganism prompted a series of studies on this phenomenon of radiation resistance, coupled with the usual studies on morphology, physiology and incidence (38; 44; 88; 100; 101; 124, p. 289-298, 161). The organism, Micrococcus radiodurans, has among its characteristics, a pink carotenoid pigment, a tetrad formation, has up until recent investigations appeared to be an obligate aerobe, has a nutritional requirement for certain sulfur-containing compounds, and produces among its end-products a sulfur-containing gas other than hydrogen sulfide.

This study is based primarily on the last two characteristics. It is known that certain sulfhydryl compounds and other agents, if present at the time of irradiation, exert a "sparking" effect on the constituents of the organisms irradiated; this could be associated with the nutritional requirement for certain sulfur compounds, especially methionine, by the microorganism. Studies with other microorganisms requiring the same sulfur compounds for growth have shown that other sulfur containing compounds could be substituted, and in some cases even by non-sulfur containing compounds. This line of study would of necessity have to be followed with M. radiodurans to see if: 1) adequate growth can be maintained in the presence of sulfur compounds other than methionine; 2) adequate growth can be maintained in the absence of methionine or other
utilizable sulfur compounds; or 3) radiation resistance is altered in cells grown in a methionine-free medium.

The sulfurous gas evolved from the metabolism of sulfur compounds could be an excess of a compound produced by the cell affording it resistance to irradiation, and if this is the case, what is the nature of the compound, and would it be of any value in the protection of other biological systems against the effects of radiation.
HISTORICAL

With the discovery by Jackson and Block in 1932 (151, p. 509) that methionine will replace cystine in the diet of the rat, methionine was elevated to the position of an essential amino acid, and subjected to countless investigations to determine the role the entire molecule plays in biological systems, and the significance of its various structural groups.

The degradative reactions of methionine in vivo initiate several major synthetic pathways: 1) the synthesis of cysteine and homocysteine; 2) the transfer of the methyl group to various acceptors, as in fatty acid synthesis (113, p. 712; 152, p. 5192-5193); 3) the oxidation of the methyl group to a $\text{C}_1$ component or components, and their subsequent utilization; and 4) the synthesis via transmethiolation of aminobutyric acid and its derivatives from the carbon chain of methionine. Most of these reactions are directly or indirectly reversible, the extent of the reversibility varying with the species of organism studied (61, p. 382-383; 90, p. 885-893; 134, p. 266-269; 135, p. 61-68).

The synthesis of methionine in vivo appears to follow at least five different pathways; 1) by direct transfer of the methyl group from N-onium compounds such as betaine; 2) by direct transfer of the methyl group from S-onium compound
such as thetins; 3) by the process of transmethiolation involving thiomethyladenosine and aminobutyric acid; 4) by the reversal of the synthetic pathway of cysteine from methionine; and 5) by synthesis de novo of the methyl group of methionine via the process of utilization of the C1 units originating from numerous metabolites by way of various pathways, including the oxidation of the methyl group of methionine, followed by its reduction (47, p. 257-258; 53, p. 1282-1283; 83, p. 293-298; 129, p. 763-773; 130, p. 244; 140, p. 134-135; 141, p. 730-735; 142, p. 169-174; 144, p. 339-334; 145, p. 558-590; 157, p. 907-914; 160, p. 651-658; 174; 179, p. 1336-1340).

Vitamin B12 does not appear to be involved in any of the processes of methionine formation with the exception of the synthesis of the methyl group de novo from the α-carbon of glycine. The nature of this involvement appears to be indirect and coupled with other deficiencies since it has been found that in animals deficient in B12 and folic acid there is a reduced ability to methylate homocysteine or betaine to form methionine and choline respectively (137, p. 21). In some cases the primary acceptor of the methyl group may be S-adenosylhomocysteine (145, p. 559) with the formation of methionine (161, p. 104).

It has been observed that certain organisms capable of utilizing both the D- and L- isomers of methionine have an active racemazing system (84, p. 629). In a species of
Pseudomonas this racemizing system is present only when growth occurs at the expense of D- or DL-methionine. Workers studying the mandelic acid racemase in Pseudomonas fluorescens noted the racemase formation was substrate induced and induction of racemase could be accomplished with either optical isomer of mandelic acid. However, the racemase functioned metabolically only when the D-isomer was the substrate (84, p. 629-630). Thorne (161, p. 104), working with Bacillus subtilis extracts, has found them to contain transaminases specific for D-amino acids, and his findings would suggest the following steps which would simulate a methionine racemase action: 1) transamination between D-methionine and pyruvate to yield D-alanine; 2) racemization of D-alanine by alanine racemase; and 3) transamination of ketomethionine and L-alanine to yield L-methionine.

It has been found that a number of methionine analogues (83, p. 293; 168, p. 273-281; 169, p. 135-141; 175, p. 51-60; 176, p. iii-iv), other sulfhydryl compounds, and vitamin B₁₂ (137, p. 19-28) can replace the methionine requirement of some methionine dependent microorganisms. The mechanism of replacement is apparently not the same in all organisms. It has also been found that methionine in excess is detrimental to growth of some organisms (6, p. 748), and that methionine dependent microorganisms capable only of aerobic growth, can with the addition of
vitamin B$_{12}$ grow anaerobically (103, p. 926). Bartnicki-Garcia and Nickerson (12, p. 142) found that for *Mucor rouxii* thiamine and nicotinic acid are required for anaerobic but not aerobic growth, and both of these constituents although not essential in the synthetic medium developed for *Micrococcus radiodurans* for aerobic growth have been included for stimulus (38, p. 30). It has also been noted that in certain vitamin B$_{12}$ dependent bacteria the dependency can be satisfied with the addition of methionine to a vitamin B$_{12}$-free medium (6, p. 748).

Of great interest to many investigators has been the study of substituting analogues, homologues, and even inorganic sulfur compounds for methionine in the medium of a methionine requiring organism, and of the sulfur metabolism of microorganisms in general. The results have shown a wide variation of results, not only among different genera and species, but even among strains of the same species.

Prescott (118, p. 725), in studying the utilization of sulfur compounds by *Streptococcus bovis*, an organism for which the primary nutritional need for sulfur is the synthesis of methionine and cystine for incorporation into protein found that among the sulfur containing amino acids methionine is completely inactive as a sulfur source; other sulfur amino acids, especially cystine and cysteine,
support good growth; several organic sulfur compounds other than amino acids are effective sulfur sources; and of the inorganic sources, sulfide and thiosulfate are utilized, with the oxidation state of the compound apparently a critical factor in the utilization. He also found autoclaved sulfur sources more effective than filter sterilized. Hase found that sulfur was required for cell division and protein synthesis in Chlorella (63, p. 131-142; 64, p. 9-24). Various organic and inorganic sulfur compounds, including methionine, were found to be satisfactory for cell division (62, p. 173-176) although Mandels (62, p. 176) had found methionine unsatisfactory.

Lockingen (104, p. 104-108), studying 22 mutants of \textit{Escherichia coli} strain B, divided them into three groups on the basis of growth response to supplements: group one grew only with methionine or methionine sulfoxide; group two with methionine, methionine sulfoxide or vitamin B$_{12}$ and group three with methionine, methionine sulfoxide or homocysteine. Davis and Mingoli (35, p. 17-28) and Kalan and Ceithaml (83, p. 293-298) found similar responses with \textit{E. coli} strain W. Methionine sulfoximine was found to inhibit both strains B and W, (104, p. 105). Molho and Molho-LaCroix (109, p. 153-172), studying a B$_{12}$ dependent strain of \textit{E. coli} strain W, felt a labile methyl group was essential for the growth of this organism, but felt that the rest of the molecule was important, and that with
methionine sulfoxide the entire molecule was incorporated by the microorganism.

McRorie (107, p. 489-497) found that methyl methionine reversed sulfonamide inhibition in E. coli Texas, and could replace the methionine for methionineless mutants of this strain, but for mutants of strain B it is not available as methionine, as a methyl donor, or as homocysteine. Methionine sulfoxide has been found suitable in the methionine dependent strains to satisfy the methionine requirement. As a converse to methyl methionine not serving as a methyl donor, Sato (132, P. 128-131) found methyl sulfoxide, which can serve as a methyl donor to yield methyl methionine, cannot serve as a methyl acceptor to yield methyl methionine. Methionine sulfoximine and methionine sulfone are two growth inhibitors which do not replace methionine in minimal medium; the inhibitions produced are partially relieved at low inhibitor concentrations in some biological systems by glutamic acid, glutamine, methionine and methionine sulfoxide. B12, homocysteine, cystationine and methyl methionine do not affect the inhibition (16, p. 573-578; 31, p. 233, 245; 168, p. 273-281; 169, p. 135-141; 175, p. 51-60; 176, p. iii-iv; 177, p. 361-374).

From the very earliest studies on the nutrition of M. radiodurans in which it was demonstrated that methionine was required for growth in a synthetic medium, work has been carried on to determine how many phases it is involved
in the metabolism of this microorganism, other than growth and reproduction, and what is the ultimate fate of the molecule in biosynthesis.

Two factors demonstrated that methionine was not only a growth requirement for the organism. These were:

1) In studies with methyl-C\textsuperscript{14}-methionine (124, p. 289-298), total C\textsuperscript{14} recovery for CO\textsubscript{2} evolved, cells and media accounted for approximately 50\% of the label.

2) Cells grown in aerated tryptone-glucose-yeast broth (TGY) proliferated and gave off sulfurous gases to a greater or lesser extent. The addition of methionine at the rate of 2 g/7 L did not increase the cellular yield but did guarantee a steady level of gas production (unpublished results).

The approximately 50\% of unaccounted-for label then could be in this other gaseous fraction, which could be just a "luxury operation" for the organism, or another form of waste products.

However, if these gases were a luxury product, could they perform any useful service for the organism when adverse conditions prevailed? This question was given consideration because sulfhydryl, or mercapto, compounds are known to exert varying degrees of radiation protection on biological systems, and possibly this luxury product was the agent that protected this microorganism so that it
could be discovered in the irradiated meat samples from which it was originally isolated (3, p. 575-577). The possibility existed that this gaseous compound was similar to, or identical with, known compounds giving radiation protection.

There is a voluminous literature on radiation-protective compounds, their mode of action, and on radiation effects in biological systems in general.

The effect of ionizing radiation on any given biological system is dependent on many different factors, including type of radiation, dose, dose-rate, irradiation temperature, biological condition at the time of irradiation, and pre- and post-radiation treatment with chemicals and other substances.

It has been found that certain substances, if present at the time of irradiation, but not necessarily at other times, can produce a modification of the radiation effect. One of the few substances which enhances the radiation effect is oxygen, although the tetrasodium salt of 2-methyl-1,4-naptha-hydroquinone diphosphate ("Synkavit") and other forms of vitamin K$_5$ produce some effect and can be used in radiation therapy (94, p. 351-353; 143, p. 78-85). The maximum sensitization by oxygen is reached at a given concentration, and further increase in the oxygen concentration can produce no further effect.
The ratio of radiation doses required to produce a given effect in a biological system with and without oxygen is about 3/1 for X- and γ-rays, but it has been found that for several systems the oxygen effect is much less with densely ionizing radiation.

Many substances have been found to give protection against radiation to biological systems. This effect has also been noticed to be less effective with densely ionizing radiations. Certain of the protective substances are relatively non-toxic and not too rapidly metabolized and could be acceptable for the treatment prior to irradiation of biological systems, or of pre-treatment of personnel who have to enter dangerously radioactive areas for short periods of time. Cystamine, the oxidized form of cysteamine (β-mercaptoethylamine)(MEA) is one of the most effective of these compounds and has been studied extensively. Other extremely effective compounds that are undergoing extensive study are aminoethylisothiuronium bromide hydrobromide (AET), 2-mercaptoethylguanidine (MEG) and 3-mercaptopropylamine (MPA). (8, p. 633-636; 10, p. 1509-1512; 17, p. 609; 18, p. 1199-1201; 19, p. 231-238; 28, p. 49-58; 34, p. 33042; 41, p. 308-322; 55, p. 3823-3827; 56, p. 3827-3734; 67, p. 165-173; 75, p. 498-499; 81, p. 406-411; 82, p. 74; 89, p. 571-584; 91, p. 94-95; 92, p. 493-496; 98, p. 1434-1435; 106, p. 23-28; 122, p. 490-492; 136, p. 62-66; 139, p. 857-870; 146, p. 1202-1204; 158, p. 76-
Although the modes of action of oxygen and the protective substances are not yet well established, they are probably closely connected. To substantiate this, there are several examples where the effect caused by a protective substance has been traced to the fact that it decreases oxygen tension, and some protective substances have been made ineffectual, or have a decreased effectivity in the presence of oxygen.

In one hypothesis which was widely accepted in the early 1950's, it was suggested that radiobiological effects were produced by the action of oxidizing free radicals, hydrogen atoms being converted to $\text{H}_2\text{O}_2$ radicals when oxygen is present, the $\text{H}_2\text{O}_2$ radicals supposedly having a very powerful biological effect. Protective substances were considered in some cases to function by decreasing oxygen tension but in most cases to act by competing with radiosensitive biological molecules for $\text{H}_2\text{O}_2$ radicals. In the case of thiols, as cysteine, which are particularly effective protective agents in most chemical systems, the thiol group is particularly susceptible to attack by free radicals, leading to oxidation (154, p. 208).

The decreased oxygen effect and the protective effect with densely ionizing radiation were ascribed to free radicals formed in dense regions of the track reacting.
differently from those in less dense regions. To explain this, it was believed that a molecule was degraded by \( \text{HO}_2 \) radicals formed from hydrogen atoms. In the presence of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), twice as many \( \text{HO}_2 \) radicals are formed because hydrogen atoms (even if they do not react with oxygen) react with \( \text{H}_2\text{O}_2 \) to give OH and both this OH and the OH from water react with \( \text{H}_2\text{O}_2 \) to give \( \text{HO}_2 \). The conception of degradation by \( \text{HO}_2 \) radicals was extended to explain the biological action of radiation and in particular the oxygen effect in radiobiology, and the action of protective agents (154, p. 172).

There are, however, two serious objections to this view. First, \( \text{HO}_2 \) radicals would be most unlikely to cause degradation if OH radicals were unable to do so. Secondly, in a system containing a polymer and \( \text{H}_2\text{O}_2 \), all OH radicals would react with the polymer rather than with \( \text{H}_2\text{O}_2 \), so that hydrogen peroxide could not give an increased yield of \( \text{HO}_2 \) radicals. That degradation is due to OH radicals is a more likely explanation. Hydrogen peroxide increases the yield of OH because the following reaction occurs:

\[
\text{H}^\cdot + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \cdot \text{OH}
\]

It has been claimed that air-free aqueous solutions of polymers (no added hydrogen peroxide) have no effect from radiation unless the polymer contained unstable peroxide groups. The unstable groups could be eliminated by heating, producing a material which would not be degraded
when irradiated in the absence of oxygen or hydrogen peroxide. These results supported the view that degradation was being caused by HØ₂ radicals. The results of later work could not be confirmed, however, which showed that degradation occurred in greater yield in the absence of oxygen than in the presence of oxygen, suggesting that oxygen stabilizes the polymer radicals to degradation in certain solvents. When polymers are irradiated in solution the attack is mainly by the free radicals formed from the solvent, but as in the solid state, crosslinking and degradation still occurs (154, p. 172).

The role of -SH compounds in decreasing radiation injury has been reviewed extensively (105, p. 564-572; 115, p. 213-214; 116, p. 549-553; 117, p. 437-474). These compounds are known to function by different mechanisms. In considering the protection of irradiated proteins the following functions seem to be most pertinent: 1) induced anaerobiosis; 2) free radical scavenger; 3) capacity to form mixed disulfides; and 4) repair of chemical damage.

Induced anaerobiosis is of great importance as a protective mechanism in irradiated animals, but should not be operative in an in vitro irradiation of proteins or enzymes in a reaction system which has been evacuated and gassed with N₂ (96, p. 679-685).
The role of -SH compounds as free radical scavengers has been supported by studies on the radiation chemistry of these compounds (7, p. 80-84; 9, p. 328-332; 41, p. 308-322). In an aqueous protein system, the -SH compounds would be readily accessible to react with free radicals produced from water. The avidity with which these compounds combine with free radicals protects even the most radiation-labile amino acids of proteins - there is a competitive reaction between the chemical protector and the protein molecules to react with free radicals.

Experimental work of Pihl et al. (117, p. 437-474) favors the theory of mixed disulfide formation with protein molecules. Results obtained by Kumta et al. (96, p. 679-685) would show that this holds true for some cases but not for all.

The fourth important mechanism is the ability of -SH compounds to repair the site of the damage. Thus if an amino acid becomes a free radical by loss of a hydrogen, it could extract hydrogen from its chemical protector. This mechanism of repair may apply to all amino acids, but it is best known for cysteine which readily undergoes free radical oxidation-reduction. The general protection of amino acids can be ascribed mainly to free radical scavenger effects. With cystine, there is evidence for the operation of disulfide formation and/or hydrogen abstrac-
tion repair mechanisms.
Kirrmann (89, p. 571 - 584), in summing up the role of sulfhydryl compounds in irradiated biological systems, came to the following conclusions, since he considered that each radioprotector could have one or several effects on irradiation according to the chemical and pharmacological properties of the compound:
1) competition with free radicals formed in water after irradiation;
2) reduction of cellular oxygen-hypoxia;
3) hypotensive action (112, p. 421 - 424);
4) hypothermia immediately after injection (102, p. 469 - 471); (both 2) and 3) can lead to hypoxia)
5) chelating action;
6) antimitotic effect;
7) creation of disulfide bonds.

Today there are as many theories as to the mechanism of action of radioprotective compounds as there have been theories proposed as to the mechanism of radiation damage since Henri discovered that ultraviolet light produced mutations in bacteria (68, p. 1032 - 1035).

Regardless of the theories of the mechanism of radiation protection, investigators have proceeded to test compounds for radioprotective properties. From this information they knew what classes of compounds gave protection,
which classes were more effective than others, what toxic qualities they possessed, and then they could develop new compounds with greater protective properties and less toxicity.

It was found that almost any organic compound, if present in sufficient concentration, is able to act as a protective agent against ionizing radiation by reducing the concentration of the OH radicals present in a solution (99, p. 307-402). This might be satisfactory in vitro, but was not desirable in a biological system. One group of compounds did prove interesting, however, - the sulfur containing amino acids, primarily cystine and cysteine, for this group as a whole gave greater protection with smaller amounts of compound (11, p. 109-124; 32, p. 439-446; 33, p. 2840-2842; 70, p. 464-470; 85, p. 23-28; 93, p. 153-175; 115, p. 213-214; 123, p. 1488-1496; 171).

Evidence was presented that certain sulfhydryl binding compounds as heavy metals, cyanide, chelating agents, N-ethylmaleimide (27, p. 467-474) and the increase of oxygen tension all could inactivate the radioprotective properties of the sulfhydryl compounds. The protectors were unable to combine with free radicals formed, to keep oxygen tension low, or to protect the sensitive sites on enzymes, especially those of the respiratory enzyme systems. Irradiation of these sulfhydryl compounds showed that in
general they were rapidly degraded by ionizing radiation, producing many free radical scavengers, or that the degradation product itself was the protective agent.

According to the compilation of present knowledge about protective compounds, certain facts stand out. In general there is a short carbon chain of two or three carbons; a nitrogen atom, which is basic; and a sulfur atom, which is, in fact or potentially, of a mercaptan character. The grouping of this general structure is N-C-C-S (85, p. 23-28). These compounds, if derivatives of amino acids, are decarboxylated, and the S- must be a free sulfhydryl (2, p. 392-415); substitution on the -NH$_2$ or -SH groups causes loss of protective properties. Results showed that the sulfhydryl group itself does not confer protective properties to a compound, and only those sulfhydryl compounds which contain an amino group are active in vivo. Some ring compounds, such as the thiazolidines, also have protective properties.

This group of compounds was found however, to be too toxic for biological systems, or toxic at the levels required for protection. Derivatives of these compounds, some biosynthetically and some chemosynthetically produced, were tested. Among the most effective found were $\beta$-mercaptoethylamine (MEA, cysteamine), 2-aminoethylisothiuronium bromide-hydro-bromide (AET), - both too toxic for general use, -2-mercaptopoethylguanidine (MEG), and 3-mercap-
topropylamine (MPA).

The protective properties of 3-mercaptopropylamine were of interest to us in our studies because: 1) it has been found as a degradation product of irradiated methionine (133, p. 330); 2) it has been produced chemosynthetically from methionine (164, p. 619-622); 3) it has been found to have a very stable complex with copper, which is being used as an index of radiation protective properties (91, p. 94-95; 92, p. 493-496), and is interesting in view of results obtained with Micrococcus radiodurans and copper (38, p. 41-42); 4) it has excellent radioprotective properties and less toxicity than mercaptoethylamine (81, p. 406-411; 92, p. 94-95); and 5) it has been identified as an end-product in the metabolic degradation of methionine (46, p. 189-227; 47, p. 257-258; 135, p. 61-68). As yet no pathways in this metabolic degradation have been studied or elucidated.

It is possible, then, that in the metabolic degradation of methionine, M. radiodurans produces 3-mercaptopropylamine which protects it from ionizing radiation, and that the gaseous end-product contains an excess that can be used to protect other biological systems.
EXPERIMENTAL STUDIES

METHIONINE UTILIZATION

In order to show more conclusively the requirement of Micrococcus radiodurans for methionine, or to show that other compounds can replace it, a series of experiments was undertaken, utilizing studies of other researchers of methionine-dependent microorganisms.

All studies were carried out on cultures grown in variations of the synthetic medium developed for M. radiodurans (38, p. 33) which contains the following constituents per 1000 ml of medium:

SYNTHETIC MEDIUM

Ammonium phosphate (dibasic, anhydrous) 500 mg
Glucose 5 g
L-methionine 10 mg
L-glutamic acid 500 mg
Thiamine hydrochloride 10 ìg
Biotin 10 ìg
Pyridoxine 200 ìg
Niacin 250 ìg
Salts A (Snell) 5.0 ml
Salts B (Snell, modified) 5.0 ml

All constituents are combined except Salts B, and diluted to 800 ml; salts B are then added and the medium is
diluted to 1000 ml. The pH is adjusted to 6.8-7.0. The medium is dispensed in 50 ml aliquots into 8-ounce prescription bottles with bakelite screw caps and sterilized for 15 minutes at 15 lbs. pressure (121°C).

Snell's Salts A and Salts B (modified) are as follows:

**Salts A**

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 25 \text{ g} \\
\text{K}_2\text{HPO}_4 & \quad 25 \text{ g} \\
\text{HOH dist. q.s. ad 250 ml} &
\end{align*}
\]

**Salts B (modified)**

\[
\begin{align*}
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 10 \text{ g} \\
\text{FeSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.5 \text{ g} \\
\text{MnSO}_4 \cdot 4\text{H}_2\text{O} & \quad 0.5 \text{ g} \\
\text{HOH dist. q.s. ad 250 ml} &
\end{align*}
\]

The sterile medium is inoculated from an actively growing culture of *M. radiodurans* and placed on a rotary shaker for 48 hrs at 30°C for satisfactory growth.

For the methionine substitution studies, the cells utilized were grown in the above medium with the methionine omitted for two successive transfers. The methionine-starved cells, which were now growing at minimum efficiency were then inoculated into synthetic medium in which the L-methionine had been substituted for by the compounds being tested, or as otherwise stated in the experimental
methods.

DeMoss and Happel (39, p. 137-141) in their studies with *Chromobacterium violaceum* found greater stimulation from autoclaved methionine than from sintered-glass filter-sterilized methionine in the medium. They felt that autoclaving may form a more active methionine derivative, as a methyl methionine sulfonium compound. They also found that methionine sulfoxide but not methionine sulfone was utilized by the organism.

**Experimental Methods:**

*M. radiodurans* was inoculated into synthetic medium containing either autoclaved or sintered-glass filter-sterilized D-, L-, or DL-methionine, placed on a rotary shaker and incubated at 30°C for 48 hours. Growth in all six cultures compared to a blank of inoculated but unincubated and unshaken synthetic medium gave a per cent transmittance of 77-79 at 630 μm, which did not bear out the preferential results obtained by DeMoss and Happel, if indeed a sulfonium compound was formed by autoclaving and was supposedly more advantageous for growth. These results also led us to the first indication of a methionine racemase activity being present.

An experiment was then set up with several methionine analogues and compounds cited in the literature as capable of replacing methionine (29, p. 517-528; 48, p. 129-130; 83, p. 293-298; 103, p. 924-933; 104, p. 104-108; 137, p.
Methionine is present in the synthetic medium at the rate of 1 mg/100 ml. All the compounds substituted for methionine were equated via their structural formulae to that of methionine for the quantities added. Sulfanilamide and \( \rho \)-aminobenzoic acid were added at the rate of 1 mg each, to media containing 1 mg/100 ml methionine.

The synthetic medium without methionine was dispensed in 50 ml aliquots into 8-ounce prescription bottles with screw cap lids. The compounds (1-19) listed below were added at half the quantity indicated into each of two pairs of 19 sets of bottles. Bottle sets #18 and #19 each had 0.5 mg L-methionine added to each bottle. One set of bottles, #20, contained L-methionine, sulfanilamide and \( \rho \)-aminobenzoic acid in the proper quantities. All bottles of media were autoclaved for 15 minutes at 15 lbs pressure (121°C).

Each bottle of sterile, cooled medium was inoculated with \( 4.12 \times 10^7 \) cells of \textit{M. radiodurans} for an initial concentration of \( 8.24 \times 10^5 \) cells per ml per bottle. Two bottles of complete synthetic medium were inoculated with the same number of cells and heat shocked to serve as inoculated controls for per cent transmittance at 630 \( \mu \) in a Bausch and Lomb spectrophotometer with \( 8.24 \times 10^5 \) = 100% T.
<table>
<thead>
<tr>
<th>Number, compound and structural formula</th>
<th>Molecular weight</th>
<th>Quantity 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) L-methionine</td>
<td>149.2</td>
<td>1 mg</td>
</tr>
<tr>
<td>(2) D-methionine</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>(3) DL-methionine (α-amino-ß-methylthiobutyric acid)</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>CH₃-S-CH₂-CH₂-CH-COOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4) DL-methionine sulfone</td>
<td>181.2</td>
<td>1.215 mg</td>
</tr>
<tr>
<td></td>
<td>(\text{O} \quad \text{NH}_2)</td>
<td>(\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CH-COOH})</td>
</tr>
<tr>
<td>(5) DL-methionine-dl-sulfoxide</td>
<td>165.2</td>
<td>1.107 mg</td>
</tr>
<tr>
<td></td>
<td>(\text{O} \quad \text{NH}_2)</td>
<td>(\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CH-COOH})</td>
</tr>
<tr>
<td>(6) DL-methionine-dl-sulfoximine</td>
<td>180.2</td>
<td>1.208 mg</td>
</tr>
<tr>
<td></td>
<td>(\text{NH} \quad \text{NH}_2)</td>
<td>(\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CH-COOH})</td>
</tr>
</tbody>
</table>
Table I - continued

<table>
<thead>
<tr>
<th></th>
<th>Chemical Name</th>
<th>Mass</th>
<th>Molar Mass</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>DL-methionine methyl sulfonium chloride</td>
<td>199.7</td>
<td>199.2</td>
<td>1.339 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH₃NH₂</td>
<td>CH₃-S-CH₂-CH₂-CH-COOH</td>
<td>Cl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(methyl methionine sulfonium chloride)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>N-acetyl-DL-methionine</td>
<td>191.0</td>
<td>190.2</td>
<td>1.280 mg</td>
</tr>
<tr>
<td></td>
<td>CH₃-C≡O</td>
<td>NH</td>
<td>CH₃-S-CH₂-CH₂-CH-COOH</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Ethionine (α-amino-γ-ethylthiobutyric acid)</td>
<td>173.2</td>
<td>173.1</td>
<td>1.094 mg</td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td>CH₃-CH₂-S-CH₂CH₂-CH-COOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>S-adenosylmethionine</td>
<td>379.0</td>
<td>379.2</td>
<td>2.544 mg</td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td>NH₂CHCOOH</td>
<td>(CH₂)₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH(CHOH)₂CH₂CH₂S-CH₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>DL-methioninol</td>
<td>135.2</td>
<td>135.1</td>
<td>0.906 mg</td>
</tr>
<tr>
<td></td>
<td>NH₂SH</td>
<td>CH₃-CH₂-CH₂-CH-CHOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table I - continued

<table>
<thead>
<tr>
<th></th>
<th>Chemical Name</th>
<th>Molecular Weight</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>DL-alanyl-DL-methionine</td>
<td>220.0</td>
<td>1.476 mg</td>
</tr>
</tbody>
</table>
|   | \[
|   | \[
|   | \[
|   | \[
|   | \[
| 13| glycyl-DL-methionine                             | 206.0            | 1.382 mg  |
|   | \[
|   | \[
|   | \[
|   | \[
|   | \[
| 14| \text{a-OH-\(\gamma\)-methylmercaptobutyric acid} | 334.1            | 1.120 mg  |
|   | \[
|   | \[
|   | \[
|   | \[
|   | \[
| 15| L-cysteine HCl                                    | 157.2            | 1.053 mg  |
|   | \[
|   | \[
| 16| S-methyl cysteine                                 | 135              | 0.906 mg  |
|   | \[
|   | \[
| 17| S-ethyl cysteine                                  | 149.2            | 1.000 mg  |
|   | \[
|   | \[


Table I - continued

<table>
<thead>
<tr>
<th>(18) sulfanilamide (ρ-sulfonic acid)</th>
<th>172.2</th>
<th>1.000 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="sulfanilamide structure" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(19) ρ-aminobenzoic acid (PABA)</th>
<th>137.1</th>
<th>1.000 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="ρ-aminobenzoic acid structure" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| (20) L-methionine  | 149.2 | 1.000 mg |
|                    | 172.2 | "        |
| sulfanilamide      |       |          |
| ρ-aminobenzoic acid| 137.1 | "        |


One pair of bottles of each set of inoculated bottles of media except the inoculated heat-shocked controls, was incubated on a rotary shaker at 30°C for a total of 144 hours, with a reading of the per cent transmittance taken at 48, 72, 96 and 144 hours. One pair of each set of inoculated bottles of media was incubated in a stationary condition in an anaerobic incubator (National Appliance Company, Portland, Oregon, Type 103) in the presence of N₂ at 30°C for 144 hours, with a reading of the per cent transmittance taken at the end of 144 hours.

*M. radiodurans* had been cultured successfully in a liquid media only with agitation in an aerobic atmosphere. Anaerobic cultures had been unsuccessful although the organism grows in hermetically sealed cans of meat which have been determined to contain no available oxygen. The cultures were incubated both aerobically and anaerobically to see if any of the above compounds would stimulate anaerobic growth.

The final readings were tabulated for six days of aerobic and anaerobic growth since alanyl-methionine and glycylmethionine had a 72 hour delay in response. Results are shown in Table II.

Kalan and Ceithaml (83, p. 293-298) had noted that a methionine dependent mutant of *Escherichia coli* is able to utilize D,L-alanine, D,L-homoserine, D,L-α-aminobutyric
TABLE II

Growth response of *M. radiodurans* to methionine analogues

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 hrs.</td>
<td>72 hrs.</td>
</tr>
<tr>
<td>(1) L-methionine</td>
<td>92.5</td>
<td>83</td>
</tr>
<tr>
<td>(2) D-methionine</td>
<td>92.5</td>
<td>85</td>
</tr>
<tr>
<td>(3) DL-methionine</td>
<td>92.5</td>
<td>83</td>
</tr>
<tr>
<td>(4) DL-methionine sulfone</td>
<td>96.5</td>
<td>93.5</td>
</tr>
<tr>
<td>(5) DL-methionine-dl-sulfoxide</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>(6) DL-methionine-dl-sulfoximine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(7) DL-methionine methyl sulfonium chloride</td>
<td>96.5</td>
<td>93</td>
</tr>
<tr>
<td>(8) N-acetyl-DL-methionine</td>
<td>98.5</td>
<td>98.5</td>
</tr>
<tr>
<td>(9) Ethionine</td>
<td>97</td>
<td>93.5</td>
</tr>
<tr>
<td>(10) S-adenosylmethionine</td>
<td>93.5</td>
<td>83</td>
</tr>
<tr>
<td>(11) DL-methioninol</td>
<td>97</td>
<td>92</td>
</tr>
<tr>
<td>(12) DL-alanyl-DL-methionine</td>
<td>98.5</td>
<td>97.5</td>
</tr>
<tr>
<td>(13) Glycyl-DL-methionine</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td>(14) α-OH-α-methylmercaptobutyric acid</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(15) L-cysteine HCl</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(16) S-methyl cysteine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(17) S-ethyl cysteine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(18) Sulfanilamide (p-sulfonic acid</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>(19) p-aminobenzoic acid</td>
<td>98</td>
<td>98</td>
</tr>
</tbody>
</table>
Table II - continued

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Aerobic 48 hrs.</th>
<th>72 hrs.</th>
<th>96 hrs.</th>
<th>144 hrs.</th>
<th>Anaerobic 144 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(20) L-methionine</td>
<td>97</td>
<td>96</td>
<td>92</td>
<td>85</td>
<td>100</td>
</tr>
<tr>
<td>sulfanilamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-aminobenzoic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Growth responses were measured after designated hours of incubation in synthetic medium, as modified, aerobically on a rotary shaker at 30°C and anaerobically and stationary at 30°C. Results are expressed as per cent transmittance at 630 μm.
acid, D,L-valine and D,L-isoleucine as the sources of the four-carbon moiety of the methionine molecule, plus inorganic sulfate of the medium; some could use D,L-homocysteine thiolactone·HCl or L,L-cystathionine as well as methionine; and some could substitute vitamin B_{12} for methionine, or vitamin B_{12} enhanced growth in the above compounds.

In order to see if *Micrococcus radiodurans* could synthesize its own methionine, it was decided to pursue the same experiment, using the same quantities of the compounds as cited by Kalan and Ceithaml. The attempt was completely unsuccessful except for the cultures containing 1) only vitamin B_{12} and 2) methionine in the quantity (10 μg/ml) that we had always used. Kalan and Ceithaml had used 20 μg/ml of D,L-methionine, and there was no apparent relationship to the quantities of the other compounds tested.

The experiment was repeated using 10 μg/ml methionine and the substituted constituents were added in proportion to their structural relationship to the methionine. Vitamin B_{12} was added at the same level we had used previously (an arbitrary amount since Kalan and Ceithaml did not designate the quantity they had used).

The synthetic medium without methionine was dispensed in 50 ml aliquots into 8-ounce prescription bottles with screw cap lids. The compounds (1-8) listed below were added, in quantities to give the concentrations per ml
### TABLE III

**Methionine precursors**

<table>
<thead>
<tr>
<th>Number</th>
<th>Compound and structural formula</th>
<th>Molecular weight</th>
<th>Quantity (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>D,L-methionine ( \text{NH}_2 )</td>
<td>149.2</td>
<td>10 20</td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_3 \text{-S-CH}_2 \text{-CH}_2 \text{-CH-COOH} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>D,L-homoserine ( \text{CH}_2 \text{-CH}_2 \text{-CH} )</td>
<td>119</td>
<td>7.9 23.8</td>
</tr>
<tr>
<td></td>
<td>( \text{NH}_2 \text{-CH-COOH} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>D,L-isoleucine ( \text{CH}_3 \text{NH}_2 )</td>
<td>131</td>
<td>8.8 26.2</td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_3 \text{-CH}_2 \text{-CH-CH-COOH} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>D,L-alanine ( \text{NH}_2 )</td>
<td>89</td>
<td>6.0 35.6</td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_3 \text{-CH-COOH} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>D,L-homocysteine-thiolactone·HCl</td>
<td>153</td>
<td>10.3 90.9</td>
</tr>
<tr>
<td></td>
<td>( \text{HCl-S} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_2 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_2 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \text{NH}_2 \text{-CH-CO} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6)</td>
<td>L,L-cystathionine</td>
<td>222</td>
<td>6.7 20.0</td>
</tr>
<tr>
<td></td>
<td>( \text{CH}_2 \text{-CH}_2 \text{-S-CH}_2 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \text{NH}_2 \text{-CH-COOH NH}_2 \text{-CH-COOH} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table III - continued

<table>
<thead>
<tr>
<th>Number</th>
<th>Compound and structural formula</th>
<th>Molecular weight</th>
<th>Quantity (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(7)</td>
<td>D,L-valine</td>
<td>117</td>
<td>7.9 23.4</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="D,L-valine structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(8)</td>
<td>D,L-α-aminobutyric acid</td>
<td>103</td>
<td>6.9 20.6</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="D,L-α-aminobutyric acid" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9)</td>
<td>Vitamin B_{12}</td>
<td></td>
<td>3 mg/100 ml</td>
</tr>
<tr>
<td></td>
<td>(cyanocobalamin)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* quantities used in our study
** quantities used by Kalan and Ceithaml
listed, to each of two pairs of 8 sets of bottles. To each of a pair of bottles from a set was added 1.5 μg of vitamin B₁₂. One pair of bottles (#9) contained only 1.5 μg vitamin B₁₂ in place of methionine. All bottles of media were autoclaved for 15 minutes at 15 lbs pressure (121°C).

Each bottle of sterile, cooled medium was inoculated with 3.08 x 10⁷ cells of M. radiodurans for an initial concentration of 6.16 x 10⁵ cells per ml per bottle. Two bottles of complete synthetic medium were inoculated with the same number of cells and beat shocked to serve as inoculated controls for per cent transmittance at 630 μ in a Bausch and Lomb spectrophotometer, 6.16 x 10⁵ = 100% T.

All the bottles except the inoculated controls were incubated on a rotary shaker at 30°C for a total of 144 hours with a reading of the per cent transmittance taken at 48, 96 and 144 hours. Results of the effect of methionine precursors and of vitamin B₁₂ on the growth of M. radiodurans are shown in Table IV.

Results:

The growth response of M. radiodurans to 14 analogues of methionine, sulfanilamide, ρ-aminobenzoic acid, 8 methionine precursors, and vitamin B₁₂ is shown in Tables II and IV.
<table>
<thead>
<tr>
<th>Compound number</th>
<th>no vitamin B₁₂</th>
<th>vitamin B₁₂ - 3 µg/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 hrs 96 hrs 144 hrs</td>
<td>48 hrs 96 hrs 144 hrs</td>
</tr>
<tr>
<td>(1) D,L-methionine</td>
<td>87 82 59</td>
<td>83 73 47</td>
</tr>
<tr>
<td>(2) D,L-homoserine</td>
<td>95 95 95</td>
<td>88 80 62</td>
</tr>
<tr>
<td>(3) D,L-isoleucine</td>
<td>94 92 92</td>
<td>84 80 46</td>
</tr>
<tr>
<td>(4) D,L-alanine</td>
<td>95 95 95</td>
<td>87 80 58</td>
</tr>
<tr>
<td>(5) D,L-homocysteine-thiolactone·HCl</td>
<td>96 96 94</td>
<td>88 84 58</td>
</tr>
<tr>
<td>(6) L,L-cystathionine</td>
<td>89 84 65</td>
<td>88 83 62</td>
</tr>
<tr>
<td>(7) D,L-valine</td>
<td>95 95 95</td>
<td>90 83 58</td>
</tr>
<tr>
<td>(8) D,L-α-aminobutyric acid</td>
<td>93 93 89</td>
<td>92 83 63</td>
</tr>
<tr>
<td>(9) Vitamin B₁₂</td>
<td>90 87 76</td>
<td></td>
</tr>
</tbody>
</table>

Growth responses were measured after designated hours of incubation in synthetic medium, as modified, aerobically on a rotary shaker at 30°C. Results are expressed as per cent transmittance at 630 μ.
Discussion:

The results shown in Table II indicate that 1) unlike the results of DeMoss and Happel, (39, p. 137-141), neither methionine sulfoxide nor methionine sulfone satisfies the methionine requirement of \textit{M. radiodurans}; 2) if indeed autoclaving of methionine results in a sulfonium compound, the sulfonium compound does not satisfy the methionine requirement; 3) methionine sulfoximine, commercially known as "A gene", a seed sterilizer, is toxic, as it is for many organisms, although Garber (52, p. 157-161) in his studies found that a methionine dependent mutant of \textit{Pseudomonas tabaci} could grow in it; 4) ethionine does have an inhibitory effect on growth; and 5) an excess of methionine is inhibitory.

The best yields were obtained from 1) the three forms of methionine, which would indicate that the organism has an active racemase; 2) S-adenosyl methionine, called "active methionine"; and 3) alanyl-methionine and glycyl-methionine which initially had a delayed response. Sulfanilamide was found to counteract the adverse influence of PABA. None of these compounds supported growth under anaerobic conditions. This study seemed to indicate that methionine is required by the organism, but that this compound does not support nor account for anaerobic growth.
The results shown in Table IV show that of the methionine precursors tested, only cystationine can replace methionine, and cystationine within its molecule contains the entire methionine molecule. The addition of vitamin B$_{12}$ increased the yield from methionine and gave the same yield with isoleucine as did methionine (isoleucine contains the methionine molecule except for the sulfur). Vitamin B$_{12}$ gave good results with other compounds but did not change the results obtained with cystathionine alone.

Vitamin B$_{12}$ alone was not completely satisfactory for good growth. One role of vitamin B$_{12}$ in metabolism involves methyl group transfer. This could be the case here with methionine being synthesized from the 4-carbon precursors and utilizing inorganic sulfates in the medium for the source of the sulfur atom in the molecule.

**ROLE OF VITAMIN B$_{12}$**

The stimulus to the growth of *M. radiodurans* by vitamin B$_{12}$, in this case possibly by methyl group transfer, brought up the question of other roles that vitamin B$_{12}$ is known to play. Lockingen (103, p. 924-933) noted, in the study of 27 coliform mutants, each with a genetic block in methionine metabolism, that with methionine they grew in the presence of oxygen, but methionine was inactive in the absence of oxygen. Vitamin B$_{12}$ stimulated mutants
showed no such oxygen dependency.

It was therefore decided to test *M. radiodurans* for anaerobic growth in the presence of vitamin B$_{12}$. Also, since one factor present in the sealed cans of meat had not been tested but could be involved in anaerobic growth, it was deemed advisable to determine the effect of hemoglobin in various concentrations on aerobic and anaerobic growth with and without methionine.

**Experimental Methods:**

The complete synthetic medium containing methionine (10µg/ml) was dispensed in 50 ml aliquots into 10 sets of 250 ml Erlenmeyer flasks, with four flasks per set. Vitamin B$_{12}$ at the rate of 3µg/100 ml was added to 6 sets of flasks. Sets #1, and #7 through #10 had no vitamin B$_{12}$ added. Set #11 had vitamin B$_{12}$ but no methionine. The flasks were stoppered with cotton plugs and autoclaved for 15 minutes at 15 lbs pressure (121°C).

A 0.1% aqueous solution of 2X crystalline beef hemoglobin (Nutritional Biochemicals Corporation, Cleveland, Ohio) was sterilized using a Zeitz filter. The hemoglobin solution was dispensed aseptically into the flasks of sterile, cooled synthetic media to give the final concentrations designated in Table V.

Each flask of sterile synthetic medium was inoculated with 4.01 x 10$^7$ of *M. radiodurans* to give an initial
**TABLE V**

Growth responses of *M. radiodurans* to hemoglobin during aerobic and anaerobic growth

<table>
<thead>
<tr>
<th>Number</th>
<th>Composition</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>CSM$^1$</td>
<td>65</td>
<td>97</td>
</tr>
<tr>
<td>(2)</td>
<td>CSM + B$_{12}^*$</td>
<td>55</td>
<td>96</td>
</tr>
<tr>
<td>(3)</td>
<td>CSM + B$_{12}^*$ + Hgb, 1 µg%</td>
<td>55</td>
<td>73</td>
</tr>
<tr>
<td>(4)</td>
<td>CSM + B$_{12}^*$ + Hgb, 2 µg%</td>
<td>55</td>
<td>71</td>
</tr>
<tr>
<td>(5)</td>
<td>CSM + B$_{12}^*$ + Hgb, 3 µg%</td>
<td>55</td>
<td>68</td>
</tr>
<tr>
<td>(6)</td>
<td>CSM + B$_{12}^*$ + Hgb, 4 µg%</td>
<td>55</td>
<td>70</td>
</tr>
<tr>
<td>(7)</td>
<td>CSM + Hgb, 1 µg%</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>(8)</td>
<td>CSM + Hgb, 2 µg%</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>(9)</td>
<td>CSM + Hgb, 3 µg%</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>(10)</td>
<td>CSM + Hgb, 4 µg%</td>
<td>65</td>
<td>85</td>
</tr>
<tr>
<td>(11)</td>
<td>SM$^2$ + B$_{12}^*$</td>
<td>55</td>
<td>96</td>
</tr>
</tbody>
</table>

* 3 µg/100 ml
$^1$ CSM = complete synthetic medium
$^2$ SM = synthetic medium, no methionine
concentration of $8.02 \times 10^5$ cells per ml per flask. One flask from each set was heat shocked to serve as an inoculated control for per cent transmittance at $630 \text{ m}_\lambda$ in a Bausch and Lomb spectrophotometer with $8.02 \times 10^5 = 100\%$ T. Turbidity and pigmentation from the hemoglobin made it necessary for each hemoglobin concentration to have a control.

One flask from each set was incubated on a rotary shaker at $30^\circ\text{C}$ for 8 days, with the per cent transmittance being taken at the end of that time. Two flasks from each set were incubated in a stationary condition in an anaerobic incubator (p. 28) in the presence of $\text{N}_2$ at $30^\circ\text{C}$ for 8 days, with the per cent transmittance taken at the end of that time. Results are shown in Table V.

Another important role involving vitamin $\text{B}_{12}$ is that of nucleic acid synthesis. Schweigert (137, p. 19-28) found that vitamin $\text{B}_{12}$ was active in the synthesis of the deoxyriboside moiety of deoxyribonucleic acid (DNA). Thymidine, plus an active purine as guanine, can replace vitamin $\text{B}_{12}$ in $\text{Lactobacillus leichmanii}$. His findings were the same for cytidine deoxyriboside and adenine deoxyriboside.

It was decided to utilize his experiment, adapted to $\text{Micrococcus radiodurans}$. Whereas per tube of media Schweigert had used:
we were using vitamin B$_{12}$ at the rate of 3 $\mu$g per 100 ml of media. We then used per 100 ml:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenine, guanine, uracil</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>vitamin B$_{12}$</td>
<td>5 $\mu$g</td>
</tr>
<tr>
<td>thymidine</td>
<td>5 $\mu$g</td>
</tr>
<tr>
<td>vitamin B$_{12}$</td>
<td>3 $\mu$g</td>
</tr>
<tr>
<td>thymidine</td>
<td>3 $\mu$g</td>
</tr>
</tbody>
</table>

Stock solutions of the above compounds were prepared so as to give the desired concentration when added to synthetic medium. Guanine was solublized in dilute potassium hydroxide before diluting to volume. Uracil was heated slightly so as to go into solution.

The synthetic medium without methionine was dispensed in 50 ml aliquots into 18 pairs of 250 ml Erlenmeyer flasks. We had also decided to determine the necessity of the glutamic acid in the medium, so flask pairs #19 and #20 were prepared with no methionine and no glutamic acid. To the flasks of synthetic medium the following compounds were added to give the final concentrations listed, so as to conform to the code given in Table VI.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>M = methionine</td>
<td>1 mg/100 ml</td>
</tr>
<tr>
<td>B = vitamin B$_{12}$</td>
<td>3 $\mu$g/100 ml</td>
</tr>
<tr>
<td>T = thymidine</td>
<td>3 $\mu$g/100 ml</td>
</tr>
<tr>
<td>G = guanine</td>
<td>0.1 mg/100 ml</td>
</tr>
</tbody>
</table>
A = adenine 0.1 mg/100 ml
U = uracil 0.1 mg/100 ml
g = glutamic acid
S = synthetic medium with none of the above

The flasks were stoppered with cotton plugs and autoclaved for 15 minutes at 15 lbs pressure (121°C).

Each flask of sterile, cooled medium was inoculated with $7.5 \times 10^6$ cells of methionine starved cells (p. 5) of *M. radiodurans* for an initial concentration of $1.5 \times 10^5$ cells per ml per flask. Two flasks of complete synthetic medium were inoculated with the same number of cells and heat shocked to serve as inoculated controls for percent transmittance at 630 nm in a Bausch and Lomb spectrophotometer, $1.5 \times 10^5 = 100\% T$.

All the flasks, except the inoculated controls, were incubated on a rotary shaker at 30°C for 96 hours with a reading of the percent transmittance taken at 96 hours. Results of the effect of vitamin B$_{12}$ in the synthesis of nucleic acids on the growth response of *M. radiodurans* are shown in Table VI.

Results:

The growth response of *M. radiodurans* to vitamin B$_{12}$ aerobically and anaerobically, with and without hemoglobin, and with active purines in nucleic acid synthesis is shown in Tables V and VI. It is shown that the organism grows
### TABLE VI

Effect of vitamin B₁₂ in the synthesis of nucleic acids on the growth response of *M. radiodurans*

<table>
<thead>
<tr>
<th>Number</th>
<th>Composition</th>
<th>% T</th>
<th>Plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>SM</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>SMB</td>
<td>34</td>
<td>4.7 x 10¹²</td>
</tr>
<tr>
<td>(3)</td>
<td>SB</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>(4)</td>
<td>SMT</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>SMBT</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>(6)</td>
<td>ST</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>(7)</td>
<td>STAGU</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>(8)</td>
<td>STAG</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>(9)</td>
<td>STAU</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>(10)</td>
<td>STGU</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>(11)</td>
<td>STA</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>(12)</td>
<td>STG</td>
<td>86</td>
<td>1.6 x 10⁷</td>
</tr>
<tr>
<td>(13)</td>
<td>STU</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>(14)</td>
<td>SBAGU</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>(15)</td>
<td>SMAGU</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>(16)</td>
<td>SMBAGU</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>(17)</td>
<td>SMBTAGU</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>(18)</td>
<td>S</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>(19)</td>
<td>SM - g</td>
<td>62</td>
<td>6.0 x 10⁸</td>
</tr>
<tr>
<td>(20)</td>
<td>SMB - g</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

Growth responses were measured after 96 hours incubation in synthetic medium, as modified, aerobically on a rotary shaker at 30°C. Results are expressed as per cent transmittance at 630 μm.
anaerobically in the presence of hemoglobin (1-4 mg%) and vitamin B$_{12}$; poorly with hemoglobin; and not at all with vitamin B$_{12}$ or methionine, alone or in combination. Purine and pyrimidine bases do not replace either methionine or vitamin B$_{12}$ so the role of vitamin B$_{12}$ is not necessarily that of nucleic acid synthesis.

Discussion:

The question of *M. radiodurans* being able to grow anaerobically in hermetically sealed cans of meat was not answered by the use of vitamin B$_{12}$. Previous work had shown that it grew in cans of pork (a source of the B vitamins), poorly in white chicken meat, but best in ground beef. Hemoglobin is an oxygen transporter in mammalian systems, and it is possible the hemoglobin present in the meat transports sufficient oxygen to be utilized by the organism.

The results of this study show that neither methionine, vitamin B$_{12}$, nor methionine plus vitamin B$_{12}$ can support anaerobic growth, although good aerobic growth is produced. Hemoglobin added to the methionine containing medium gives good growth aerobically, and some anaerobically; the addition of both hemoglobin and vitamin B$_{12}$ to the methionine-containing medium gives substantial growth anaerobically.

These results gave rise to another possible property of this organism - anaerobic photosynthetic growth.
The non-involvement of vitamin $B_{12}$ primarily in nucleic acid synthesis is borne out by our results.

**PHOTOSYNTHESIS**

The pigmented nature of *M. radiodurans* and the nutritional requirement for sulfur containing compounds had long raised the question of the possibility of a photosynthetic nature also existing in this microorganism. The fact that we had now obtained anaerobic growth again brought up this possibility. This possibility also brought up two more questions. If anaerobic photosynthetic growth were achieved, would the organism still be as highly pigmented and would there be any change in the radiation resistance of the microorganism.

**Experimental Methods:**

The complete synthetic medium (p. 21) plus vitamin $B_{12}$ was dispensed in 300 ml aliquots into 3 gas washing bottles (Drechsel, tall form, Kimax(Kimble #15060)) with a Teflon® covered stirring bar added to one tower. The towers were stoppered with gauze-covered cotton plugs, covered with caps of wrapping paper which were tied in place and autoclaved 20 minutes at 15 lbs pressure (121°C).

To the exterior glass tubing of the fritted glass-stoppered fittings of the gas washing bottles were attached
4 inch pieces of new surgical rubber tubing, I.D. $\frac{1}{3}$"; the ends of the rubber tubing on the outlet tube were plugged with gauze-covered cotton plugs and wrapped with paper which was tied in place; cotton-plugged Nalgene (Calcium chloride drying tubes #6201, The Nalge Co., Rochester, N. Y.) filters were attached to the rubber tubing on the inlet tubes. The glass-fittings with their attachments were wrapped in paper, tied with string and autoclaved 15 minutes at 15 lbs pressure ($121^\circ$C). A 1% solution of sodium sulfite ($\text{Na}_2\text{SO}_3$) was sterilized, using a sintered-glass filter and stored in screw-capped tubes.

To the sterile, still hot synthetic medium, 2 ml of sterile 1% $\text{Na}_2\text{SO}_3$ was added. The sterile glass fittings were set in place in the towers and Plasticine® (Harbutt's Plasticine Ltd, Bath, England) was molded around the neck fitting to seal it. While the medium was still hot, it was flushed with $\text{N}_2$ through the sterile airway filters. While still under positive pressure, the inlet and outlet tubing were clamped off with screw clamps and the ends further sealed with molded Plasticine®.

When the medium was cooled it was inoculated aseptically and presumably anaerobically with 2 ml of a culture of $\text{M. radiodurans}$, with the procedure as follows: The surgical tubing on the inlet side was scrubbed with 70% ethanol. It was draped with a sterile cloth to allow it
to dry. Into a sterile 10 ml syringe fitted with a sterile 1\(\frac{1}{2}\)" - 20 gauge needle was drawn 10 ml of a culture of *M. radiodurans*. The 20 gauge needle was replaced with a sterile 1" - 25 gauge needle. With the needle pointed upright, 2 ml of the cell suspension was expelled into a sterile pledget of cotton. The needle was then inserted through the sterilized surface of the wall of the surgical tubing on the inlet tube and 2 ml of the cell suspension inoculated into the flask; the plunger was pulled back and the N\(_2\)-pocket was used to force any cell inoculum still in the tubing down into the medium. The needle was withdrawn and the procedure was repeated with the other flasks.

The flask containing the Teflon\textsuperscript{®} stirring bar was placed on a Magnestir, (Labline, Inc. Chicago, Ill. #1250) which was set for moderate agitation. All three flasks were incubated at 30°C continuously illuminated by three lamps, each with a 60 watt light bulb. At the end of 25 days a slight turbidity was noted in the two stationary flasks, but none in the agitated flask. At the end of 30 days incubation pink colonies were noted on the bottom of the stationary flasks and smaller colonies adhering to the walls. That the flasks had maintained anaerobic conditions (no leaking-in of air) was evident from the inrush of air when the Plasticine\textsuperscript{®} plugging the inlet tube and the clamp were removed.
Since it was desirable to test the reproducibility of this phenomenon, to obtain as many cells as possible for other tests, and to have an easier method of cultivation, the procedure was modified as follows.

Eight-ounce prescription bottles with entire, smooth lips were used as culture flasks. Previously unused screw cap lids with soft plastic liners were used as closures. Each bottle when completely full holds almost 300 ml. The complete synthetic medium with vitamin B\textsubscript{12} was dispensed in 150 ml aliquots into each of 20 bottles and the bottles closed with screw cap lids. The medium was autoclaved for 15 minutes at 15 lbs pressure (121°C).

While the sterile medium was still warm (47-50°C), 10 bottles were inoculated with 10 ml of the "photosynthetic" culture. Additional warm sterile synthetic medium from the other bottles was added until the bottles lacked about 1 ml of being completely full; a sterile 1% solution of Na\textsubscript{2}SO\textsubscript{3} was added until the bottles overflowed and the screw cap lids were put on as tightly as possible. Nine bottles were incubated with illumination as before; 1 bottle was incubated in the dark.

Turbidity appeared in the illuminated bottles on the fifth day; minute colonies adhering to the walls of the bottles were apparent by the seventh day; on the tenth day larger colonies (aggregates of colonies falling off the walls) were clearly visible on the bottoms of the bottles.
The unilluminated control showed no growth; it was kept for six weeks at the end of which time it still showed no growth. The cultures were continued until the end of 14 days at which time they were harvested for subcultures, as above, and for study. The massed cells were paler in color than the heterotrophically grown cells, a pink-beige rather than a coral pink. Gram stains of smear showed the characteristic morphology of *M. radiodurans* - large, gram-positive cocci occurring singly, in pairs, tetrads, and sarcina-like packets.

The next procedure was to determine the radiation sensitivity of this de-pigmented *M. radiodurans*. The controls used and their designations in Table VII are as follow:

I - R₁-P - *M. radiodurans* grown photosynthetically
II - R₁-h- " " grown heterotrophically
III - U₁-p- " " originally isolated from an unirradiated source
IV - U₁-np- an organism isolated by another investigator who believed it to be a non-pigmented variant of *U₁*.
V - R₄R-p- *Brevibacterium oregonium*
VI - R₄R-np-an organism isolated by another investigator who believed it to be a non-pigmented variant of *B. oregonium*.

Cells I were pooled from several photosynthetic cultures. Cells II - VI were grown in tryptone-glucose-yeast extract broth (TGY):
Tryptone-Glucose-Yeast Extract Broth

Tryptone (Difco)  5 g
Yeast Extract (Difco)  1 g
Glucose (anhydrous)  1 g
Dilute to 1000 ml with tap water

The medium (TGY) is dispensed in 50 ml aliquots into 250 ml Erlenmeyer flasks. The flasks are stoppered with cotton plugs and autoclaved 20 minutes at 15 lbs pressure (121°C). Each of the cultures listed above, II - VI, were inoculated into two flasks of sterile, cooled TGY. The flasks were incubated aerobically on a rotary shaker at 30°C for 48 hours.

All cells were harvested aseptically by centrifugation in sterile round 50 ml Nalgene centrifuge tubes with caps (#3110, The Nalge Co., Rochester, N. Y.), using an International #1 centrifuge; washed 3 times with sterile 0.067M phosphate buffer; and each was dispensed in duplicate into 4 15 ml screw-capped irradiation vials (8 vials per culture; 48 vials per run). The vials were irradiated at the Co\(^{60}\) source, U. S. Bureau of Mines, Albany, Oregon at levels of 0, 250,000, 500,000 and 700,000 rad.

The irradiated cells and unirradiated controls were diluted in sterile 99 ml water blanks to dilutions of 10\(^6\); the dilutions were plated in duplicate from 10\(^2\) to 10\(^7\) in TGY agar.
Tryptone-Glucose-Yeast Extract Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Difco)</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast Extract (Difco)</td>
<td>1 g</td>
</tr>
<tr>
<td>Glucose (anhydrous)</td>
<td>1 g</td>
</tr>
<tr>
<td>Agar (Difco)</td>
<td>15 g</td>
</tr>
</tbody>
</table>

Dilute to 1000 ml with tap water

The medium was heated in a steamer until the agar had gone into solution; dispensed in 200 ml aliquots into 8-ounce prescription bottles with screw cap lids and autoclaved for 20 minutes at 15 lbs pressure (121°C).

The plates were incubated 72 hrs at 35°C and counted on Darkfield Quebec Colony Counter (American Optical Company, Buffalo, New York, #3330). Survival results are shown in Table VII.

Results:

The results seem to show that *M. radiodurans* can be induced to grow photosynthetically. The photosynthetically grown cells are not more radiation sensitive than are heterotrophically grown cells, so it is possible that the pigment is not related to the radiation resistance of *M. radiodurans*. The apparently greater survival of the photosynthetically grown cells is believed due to the fewer number of cells irradiated, a phenomenon previously shown by Duggan (44). The non-pigmented cells are either radiation sensitive variants or are not the same organism.
### TABLE VII

Survival rates of variants of radiation resistant microorganisms

<table>
<thead>
<tr>
<th>Number Cells</th>
<th>Radiation Dose in Rads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>R1P</td>
</tr>
<tr>
<td>II</td>
<td>R1h</td>
</tr>
<tr>
<td>III</td>
<td>U1p</td>
</tr>
<tr>
<td>IV</td>
<td>U1np</td>
</tr>
<tr>
<td>V</td>
<td>R4Rp</td>
</tr>
<tr>
<td>VI</td>
<td>R4Rnp</td>
</tr>
</tbody>
</table>
Discussion:

The results of this work, if borne out by further work being done in our laboratory, demonstrate that M. radiodurans is a very versatile microorganism. This, together with results obtained by another investigator on anatomical structure may lead to reclassification of this organism as to family, genus and species.

THE GASEOUS END-PRODUCT OF METHIONINE METABOLISM

Our studies have shown that methionine as such is required for the growth of M. radiodurans in a synthetic medium. We had observed also that in a non-synthetic medium, (TGY broth, p. 47) the organism, when grown in large (7 liter) quantities and sparged vigorously, produced a mercaptan-like gas as well as hydrogen sulfide, ammonia, and carbon dioxide. The production of this mercaptan-like gas was dependent on the presence of an adequate amount of methionine, but an excess of methionine hindered gas production. We found that the addition of 2 g of methionine per 7 L of TGY gave good gas production with vigorous sparging, starting about 20-24 hours after inoculation. 5 g per 7 L was detrimental to both gas production and to growth. The omission of the added methionine gave good growth, and sometimes gas was produced. We therefore felt that this unknown gas was an end-product of methionine catabolism, and if our
Figure 1. Proposed pathway for catabolism of methionine to \(\beta\)-mercaptoethylamine (MEA).
Figure 2. Proposed pathway for catabolism of methionine to 3-mercaptopropylamine (MPA).
assumptions were correct, this gas could possibly be similar to certain radiation protective compounds, such as \( \beta \)-mercaptoethylamine (I) (MEA, cysteamine) or 3-mercaptopropylamine (II) (MPA),

(I) \[ \text{HS-CH}_2\text{-CH}_2\text{-NH}_2 \]

(II) \[ \text{HS-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH}_2 \]

both of which are structurally in accord with the requirements of a good radiation protective compound, "...there is a short carbon chain of two or three carbons; a nitrogen atom, which is basic; and a sulfur atom, which is, in fact or potentially, of a mercaptan character" (p. 19).

It would be possible for methionine to be metabolized to \( \beta \)-mercaptoethylamine or 3-mercaptopropylamine via homocysteine, by way of two pathways known to occur in higher animals and in some microorganisms.

Pathway II with part of the homocysteine going to \( \text{NH}_3 \), \( \text{H}_2\text{S} \) and pyruvic acid, and part being decarboxylated and going to mercaptopropylamine is a pathway that fits in with our results. Unfortunately the enzyme systems in this pathway are still unknown (135, p. 61-68).

Both MEA and MPA are very effective in preventing radiation damage. Knoblock and Purdy (91, p. 94-95; 92, p. 493-496) have shown a correlation between the radiation protective capacity of an agent and the instability constant formed between the agent and divalent copper. Their data
show that some relation may exist between a compound's chelating power and its protective power against ionizing radiation. There may be some correlation between this fact and the copper tolerance (15,630 ppm CuSO$_4$) of _M. radiodurans_ (38, p. 41).

**Experimental Methods:**

Prior to successful attempts to trap the gaseous compound in a solid state, we had demonstrated the presence of sulfhydryl compounds using gas partition chromatography (37, p. 932-941). Columns used were:

1) Benzyl ether (Beckman 17297) - 6 ft. column  
2) Carbowax 600 - 6 ft column

The compound was condensed in a gas trap using a dry-ice alcohol bath and read in a Beckman GC-2 Gas Chromatograph. The chromatograph demonstrated the possibility of two fractions coming off, a fact substantiated by paper chromatography using N-ethylmaleimide and alkali, in which H$_2$S and a thiol were demonstrated.

Sulfhydryl compounds react readily with metals, such as mercury, sodium and cadmium, but the resulting complexes are difficult to purify and to assay. Many investigators feel that vigorous treatment of the complexes alters them, so that the end-product may not be the product one is attempting to study.

The classical method for trapping mercaptans is that
of Wertheim(172, p. 3661-3664) utilizing an alcoholic solution of mercuric cyanide. The trapping solution developed was:

**MERCURIC CHLORIDE TRAPPING SOLUTION**

\[
\begin{align*}
\text{Hg(CN)}_2 & \quad 8 \text{ g} \\
\text{EtOH - 95\%} & \quad 100 \text{ ml} \\
\end{align*}
\]

Dissolve crystals in warm EtOH; filter to remove any residue.

The solution was placed in a gas washing bottle. To the inlet tube of the fritted glass fittings of the bottle was added a sintered glass air-dispersion tip.

*M. radiodurans* was cultured in 7 L quantities in TGY for gas production. The modification of the medium for maximum gas production and the preparation of the equipment was as follows:

**TGY BROTH FOR GAS PRODUCTION**

\[
\begin{align*}
\text{Tryptone (Difco)} & \quad 35 \text{ g} \\
\text{Yeast Extract (Difco)} & \quad 7 \text{ g} \\
\text{Glucose (anhydrous)} & \quad 7 \text{ g} \\
\text{D,L-methionine (NBCo)} & \quad 2 \text{ g} \\
\end{align*}
\]

The dehydrated constituents were placed in a 2½ gal Pyrex (Corning #1595) carboy and dissolved in 7 L tap water. Anti-foam A (Dow-Corning) was sprayed on the surface of the media to minimize foaming during aeration. The carboy was stoppered with a gauze-covered cotton plug, capped with paper which was tied in place with string, and the medium was autoclaved 60 minutes at 15 lbs pressure (121°C).

A #12 - 2-holed rubber stopper was fitted with glass
tubing. The inlet tube was fitted on the inner end with a fritted-cylinder tipped gas dispersion tube to reach almost to the bottom of the carboy. A cotton-plugged Nalgene® airway filter was fitted on the outer end of the inlet tube. The outlet tube was fitted with surgical tubing on the outer end. The entire apparatus was wrapped in paper, tied with string and autoclaved 20 minutes at 15 lbs pressure (121°C).

The sterile, cooled medium was inoculated with 50 ml of an actively growing culture of M. radiodurans in synthetic medium (p. 21). The stopper-sparger-airway set-up was aseptically inserted in the carboy, and the stopper was held in place with a heavy metal clamp. The inoculated carboy of media was placed in a water bath at 30°C; the airway was attached to an air pump, and the air flow was regulated to give moderate aeration of the culture. Gas production generally started 20-24 hours after inoculation of the medium, and lasted until 48-54 hours after inoculation.

The outlet tube from the culture flask was attached to the inlet tube of a gas washing bottle containing the alcoholic Hg(CN)₂. A yellow-green precipitate started to form in 2 hours. When the solution appeared saturated, it was removed and a fresh solution of alcoholic Hg(CN)₂ was added. Fresh cultures and Hg(CN)₂ solutions were kept going continuously until a moderate amount of crystalline
material had been accumulated. The crystals were washed 3 times in ethanol and dried in a dessicator. Attempts were then made to analyze the dried crystals.

The capillary melting point of the crystals was tested in the following manner: The crystals were placed in a capillary tube sealed at one end which was placed in a beaker of cottonseed oil (Wesson Oil) (which is smokeless to 221°C) on a Temco hot plate, type 1900 (Thermo Electric Mfg. Co., Dubuque, Iowa). The bottom of the tube containing the crystals was at the same level as the bulb of a thermometer. The oil was stirred continuously to keep the temperature uniform. No melting point was achieved. Reference melting points were:

- methyl mercaptan, mercuric salt M.P. 176°C
- ethyl mercaptan, mercuric salt M.P. 76°C
- mercuric cyanide disintegrates with heat

Mercuric derivatives of known sulfur compounds were prepared. H₂S gave a black precipitate and MEA gave a greenish precipitate.

The presence of sulfur was determined using Grote's method (58, p. 25-30). We had used this method because it supposedly gave different reactions for different sulfur linkages. However, this procedure, although it does show the presence of sulfur, is not as specific nor as sensitive as the reference states. The chromatographic technic
of Benesch (15, p. 981-982), using N-ethylmaleimide and alkali gave positive results for the presence of -SH and of H₂S. Attempts at quantitative analysis for carbon, sulfur and nitrogen were singularly unsuccessful when attempted by 1) Schwarzkoff Analytical Laboratories, Woodside, N.Y., 2) Weiler and Strauss Laboratories, Oxford, England, and 3) our laboratory.

The methods we had tried were several modifications of the microkjeldahl, including those for metal derivatives (165, p. 238; 167, p. 637); a micro-sulfur wet-combustion method utilizing the formation of BaSO₄ (170, p. 1516-1517); and a carbon wet-combustion method utilizing the formation of BaCO₃ (165, p. 15-17).

The solubility of the mercuric salt was tested in 15 solvents used for infrared spectroscopy, some of them being specific for sulfur compounds (4, p. 221). The solvents tested were carbon tetrachloride, redistilled methanol, carbon disulfide, ethanolamine, chloroform, n,n-dimethylformamide, dimethoxypropane, 2,2,4-trimethylpentane, propanol-1, propanol-2, acetone, anhydrous ether, cyclohexane, acetonitrile and formamide. The salt was insoluble in all of them. The salt was also insoluble in ammonia, ethylene chloride, benzene, water and butanol, but soluble in hot ethanol, 95%.

A potassium bromide pellet and a Nujol mull were made of the crystals (120, p. 360-374) and infrared spectra run
on them using a Beckman IR-5 Infrared Spectrophotometer. The spectrum showed the possible presence of a mercapto group, an amino or imino group and a short carbon chain with the possibility of a C=C or a C=O. Analysis of this latter fact showed that neither was present.

An alcoholic sodium solution was tried for trapping the mercapto compound, (72, p. 4756-4757) but the CO₂ being respired by M. radiodurans soon saturated the solution, and made the procedure useless.

A cadmium derivative was tried next. The final procedure used in obtaining the derivative was as follows:

The culture was set up as on p. 60. When the mercaptide gas was being produced (as shown by a yellowish ring in an alcoholic Hg(CN)₂ solution) a series of 3 gas washing bottles was substituted for the Hg(CN)₂ solution.

**Bottle #1** - to remove H₂S and CO₂

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CdO</td>
<td>5 g</td>
</tr>
<tr>
<td>HOH, dist.</td>
<td>500 ml</td>
</tr>
<tr>
<td>CH₃COOH, glacial</td>
<td>8 ml</td>
</tr>
</tbody>
</table>

The acetic acid allows the insoluble CdO to go into solution. Both H₂S and CO₂ had to be removed from the gaseous mixture because they were readily trapped in the next solution also.

**Bottle #2** - to trap the mercaptide

<table>
<thead>
<tr>
<th>Substance</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd(Cl)₂</td>
<td>5 g</td>
</tr>
<tr>
<td>EtOH - 95%</td>
<td>500 ml</td>
</tr>
</tbody>
</table>
Bottle #3

Hg(CN)₂ 5 g
EtOH - 95% 100 ml

Dissolve crystals in warm EtOH; filter to remove any residue

Bottle #3 was a fresh alcoholic Hg(CN)₂ solution in case the mercaptide was not trapped in bottle #2.

Trap #1 removed CdS, an insoluble yellow precipitate, and Cd(CO₃)₂, a whitish precipitate. Trap #2 formed a creamy precipitate of the cadmium mercaptide. Cd(CO₃)₂ and CdS are insoluble in hot water; Cd mercaptides are soluble. To remove any trace of Cd(CO₃)₂ and CdS, the precipitate from trap #2 was centrifuged out; resuspended in water and centrifuged; resuspended in hot water and filtered through an ultra-fine sintered-glass filter. The filtrate was then treated with 4% AgNO₃ until the solution, passing through an ultra-fine sintered-glass filter no longer gave a positive test for chlorides. The filtrate was then dried in a vacuum oven at 70°C.

A potassium bromide pellet and a Nujol mull were made of the cadmium mercaptide since this compound also gave poor results with the solvents for infrared spectroscopy. The results of the infrared scan were similar to those of the mercury salt. The yellow precipitate from trap #1 gave an identical scan with known CdS. A Nujol mull was also made from a cadmium derivative of MEA and this also gave a scan resembling the unknown.
The supernatant fluid of trap #2 had a strong sulfurous odor. It was possible that only an excess of the mercaptide had precipitated out and that there was some in solution. After removing the precipitate by centrifugation and evaporating the supernatant not quite to dryness, an oily residue was noticed. A few drops of this oily material was placed between salt crystals and an infrared scan run. The tracing was very like that of the known mercaptides, and the mercury and cadmium derivatives.

To remove any remaining water, the mixture was placed in a separatory funnel with petroleum ether and shaken. The globules were in the aqueous phase at first but gradually rose to the ether phase. After 24 hours, the mixtures were separated; the ether phase vacuum dried, followed by two more ether extractions and dryings, leaving a yellowish oil.

Chemical analysis of the cadmium mercaptides was also unsatisfactory.

Considering the isolation of a soluble compound from the supernatant of trap #2, it was felt possible that the gas might be soluble in the aqueous phase of the medium, especially since the gas production appears to decline after 48 hours, and if this is a radiation protective compound it should still be present, because *M. radiodurnas* is still very radio-resistant beyond this time (44).
Also, it might be possible to obtain the compound in greater yield from the medium. The cells from 72 and 96 hour cultures in TGY were harvested, using a Sharples high speed continuous flow centrifuge and the two lots of media retained separately. The whole volume of each was extracted with petroleum ether in separatory funnels and the extracts taken to dryness \textit{in vacuo}. The residues each were extracted 3 times more in petroleum ether and dried. Infrared scans of each residue showed a similarity to the oily residue, and the presence of impurities. The yield was not great, but we now knew that this end-product of metabolism was soluble in the medium and non-toxic to the cells producing it.

Proctor (121) had used buffered N-ethylmaleimide (NEM) to trap mercaptan-like compounds from irradiated meats. The irradiated meat distillates were collected in buffered NEM and allowed to stand overnight; the solution was saturated with sodium chloride and extracted with ether yielding a yellow liquid oil, which gave the characteristic red thiol color with the addition of alkali. Benesch (15, p. 981-982) had stated that NEM-sulfhydryl complex was not pH dependent, but Morrell (111, p. 1057-1061) said the reaction was faster, more complete and more stable at pH 6.8, whereas there was rapid reduction of rate, completion and stability below pH 6.1 and above pH 7.3.
The procedure was adapted to trap the gas being produced by *M. radiodurans*.

**Phosphate buffer**

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 4.5 \text{ g} \\
\text{K}_2\text{HPO}_4 & \quad 11.6 \text{ g} \\
\text{HOH dist.}, \text{ q.s. ad } 1000 \text{ ml} & \\
\end{align*}
\]

**N-ethylmaleimide solution**

\[
\begin{align*}
\text{NEM (0.01 mol)} & \quad 1.25 \text{ g} \\
\text{PO}_4 \text{ buffer} & \quad 300 \text{ ml} \\
\text{adjust pH to 6.8-7.0} & \\
\end{align*}
\]

A 7 L culture of *M. radiodurans* was set up as on p. 60. At 24 hours a gas washing bottle was attached via Tygon tubing to the outlet tubing of the culture. Successive cultures (five) were used to replace the original culture until the buffered-NEM solution seemed saturated as evidenced by a sulfurous gas emerging from the trap, and the trap became turbid. The trap was left at room temperature overnight; saturated with NaCl; the mixture was transferred to a separatory funnel, and 50 ml of anhydrous ether added. The mixture was shaken thoroughly and placed in a refrigerator for 6 hours for the two phases to separate. The aqueous phase was removed; the other phase placed in a flask and evaporated in vacuo. A yellow oil was obtained which gave the characteristic red color for thiols with the addition of potassium hydroxide.

A buffered-NEM preparation was made from a sample of MEA (oil) obtained from the Delta Chemical Corporation.
Infrared spectra were run on this compound and on the unknown. The unknown compared favorably with the metal derivatives and had some similarity to MEA.

The combining of N-ethylmaleimide with thiols is dependent on the presence in the compound of a free sulfhydryl which attaches to the ring of the NEM, producing a relatively stable complex in which the only change from the parent compound is the breaking of the double bond of the imide ring of the NEM to a single bond. Disulfides and compounds with substitutions on the sulfhydryl would be unreactive. The color reaction for NEM and thiols on the addition of alkali (10% KOH in isopropyl alcohol) is a red-purple; with H2S the color reaction is a blue-violet.

\[
\begin{align*}
\text{HC} & \quad \text{CH} \\
\text{C}_2\text{H}_5 & \quad \rightarrow \\
\text{N} & \quad \text{C} \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{CH} \quad \text{S} \quad \text{R} \\
\text{C}_2\text{H}_5 & \quad \rightarrow \\
\text{N} & \quad \text{C} \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
\end{align*}
\]

N-ethylmaleimide
M.W. 125

thicol complex

Data for N-ethylmaleimide complexes with the two proposed end-products would be:

(I) mercaptoethylamine (MEA) M.W. 77

\[
\begin{align*}
\text{HC} & \quad \text{CH} \\
\text{C}_2\text{H}_5 & \quad \rightarrow \\
\text{N} & \quad \text{C} \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{CH} \quad \text{S} \quad \text{-(CH}_2)_2\text{-NH}_2 \\
\text{C}_2\text{H}_5 & \quad \rightarrow \\
\text{N} & \quad \text{C} \quad \text{N} \quad \text{C} \quad \text{C} \quad \text{O} \\
\end{align*}
\]
The molecular weight of the MEA-NEM complex is 202, which consists of:

- Carbon: 47.52%
- Hydrogen: 6.93%
- Oxygen: 15.84%
- Nitrogen: 13.86%
- Sulfur: 15.84%

(II) Mercaptopropylamine (MPA) M.W. 91

\[
\text{HC} \equiv \text{CH} + \text{HS-(CH}_2\text{)}_3\text{-NH}_2 \rightarrow \text{H}_2\text{C} \equiv \text{CH-S-(CH}_2\text{)}_3\text{-NH}_2
\]

The molecular weight of the MPA-NEM complex is 216, which consists of:

- Carbon: 50.00%
- Hydrogen: 7.40%
- Oxygen: 14.81%
- Nitrogen: 12.96%
- Sulfur: 14.81%

Chemical analysis for carbon, nitrogen and sulfur of the NEM-thiol complex were made.

1. Carbon Determination

Carbon determinations were made using a persulfate oxidation method in a closed system (165, p. 15-17).

Materials:

1. Silver nitrate - a fresh solution of 4% AgNO₃
Figure 3. Special reaction flasks for closed combustion of carbon. a.1.) 125 ml Pyrex flask; a.2.) center well for base, with evacuating side arm; a.3.) stopper with vent. b) reaction vessel assembled, vent open; c) reaction vessel assembled, vent closed.
2. Persulfate, finely ground
3. Sulfuric acid - 6N-H₂SO₄
4. Sodium hydroxide - 5N - NaOH, CO₂ free
5. Distilled water, CO₂ free
6. Barium chloride solution - 5% BaCl₂, aged at least one week.
7. High-vacuum grease (Dow Corning)

Apparatus:
1. Special combustion flasks - Figure 3
2. Oven

Procedure:
1. 10 mg of sample - resuspend in 5-10 ml dist. water
2. 1 ml of above in special reaction flask
3. AgNO₃ - 4% - 1 ml (use more if precipitate forms)
4. 0.5-0.6 g persulfate, finely ground
5. H₂SO₄ to make solution 0.5 - 5 N
6. Dist. HOH, q.s. ad 15 ml
7. Base in center well - 1 ml - 5N-NaOH, CO₂ free
8. Stopper with all stoppers greased with high vacuum grease; pull vacuum; swirl to mix.
9. Place flask in oven 73-76°C until clear - agitate occasionally (takes about 1 hr)
10. Release vacuum
11. Remove sample; add precipitation mixture - 5% BaCl₂
12. Filter through previously dried and weighed sintered-glass filters (M); wash flasks and filter with CO₂-free dist. HOH; dry in oven at 180°C for at least 2 hours; weigh.
13. Calculate amount of carbon present

(1) Unknown:

10 ml vol. flask & stopper, dried = 13.0122 g
sample = 13.0310 g
sample = .0188 g

(2) Control - L-methionine:

10 ml vol. flask & stopper, dried = 13.5055 g
control = 13.5165 g
control = .0110 g

(3) Reagent blank:

In ml of the diluted unknown was placed in each of 3 reaction flasks; 1 ml of the diluted methionine was placed in each of 2 reaction flasks; 1 reaction flask was used as a reagent control. Results are shown in Table VIII.

2. Nitrogen Determination

Due to the minute amount of the compound and to the large amount of sample necessary for a micro-kjeldahl, it was necessary to use an ultra-micro-analytical method for the nitrogen. A microkjeldahl method for nitrogen measurement which included a microdiffusion procedure described by Seligson and Seligson (138, p. 324-333) for use in clinical investigations was modified and adopted for use. The sensitivity range of the method is 2 to 25 micrograms. If the procedure were followed using an acid digestion mixture a faint reaction was produced on Nesslerization; a
# TABLE VIII

Carbon determination of an N-ethylmaleimide-thiol complex

<table>
<thead>
<tr>
<th>Sample</th>
<th>Filter</th>
<th>Filter+BaCO₃</th>
<th>BaCO₃</th>
<th>C/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>12.9400 g</td>
<td>12.9552 g</td>
<td>15.2 mg</td>
<td>9.25 mg</td>
</tr>
<tr>
<td>(1)</td>
<td>13.0805 g</td>
<td>13.0959 g</td>
<td>15.4 mg</td>
<td>9.38 mg</td>
</tr>
<tr>
<td>(1)</td>
<td>13.2280 g</td>
<td>13.2430 g</td>
<td>15.0 mg</td>
<td>9.12 mg</td>
</tr>
<tr>
<td>(2)</td>
<td>13.1418 g</td>
<td>13.1491 g</td>
<td>7.3 mg</td>
<td>4.45 mg</td>
</tr>
<tr>
<td>(2)</td>
<td>13.0254 g</td>
<td>13.1324 g</td>
<td>7.0 mg</td>
<td>4.43 mg</td>
</tr>
<tr>
<td>(3)</td>
<td>13.3700 g</td>
<td>13.3701 g</td>
<td>0.1 mg</td>
<td>0.06 mg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total Carbon Found</th>
<th>Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 9.25 mg = 49.25%</td>
<td>(I) 8.93 mg = 47.5%</td>
</tr>
<tr>
<td>(II) 9.40 mg = 50.0%</td>
<td></td>
</tr>
<tr>
<td>(2) 4.38 mg = 39.8%</td>
<td>4.43 mg = 40.3%</td>
</tr>
</tbody>
</table>
digestion procedure using concentrated $K_2CO_3$ produced no reaction. It was then suggested to try direct Nesslerization, this procedure being effective if a free $NH_2$ group were present. Since the proposed compounds had one free $NH_2$ group, and a nitrogen bound in the imide ring, direct Nesslerization would give us half the theoretical nitrogen. The analysis was run against an $NH_3$ standard, $1 \text{ ml} = 100 \text{ g N}$.

**Materials:**

1. Nitrogen standard - ammonium sulfate, 0.4716 g per liter, in deionized water. This solution provides 100 $\mu g$ N per ml

2. Acid digestion mixture

   Sulfuric acid, conc. C.P. 160 ml
   Potassium sulfate, anhydrous 104.4 g
   Mercuric sulfate 5.5 g

   The salts were dissolved in 100-200 ml of deionized water, the sulfuric acid added cautiously, and the mixture diluted to 1000 ml, using deionized water.

3. Alkaline wash solution

   Sodium thiosulfate, anhydrous 50 g
   Sodium hydroxide, reagent grade 40 g
   Deionized water to 1000 ml

4. Koch-McMeekin Nessler's Solution (95, p. 797)
Nessler's solution was diluted 1:10 with deionized water for use.

5. Sulfuric acid, approximately 1N, in dropper bottle.
6. Sodium hydroxide pellets.

Apparatus:
1. Small, hard-glass bottles of about 25 ml capacity, of a type commonly used for packaging antibiotic solutions for parenteral use. Each was fitted with a tight rubber stopper having a center depression into which was fitted a short glass rod. When the stopper was in place, the rod reached to about one centimeter from the bottom of the bottle. The lower end of the rod was flared or footed and roughened with sandpaper so it would retain a thin film of acid.

2. Marbles

3. An electric hot plate provided with a continuous dial-type heat control; Temco, type 1900 (Thermo Electric Mfg. Company, Dubuque, Iowa).

4. A Beckman model B spectrophotometer, with standard cuvettes.

5. A motor-driven revolving drum for rotation of bottles during the microdiffusion process.

Method:
1. A sample, 0.2-1.0 ml in volume, was pipetted into a bottle and the volume adjusted, if less than 1.0
ml, to 1.0 ml by the addition of deionized water.

2. 1 ml of acid digestion mixture was added and the bottle was placed on the hot plate and warmed to drive off all water. The temperature control was kept below 2 so that the solution did not boil.

3. When all water was gone, as indicated by the dry inner walls of the bottle, a marble was placed over the mouth of the bottle and the temperature was gradually increased until the mixture turned slightly brown and white fumes appeared in the bottle.

4. Strong heating at 5 on the control dial was continued for about 2 hours until the acid could be seen refluxing on the inner walls approximately to the shoulder of the bottle. Heating was continued for 20 minutes more, the heat turned off, and the bottle allowed to cool in place.

5. 2 ml of alkaline wash solution and 3 NaOH pellets were added and the bottle quickly stoppered using the rubber stopper-glass rod assembly described. (If a number of determinations were being done, addition of alkaline wash solution, NaOH pellets, and stoppering were completed for one bottle at a time, to avoid loss of ammonia).

6. Just before insertion of the stopper and rod into
each bottle, the roughened portion of the rod was dipped into 1N sulfuric acid to coat it with a thin film of acid for collecting the ammonia that would be evolved in the microdiffusion process.

7. The stoppered bottles were rotated several times to help dissolve the NaOH pellets and then placed in a horizontal position on the revolving drum (12 cycles per hour) and rotated overnight in an incubator at 35°C.

8. Each determination of unknown samples was accompanied by a set of standards consisting of bottles containing 5, 10, 15 and 20 μg of nitrogen provided by the standard ammonium sulfate solution and a blank consisting of 1 ml of deionized water. Standards and blank were digested and treated like the unknown samples.

9. After the microdiffusion process, the bottles were cooled to room temperature; each was opened, the glass rod carefully removed and dipped in a small beaker containing 10 ml of the diluted Nessler's solution.

10. After 5 minutes, the yellow-colored solutions were read in a Beckman model B spectrophotometer at 420 mμ, with sensitivity setting at "1" and the reagent blank at 100% T, O.D. = 0.0
11. A standard curve was prepared from the optical density values and micrograms of nitrogen of the standards.

12. Nitrogen values for unknowns were obtained from the curve.

This method was utilized for the standard curve, but was unsatisfactory for the unknown samples because the ammonia was lost at step #2. Direct Nesslerization of the unknowns was used, beginning with step #9 - adding the diluted sample directly to the Nessler's solution (and unfortunately introducing an error in dilution).

Since we were expecting to obtain only half the theoretical nitrogen value with the direct Nesslerization, in order to have our results fall in the optimum range of 10-15 μg, we used 20.1 mg of sample, rather than 10 mg. 20.1 mg of sample was diluted 1:10 with deionized water; 1 ml of this dilution was diluted 1:10 with deionized water, with 1 ml now theoretically containing 13.93 μg N for MEA (I), or 13.04 μg N for MPA (II).

The results of the nitrogen determination are shown in Table IX.

3. Sulfur Determination

Sulfur determinations were made utilizing a micro-sulfur method (170, p. 1516-1517) which has a sensitivity range of 0-20 mg sulfur.
Figure 4
TABLE IX
Nitrogen determination of an N-ethylmaleimide-thiol complex

<table>
<thead>
<tr>
<th>Sample</th>
<th>O.D.</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µg standard</td>
<td>.091</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>10 µg</td>
<td>.172</td>
<td>10.0 µg</td>
</tr>
<tr>
<td>15 µg</td>
<td>.250</td>
<td>15.0 µg</td>
</tr>
<tr>
<td>20 µg</td>
<td>.338</td>
<td>20.0 µg</td>
</tr>
<tr>
<td>0.2 ml of 1:100 of unknown</td>
<td>.041</td>
<td>2.5 µg</td>
</tr>
<tr>
<td>0.4 ml</td>
<td>.086</td>
<td>5.0 µg</td>
</tr>
<tr>
<td>0.5 ml</td>
<td>.102</td>
<td>6.0 µg</td>
</tr>
<tr>
<td>0.6 ml</td>
<td>.123</td>
<td>7.5 µg</td>
</tr>
<tr>
<td>0.8 ml</td>
<td>.155</td>
<td>9.0 µg</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>.212</td>
<td>12.5 µg</td>
</tr>
</tbody>
</table>

Results are expressed in O.D. read at 420 µm.

<table>
<thead>
<tr>
<th>Total Nitrogen</th>
<th>Found</th>
<th>Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.44%</td>
<td>(I) 13.86%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(II) 12.96%</td>
</tr>
</tbody>
</table>
Materials:
1. Oxidant - 3 volumes of concentrated HNO$_3$ to 1 volume of 60% perchloric acid
2. Copper wire
3. Barium chloride solution - 5% - aged at least 1 week before use
4. Tween 80, polyoxyethylene sorbitan mono-oleate (Atlas Powder Co.) in 2 different dilutions: 2 and 20%
5. Nitric acid, 0.05 N
6. Hydrochloric acid, 1N
7. Ethyl alcohol, 50%
8. Sulfuric acid, 0.1N, standardized volumetric solution.

Apparatus:
1. Centrifuge tubes, 40 ml round bottomed Pyrex® (A.H. Thomas Co. #3121-D) modified by poking in the flame softened bottom with a blunt pencil point - Figure 5.
2. Sand bath
3. Steam bath

Method:
1. To sample (containing up to 20 mg sulfur) in a
Figure 5. Modified centrifuge tube.
modified tared 40 ml centrifuge tube add 15 ml of oxidizing agent

2. After foaming, if any, subsides, add 50 mg copper wire which is adequate for up to 22 mg sulfur

3. When reaction ceases, immerse reaction tube to 4/5 of its height in a sand bath at 180°C in a hood.

4. If solution is not entirely clear after 1 hour, add 5 ml of oxidant

5. Take to dryness (3-5 hours) and raise temperature of sand bath to 280°C (took 12 hours)

6. When fumes no longer appear (took 3 hours), remove tubes from sand bath, cool below 100°C and add 5 ml of 1N HCl to the residue which is either white, green or black at this stage (if blue continue heating at 280°C before adding HCl)

7. Warm until a clear solution is obtained

8. To obtain a final pH between 0.5 and 1.0 add 20 ml distilled water.

9. Heat the solution to boiling and add dropwise an excess of 5% BaCl₂ solution

10. Transfer the tube to a steam bath for 2 hours.

11. After adding 2 drops of 20% Tween 80 to the sides of each tube and washing in with a few drops of water from a wash bottle, centrifuge
tubes at 2500 r.p.m. for 10 minutes.

12. Carefully pour off supernatant fluid and add 4 drops of 2% Tween 80 around the circumference of the tube.

13. With a strong jet of 0.05N nitric acid from a wash bottle, resuspend and centrifuge the precipitate.

14. The second wash is identical with the first except the suspension is digested for 20 minutes in a steam bath before centrifugation.

15. Hot washes are repeated until the test for chloride is negative (took 4 hot washes), following which the precipitate is washed twice by suspension in 50% ethanol and centrifugation to remove Tween 80.

16. After the outside of the tube has been carefully rinsed with distilled water it is placed in a slanting position (to prevent loss of material by bumping) in an oven at 180°C for at least 2 hours.

17. The cooled tubes are weighed and the amount of sulfur present calculated.

Tubes #1-4 are the unknown sample; tubes #5-6 are sulfur controls of cysteine hydrochloride - 20.35% sulfur; tube #7 is a reagent control. Results are shown in Tables X and XI.
### TABLE X

Sulfur determination of an N-ethylmaleimide-thiol complex

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Weight</th>
<th>Weight</th>
<th>Weight of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dried tube</td>
<td>Tube + sample</td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>38.9621 g</td>
<td>39.0135 g</td>
<td>51.4 mg</td>
</tr>
<tr>
<td>(2)</td>
<td>39.9314 g</td>
<td>40.0226 g</td>
<td>91.2 mg</td>
</tr>
<tr>
<td>(3)</td>
<td>39.3759 g</td>
<td>39.4362 g</td>
<td>60.3 mg</td>
</tr>
<tr>
<td>(4)</td>
<td>39.2612 g</td>
<td>39.3173 g</td>
<td>56.1 mg</td>
</tr>
<tr>
<td>(5)</td>
<td>38.5844 g</td>
<td>38.6329 g</td>
<td>48.5 mg</td>
</tr>
<tr>
<td>(6)</td>
<td>38.5496 g</td>
<td>38.6388 g</td>
<td>89.2 mg</td>
</tr>
<tr>
<td>(7)</td>
<td>38.5929 g</td>
<td>38.5929 g</td>
<td>0.0 mg</td>
</tr>
</tbody>
</table>
TABLE XI

Sulfur determination of an N-ethylmaleimide-thiol complex
(cont.)

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Weight of Tube</th>
<th>Weight of Tube+BaSO$_4$</th>
<th>mg S*</th>
<th>%S of Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>38.0621 g</td>
<td>39.0157 g</td>
<td>53.6 mg</td>
<td>7.35</td>
</tr>
<tr>
<td>(2)</td>
<td>39.9314 g</td>
<td>40.0265 g</td>
<td>95.1 mg</td>
<td>13.04</td>
</tr>
<tr>
<td>(3)</td>
<td>39.3759 g</td>
<td>39.4384 g</td>
<td>62.5 mg</td>
<td>8.56</td>
</tr>
<tr>
<td>(4)</td>
<td>39.2612 g</td>
<td>39.3202 g</td>
<td>59.0 mg</td>
<td>8.08</td>
</tr>
<tr>
<td>(5)</td>
<td>38.5844 g</td>
<td>38.6541 g</td>
<td>69.7 mg</td>
<td>9.55</td>
</tr>
<tr>
<td>(6)</td>
<td>38.5496 g</td>
<td>38.6797 g</td>
<td>130.1 mg</td>
<td>17.82</td>
</tr>
<tr>
<td>(7)</td>
<td>38.5929 g</td>
<td>38.5929 g</td>
<td>0.0 mg</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Total Sulfur

<table>
<thead>
<tr>
<th></th>
<th>Found (av)</th>
<th>Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>14.3%</td>
<td>(1) 15.84%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(II) 14.81%</td>
</tr>
<tr>
<td>Cysteine·HCl</td>
<td>19.8%</td>
<td>20.35%</td>
</tr>
</tbody>
</table>

*BaSO$_4$ = 13.7% S
A molecular weight determination was done using an Osometer (Mechro Lab). The reading was at first 206, but this gradually dropped to 181, and then to 152. It was felt that the NEM thiol complex was too unstable for this method of molecular weight determination.

**Results:**

These results indicate that the sulfurous gas produced by *M. radiodurans*, like mercaptans in general, complexes strongly with metals, as with mercury and cadmium. Analyses for carbon, nitrogen, sulfur and molecular weight give the results: carbon - 49.25%; nitrogen 12.44%; sulfur - 14.3%; molecular weight - 206. The analysis for β-mercaptoethylamine (I) is: carbon - 47.5%; nitrogen - 15.8%; sulfur - 13.8%; molecular weight - 202. The analysis for 3-mercaptopropylamine (II) is: carbon - 50.0%; nitrogen - 14.8%; sulfur - 12.9%; molecular weight - 216. There is no indication of a C=C or of a C=O present in any of the derivatives.

**Discussion:**

These experiments seem to indicate that the sulfurous gas produced by *M. radiodurans* is an end-product of methionine metabolism. The chemical analyses of the NEM-thiol complex indicate the presence of a free sulfhydryl group and of a free amino group. The analyses for carbon,
nitrogen, sulfur and molecular weight are compatible with those for 3-mercaptobutylamine, and possibly for $\beta$-mercaptoethylamine. The manner in which this gas is produced could be along the lines indicated in pathway (II) (p. 56) or possibly pathway (I) (p. 55). The compound is to be tested for the radiation protection of other biological systems.

**RADIOPROTECTIVE CAPACITY OF A GASEOUS END-PRODUCT**

Considering the proposed nature of the gaseous compound that is a metabolic end-product of methionine produced by *M. radiodurans*, it was felt essential to determine the radioprotective capacity, if any, on other biological systems. However, the compound had been trapped successfully only as heavy metal and N-ethylmaleimide complexes and these trapping agents are very toxic to biological systems. It had been noted (p. 66) that a certain amount of gas was dissolved in the TGY broth of an actively growing culture. With this fact in mind, it was decided to saturate cultures of microorganisms in TGY broth with the gas so that some of the gas might go into solution, be taken up by the cells, and increase the radiation resistance of these organisms.

It was decided to use three different microorganisms with varying degrees of sensitivity to radiation damage.
The cultures of microorganisms utilized, and the levels of radiation listed by Frazier (51, p. 153) as LD_{100} in nutrient broth were:

- **Sarcina lutea**: 400,000 rep
- **Staphylococcus aureus**: 150,000-500,000 rep
- **Pseudomonas fluorescens**: 250,000 rep

All irradiation with these organisms was with the Co^{60} source at the U. S. Bureau of Mines, Albany, Oregon. Radiation doses in air employed and the length of time required to reach this dose were:

<table>
<thead>
<tr>
<th>Rad</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>62,500</td>
<td>2.77 minutes</td>
</tr>
<tr>
<td>125,000</td>
<td>5.5 minutes</td>
</tr>
<tr>
<td>250,000</td>
<td>11.1 minutes</td>
</tr>
<tr>
<td>375,000</td>
<td>16.65 minutes</td>
</tr>
<tr>
<td>500,000</td>
<td>22.2 minutes</td>
</tr>
</tbody>
</table>

In all, three major runs were utilized for the experiment, with the procedures for all runs almost identical, any variation being dependent on the results of the previous run. A total of 256 vials of cells were irradiated, and there were 30 unirradiated controls.

**Experimental Methods:**

M. radiodurans was cultured in 7 L quantities in TGY broth for gas production as on p. 60.
The cultures to be tested were grown in TGY broth (p. 51). The media was dispensed in 500 ml aliquots into 1 L Pyrex side-arm filter (Corning 5340) flasks. Anti-foam A (Dow-Corning) was added to the side-arms of the flasks and the end of the tubing then plugged with gauze-covered cotton, wrapped with paper and tied with string. The mouths of the flasks were plugged with gauze-covered cotton stoppers, wrapped with paper and tied with string. The flasks of media were autoclaved 20 minutes at 15 lbs pressure (121°C).

For each flask, a #8 1-holed rubber stopper was fitted with glass tubing and a sintered-glass air-dispersion tip was fitted to the tubing. A cotton-plugged Nalgene airway filter was attached with rubber tubing to the exterior glass tubing. The assembly was wrapped with paper, tied with string, and autoclaved 15 minutes at 15 lbs pressure (121°C).

When the 7 L culture of M. radiodurans began to produce the gas, 2 suction flasks each were inoculated with S. lutea, S. aureus and Ps. fluorescens, and 1 suction flask was inoculated with M. radiodurans. Each flask was fitted aseptically with a rubber-stopper-sparger assembly. One culture flask each of S. lutea, S. aureus, and Ps. fluorescens was attached to a tube carrying away the gas produced by the 7 L culture of M. radiodurans, and these cultures were aerated by this gas. One culture flask each of
S. lutea, S. aureus, Ps. Fluorescens and M. radiodurans was attached to the air pump and aerated with air. New cultures of M. radiodurans were kept growing so that when one culture stopped producing gas, another could be substituted. Three cultures were continuously sparged with the mercaptan-like gas for 48 hours; four cultures were continuously sparged with air for 48 hours.

A portion of each culture in TGY was saved for the irradiation of the cells in broth. The cells from all cultures were harvested individually and aseptically in sterile Nalgene centrifuge tubes (40 ml cap) with caps in International #1 centrifuge, at 4000 r.p.m. The cells were resuspended in sterile 0.067M phosphate buffer and again centrifuged. This washing was repeated 3 times.

Portions of all cells in broth, and washed cell suspensions were dispensed in 10 ml aliquots into 15 ml screw-capped irradiation vials, 11 vials each of broth suspension and of phosphate buffer suspension per culture. A total of 130 vials were irradiated and there were 13 unirradiated controls. Single vials each of the broth and phosphate buffer suspended cells were unirradiated controls. Duplicate vials of broth and of phosphate buffer suspended cells were irradiated at each level of irradiation - 62,500, 125,000, 250,000, 375,000 and 500,000 rads.
Coding of the vials as to cells contained and suspending media was:

I - \textit{M. radiodurans} in PO\textsubscript{4}

II - \textit{S. lutea} in TGY

III - \textit{S. lutea} in PO\textsubscript{4}

IV - \textit{S. lutea} + gas in TGY

V - \textit{S. lutea} + gas in PO\textsubscript{4}

VI - \textit{S. aureus} in TGY

VII - \textit{S. aureus} in PO\textsubscript{4}

VIII - \textit{S. aureus} + gas in TGY

IX - \textit{S. aureus} + gas in PO\textsubscript{4}

X - \textit{Ps. fluroescens} in TGY

XI - \textit{Ps. fluroescens} in PO\textsubscript{4}

XII - \textit{Ps. fluroescens} + gas in TGY

XIII - \textit{Ps. fluroescens} + gas in PO\textsubscript{4}

Cells in the odd-number vials are phosphate butter washed cells of the preceding even-numbered TGY suspensions. Cultures IV and V, VIII and IX, and XII and XIII were sparged during growth with gas from an actively growing, gas-producing culture of \textit{M. radiodurans}.

Coding of the vials as to radiation dose level was:

0 = 0

1 = 62,500 rad

2 = 125,000 rad

3 = 250,000 rad
4 = 375,000 rad
5 = 500,000 rad

The irradiated cells and the unirradiated controls were diluted in 99 ml water blanks to dilutions of $10^6$; pour plates in TGY agar (p. 52) were made in duplicate of the dilutions from $10^2$ to $10^7$. Some of the cells that had been found to be extremely sensitive to radiation in previous runs were also plated in pour plates in duplicate undiluted and at $10^1$.

The plates were incubated 72-96 hours at 35°C and counted on a Darkfield Quebec Colony Counter. Survival results of the plate counts are shown in Table XII. Graphs of the logs of the % survival, based on the unirradiated counts, are shown in Figures 6, 7 and 8.

**Results:**

The results of these experiments show a greater survival of microorganisms irradiated in broth than those irradiated in phosphate buffer, a fact that is already well known. They also show that the gas-treated cells when irradiated have a greater survival rate than do the untreated cells.

**Discussion:**

It appears that the mercaptan-like compound, produced by *M. radiodurans* from methionine metabolism, is capable in the manner used here, of affording some degree of
<table>
<thead>
<tr>
<th>Number</th>
<th>Radiation Dose Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1.9x10⁷</td>
<td>1.1x10⁷</td>
</tr>
<tr>
<td>1.1x10⁷</td>
<td>1.2x10⁷</td>
</tr>
<tr>
<td>7.9x10⁷</td>
<td>6.1x10⁷</td>
</tr>
<tr>
<td>4.8x10⁷</td>
<td></td>
</tr>
<tr>
<td>1.1x10⁷</td>
<td>5.2x10⁷</td>
</tr>
<tr>
<td>3.1x10⁷</td>
<td>5.4x10⁶</td>
</tr>
<tr>
<td>2.0x10⁵</td>
<td>3.1x10³</td>
</tr>
<tr>
<td>8.6x10⁶</td>
<td>9.3x10⁶</td>
</tr>
<tr>
<td>1.4x10⁵</td>
<td>4.8x10²</td>
</tr>
<tr>
<td>8.5x10⁰</td>
<td>0</td>
</tr>
<tr>
<td>7.1x10⁷</td>
<td>3.4x10⁷</td>
</tr>
<tr>
<td>2.0x10⁷</td>
<td>2.3x10⁶</td>
</tr>
<tr>
<td>1.6x10⁵</td>
<td>3.1x10³</td>
</tr>
<tr>
<td>10.7x10⁷</td>
<td>1.9x10⁶</td>
</tr>
<tr>
<td>5.3x10⁵</td>
<td>1.3x10³</td>
</tr>
<tr>
<td>8.8x10⁴</td>
<td>5.5x10⁰</td>
</tr>
<tr>
<td>23.8x10⁸</td>
<td>17.5x10⁷</td>
</tr>
<tr>
<td>12.6x10⁷</td>
<td>9.8x10²</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>28.0x10⁸</td>
<td>9.6x10²</td>
</tr>
<tr>
<td>3.0x10¹</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18.5x10⁵</td>
<td>12.9x10⁷</td>
</tr>
<tr>
<td>12.1x10⁶</td>
<td>5.9x10³</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>26.9x10⁵</td>
<td>6.9x10⁴</td>
</tr>
<tr>
<td>3.8x10²</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>35.8x10⁸</td>
<td>19.7x10²</td>
</tr>
<tr>
<td>4.0x10²</td>
<td>1.3x10⁰</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>24.5x10⁸</td>
<td>13.5x10²</td>
</tr>
<tr>
<td>8.8x10⁰</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6. Survival of *S. lutea*. 
Figure 7. Survival of *S. aureus*. 
Figure 8. Per cent survival of *Ps. fluorescens*. 
radiation protection to more radiation sensitive microbial systems. In this case it is probable that the cells have taken up the compound from the medium rather than the compound having reduced the oxygen tension of the suspending medium, since treated cells suspended in buffer and irradiated have increased radiation resistance as well as those treated cells suspended in TGY broth and irradiated.
Studies on the utilization of methionine, methionine analogues and methionine precursors by *M. radiodurans* have demonstrated several facts: 1) methionine is required by the organism in synthetic medium and that it can utilize D-, L-, and D,L- forms equally well, and that an excess of methionine is detrimental to growth; 2) of the methionine sulfonium compounds, S-adenosylmethionine can be substituted for methionine, but S-methyl-L-methionine cannot replace it; 3) cyanocobalamine (vitamin B₁₂) can replace the methionine requirement, the organism probably utilizing inorganic sulfate from the medium for its sulfur requirement, or enhance the growth of the organism in the presence of methionine precursors; 4) purine and pyrimidine bases do not replace either methionine or vitamin B₁₂, so that the role of vitamin B₁₂ is not necessarily that of nucleic acid synthesis; 5) the organism grows anaerobically in synthetic medium in the presence of hemoglobin and vitamin B₁₂, poorly with hemoglobin alone, and not at all with vitamin B₁₂ or methionine, alone or in combination; and 6) the organism when growing in an actively aerated nutrient broth culture with adequate methionine produces a sulfurous gas in addition to H₂S, this gas not being produced if the methionine content is too low or too high.
Anaerobic photosynthetic growth was demonstrated which may necessitate reclassification of this organism. The photosynthetically grown cells were less pigmented than the aerobic heterotrophically grown cells, but their resistance to γ-radiation was not diminished.

Proposed pathways of methionine degradation by the microorganism point to the possible production of two mercapto compounds having radiation protective capacities. These compounds are 3-mercaptoethylamine (I) (MFA, cysteamine) and 3-mercaptopyrrolamine (II)(MPA). Chemical analyses of an N-ethylmaleimide derivative of the gas produced by this organism are compatible with (II) and possibly with (I).

Radiation sensitive organisms, *S. lutea*, *S. aureus* and *Ps. fluorescens*, grown while being aerated with the gas respired by *M. radiodurans* show a slightly increased resistance to γ-radiation.


63. Role of sulfur in the cell division of Chlorella with special reference to the sulfur compounds appearing during the process of cell division. I. Plant and Cellular Physiology 1:131-142. 1960.

64. Role of sulfur in the cell division of Chlorella with special reference to the sulfur compounds appearing during the process of cell division. II. Plant and Cellular Physiology 2:9-24. 1961.


108. Meisel, M. N. The aims of radiation microbiology with reference to the 1959-1965 plan of development of the people's economy in the USSR. Microbiology 28:159-161.


