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The Use of *Tetrahymena* to Evaluate the Effects of Gamma Radiation on Essential Nutrilites*

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SUMMARY. The complete synthetic medium for the growth of *Tetrahymena pyriformis* E was subjected to gamma-radiation from cobalt^{60}. A dose-response curve indicated the range of radiation which damaged the medium so that growth of *Tetrahymena* was inhibited.

The essential vitamins and amino acids which comprise the medium were individually irradiated in solution. Media were prepared with single irradiated components and tested for their ability to support growth of the protozoa.

Thiamine, riboflavin, pantothenate, pyridoxine, folic acid, and thioctic acid were destroyed by less than 1 x 10^6 rep, while 2 x 10^6 rep was required to inactivate nicotinic acid. Most amino acids proved to be relatively radiation-resistant. At the high radiation levels of 1 x 10^7 rep and 2.3 x 10^7 rep respectively, only serine and methionine were damaged. All other amino acids remained biologically active even after receiving 2.3 x 10^7 rep, the highest level of radiation employed. Organoleptic observations, however, showed radiation-caused color and odor changes in many of those amino acids whose biological activity for *Tetrahymena* was unaffected. The nucleotide, guanylic acid, was also inactivated at 2.3 x 10^7 rep.

THE PHYSICAL and chemical changes occurring in amino acids and vitamins exposed to radiations of all types have been rather extensively studied. Biological damage to these nutrilites as demonstrated by their ability to supply the nutritional needs of animals is not so well known. This lack of information stems from the unavailability of adequate radiation sources and from the fact that nutritional studies on conventional laboratory animals (mouse, rat, chick), where chemically pure nutrients are employed, are costly and time consuming.

Recently there has been considerable attention given to the utilization of radiations from fission products. This interest has been stimulated by the availability of excellent sources of gamma radiation, one of the best being cobalt^{60}. Moreover, the ciliate protozoan, *Tetrahymena pyriformis,* with its well-known mammalian-like nutritional requirements, has come into prominence as a microorganism ideally suited for studies of nutrition. It has the advantages inherent in microbiological research, the most salient being precise and rapid results with small samples and consequent low cost.

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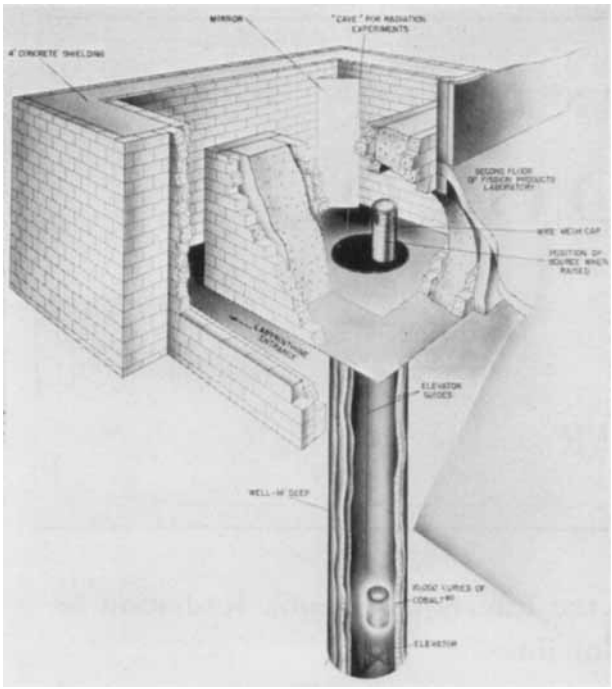

FIG. 1. Cutaway drawing of radiation cave for 10 kilo curie cobalt^{60} source.

With a cobalt^{60} source of gamma radiation at hand, together with some facility in handling the test organism, it seemed well worthwhile to initiate an investigation of the effect of gamma radiation on the essential nutrilites of this organism.

MATERIAL AND METHODS

The materials were irradiated in the 10 kilocurie cobalt^{60} source located on campus in the Fission Products Laboratory which is jointly sponsored by the Michigan Memorial-Phoenix Project and the Engineering Research Institute. Materials were irradiated inside the "cave" (Fig. 1) at a specified distance from the source in the raised position to obtain the desired dose. Flux intensity is dependent on distance from the source and length of exposure. The radiation intensity inside the cage containing cobalt^{60} rods averages 240,000 rep/hr. Fig. 2 is a calibration of the radiation flux outside the cage and was made along a horizontal plane passing through the center of the source. The calibrations were made by a ferrous sulfate dosimetry technique(23) which is compared with instrument calibrations. The ferrous sulfate measurements are considered to be the most reliable. In all cases the rep is defined as 93 ergs/gram of energy absorption from radiation(15).

Stock cultures of *Tetrahymena pyriformis* E, were grown in a 1% tryptone-proteose-peptone broth. The inoculum for experimental studies was obtained by harvesting cells from 48-hour stock cultures by centrifugation and aseptically washing the cells three or four times in redistilled water. Washed cells were resuspended in distilled water and diluted to give an optical density of 0.032 in the Lumetron colorimeter, model 401, with the blue (420 mμ) filter. Such a suspension contains approximately 25,000 cells/ml. Subsequently, 0.2 ml. of this suspension was pipetted into 5 ml. amounts of sterile, chemically defined medium(9, 10,11) in 15 x 125 mm. Pyrex culture tubes. The composition of the medium is indicated in Table I. Following inoculation the cultures were incubated vertically at 25° C. in the dark. Daily growth measurements were made turbidimetrically with the Lumetron by adapting the method described by Elliott(8). The pH of the medium was routinely set at 7.6 before autoclaving, after which the readings were 7.2 to 7.4, except in irradiated complete liquid medium where the pH dropped as low as 6.7 at the highest radiation level. A consideration of the significance of this pH decline is presented in the discussion.

All media were prepared with glass-redistilled water, and the glassware was routinely given a final rinse in glass-redistilled water before drying for use in an experiment.

Results reported are the averaged data from at least two experiments, each made in duplicate or triplicate tubes. Any deviation from this procedure is described with the individual experiments in question.

RESULTS

Irradiation of complete defined medium. Preliminary irradiation was performed to determine the effec-

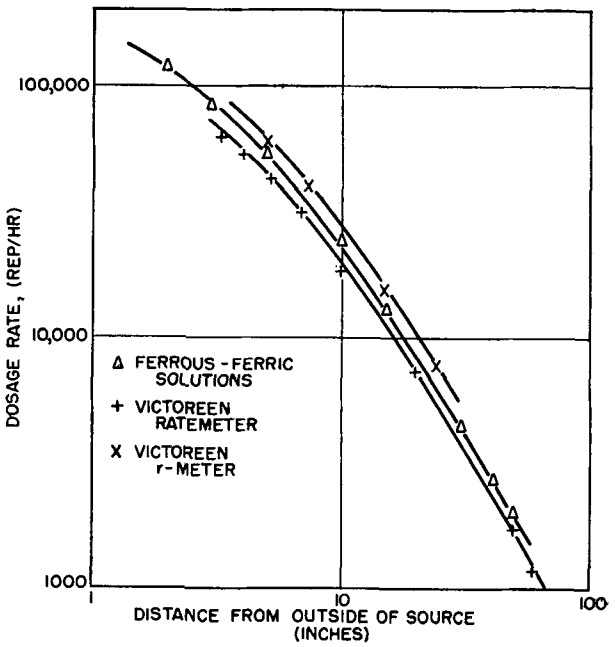

FIG. 2. Gamma flux outside the cylinder of the 10 kilo curie cobalt^{60} source.

TABLE I. Basal chemically defined medium.

Compounds	Micrograms per milliliter	Molar equivalent concentrations used for irradiation
L-Arginine · HCl	150	0.05
L-Histidine · HCl · H_2O	110	0.05
DL-Isoleucine	100	0.05
L-Leucine	70	0.05
L-Lysine · HCl · H_2O	35	0.048
DL-Methionine	35	0.046
DL-Phenylalanine	100	0.05
DL-Serine	180	0.05
DL-Threonine	180	0.05
L-Tryptophan	20	0.05
DL-Valine	60	0.05
Dextrose	1000	
Sodium acetate	1000	
Adenylic acid	25	8.8×10^{-3}
Cytidylic acid	25	9.6×10^{-3}
Guanylic acid	25	8.7×10^{-3}
Uracil	25	9.1×10^{-3}
Ca pantothenate	0.1	2.0×10^{-4}
Nicotinic acid	0.1	2.03×10^{-4}
Pyridoxine · HCl	2.0	2.0×10^{-4}
Riboflavin	0.1	2.0×10^{-4}
Folic acid (PGA)*	0.01	2.0×10^{-5}
Thiamine · HCl	1.0	1.98×10^{-4}
Thioctic acid	0.001	2.0×10^{-4}
K_2HPO_4	100.0	
$MgSO_4 \cdot 7H_2O$	10.0	
$Zn(NO_3)_2 \cdot 6H_2O$	5.0	
$FeSO_4 \cdot 7H_2O$	0.5	
$CuCl_2 \cdot 2H_2O$	0.5	

* Folic acid could not be prepared at the concentration of the other vitamins since such a concentration is above its limit of solubility.

tive range of radiation and the comparative radiation effects on dry and liquid media. The defined medium used to culture *T. pyriformis* was irradiated both in solution as well as in the dry state (except for thioctic acid which was used as a concentrated solution of sodium DL-6-thioctate[1]). Dry, crystalline amino acids, nucleic acid components, glucose, sodium acetate, and inorganic salts were mixed and irradiated in aluminum-foil-capped 50-ml. Erlenmeyer flasks. Vitamins were not included in the dry mix because of the mechanical impossibility of weighing the small amounts which would be necessary. Therefore, except for thioctate, they were separately irradiated in 5-ml. glass screw-cap vials, then made up in concentrated solution and dispensed to prepare the media. The liquid medium was prepared, sterilized and irradiated in 500 or 1000 ml. Pyrex reagent bottles. At selected radiation intervals aliquots were aseptically removed and transferred to culture tubes for inoculation.

A range of radiation from 1×10^5 to 4×10^6 rep was employed in the initial screening experiment for both the dry and liquid media. Damage to the irradiated materials was tested by the ability of *Tetrahymena* to grow in the irradiated liquid medium or in medium prepared from irradiated dry constituents. Even the highest radiation dose failed to influence noticeably the dry components since the protozoa grew equally well in medium prepared from the irradiated mix and in the non-irradiated medium. On the other hand, the irradiated liquid medium failed to support growth of the ciliates when the radiation dose was 5×10^5 rep or over. When non-irradiated medium was added back to these inhibited cultures, growth recovery was achieved which was inversely related to the radiation level. Thus, recovery was greater when fresh medium was added to that which received only 5×10^5 rep than when administered to medium irradiated by 4×10^6 rep. The smaller growth recovery at the higher radiation level may be due to (a) increased toxicity with increased radiation, or (b) more nutrilites destroyed at higher doses of radiation. A dose-response curve (Fig. 3), based on experimental evidence, shows that the critical range of radiation for the complete liquid medium is from 1.5×10^5 to 3×10^5 rep. The complexity of events which may occur in complete medium makes it difficult to pin-point the components being affected. Therefore, the direct approach of irradiating individual medium components was undertaken.

Irradiation of individual components of the medium. In the first experimental series in which separate components were to be tested for the effects of irradiation, each was irradiated in the concentration which usually is employed in the preparation of the medium (Table I). Media were prepared by omitting one or several of the components in groups. The five groupings used were: (1) amino acids; (2) vitamins; (3) nucleic acid components; (4) glucose; and (5) sodium acetate. Each component was individually irradiated at 1×10^6 rep. The irradiated compounds were then pooled in the described groups and were added to that experimental medium which was deficient in the test group. Upon testing the growth of *Tetrahymena* in such media, it was found that only the medium containing irradiated vitamins failed to support growth. All other media yielded normal growth, except a control medium prepared entirely from irradiated components in which growth also was negative.

In order to determine toxicity effects of the irradiated vitamins, another experiment was arranged containing complete non-irradiated synthetic medium to which the irradiated vitamin group was added. Growth of the protozoa in this medium was equal to the control growth, demonstrating that the irradiated vitamins were not toxic, though they may have been destroyed by gamma rays.

At this point in the investigation it became clear,

[1] Na-DL-6-thioctate was generously supplied by the Lederle Laboratories, Pearl River, New York.

TABLE II. Organoleptic changes in aqueous solutions of vitamins, amino acids, and nucleic acid components subjected to gamma radiation from Co^{60}.

Compound	Irradiation* (rep)	Color	Odor
L-Arginine • HCl	2.3×10^7	none	faintly sweet
L-Histidine • HCl • H_2O	1×10^7	light amber	none
	2.3×10^7	light amber	dilute ethyl alcohol
DL-Isoleucine	1×10^6	none	faintly sweet
	1×10^7	none	musty fruit
	2.3×10^7	none	musty fruit
L-Leucine	1×10^6	none	faintly sweet
	1×10^7	none	musty fruit
	2.3×10^7	none	musty fruit
L-Lysine • HCl	2.3×10^7	yellow	faintly sweet
DL-Methionine	1×10^6	none	boiled cabbage
	1×10^7	turbid	boiled cabbage
	2.3×10^7	turbid-light straw	boiled cabbage
DL-Phenylalanine	1×10^7	yellow	mild geranium
	2.3×10^7	dark amber	geranium
DL-Serine	1×10^7	straw	faintly sweet
	2.3×10^7	none	none
DL-Threonine	1×10^7	light straw	faintly sweet
	2.3×10^7	light straw	dilute ethyl alcohol
L-Tryptophan	1×10^6	light brown	none
	1×10^7	dark brown	mild indole
	2.3×10^7	dark brown	indole
DL-Valine	1×10^6	none	faintly sweet
	1×10^7	none	sweet
	2.3×10^7	none	musty fruit
Adenylic acid	1×10^7	light straw	none
	2.3×10^7	light straw	none
Cytidylic acid	1×10^7	light straw	none
	2.3×10^7	light straw	none
Guanylic acid	2.3×10^7	light straw	none
Uracil	2.3×10^7	none	none
Thiamine • HCl	1×10^6	turbid	H_2S
	2×10^6	turbid	H_2S
Riboflavin	1×10^6	light yellow	faintly sweet
	2×10^6	none	faintly sweet
Ca pantothenate	2×10^6	none	none
Nicotinic acid	2×10^6	none	none
Pyridoxine • HCl	2×10^6	none	none
Folic acid (PGA)	2×10^6	none	none
Na-DL-6-Thioctate	2×10^6	none	none

* Radiation doses lower than those shown had no effect on either color or odor.

since certain organoleptic changes had occurred in the irradiated compounds (Table II) yet some retained normal biological activity, that it was necessary to run dose-response curves on all of the components of the medium (except the inorganic salts, glucose, and sodium acetate) to determine at which level of radiation each was rendered useless as a nutrient for *Tetrahymena*. It was decided to restrict radiation effects to the range where a linear relationship occurs. As indicated by Allsopp(2), this effect takes place in dilute aqueous solutions not lower than 10^{-4}M. Therefore the medium components were prepared and irradiated in the concentrations listed in Table I. The following radiation levels were used: $1x10^5$, $2.5x10^5$, $5x10^5$, $7.5x10^5$, $1x10^6$, $1.5x10^6$, $2x10^6$, $3x10^6$, $4x10^6$, $1x10^7$, and $2.3x10^7$ rep.

Radiation of the vitamins. Preliminary experiments had shown that media containing irradiated vitamins failed to support growth of the ciliate. In order to determine which of the vitamins were destroyed at this level of radiation ($1x10^6$ rep), an omission experiment was performed in which single vitamins prepared as shown in Table I were left out of the medium. The omitted vitamin was then replaced by the irradiated vitamin. Growth experiments in this series indicated that pyridoxine and thiamine were refractory to radiation damage, whereas thioctate, folic acid, riboflavin, nicotinic acid (niacin), and pantothenate were *all* partially destroyed. Lack of toxicity of all of the irradiated vitamins was demonstrated by supplementing non-irradiated control medium with single irradiated vitamins and obtaining growth which was equivalent to that in the control medium.

It was obviously important to determine, if possible, dose levels at which each of the vitamins was rendered useless to *Tetrahymena* as a growth factor. Employing the concentration levels described in Table I, media were again prepared with single vitamin omissions. The deleted vitamin was then added back after it had received the graded series of radiation doses stated above. Tubed media were then sterilized, inoculated, and checked for growth of the test or-

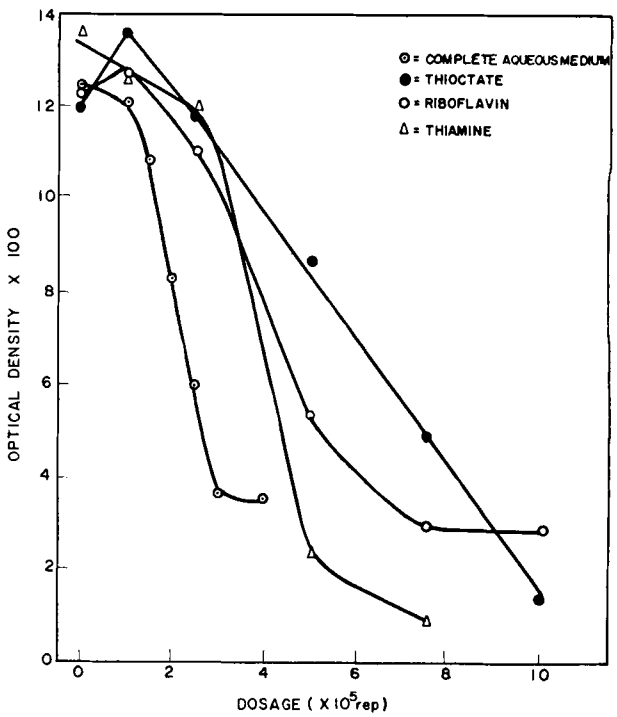

FIG. 3. Dose-response of complete medium and the vitamins: Thioctate, Thiamine, and Riboflavin.

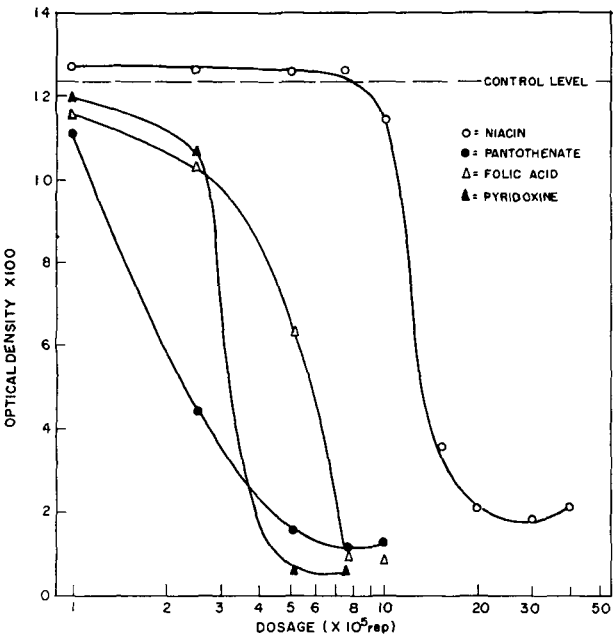

FIG. 4. Dose-response with irradiated vitamins; Nicotinic acid (Niacin), Pantothenate, Folic Acid, and Pyridoxine.

ganism. Positive damage to all vitamins except thiamine and thioctate was observed. The inconsistencies noted for pyridoxine and thioctate between the preliminary study and these results are explained on the basis of the different molar concentrations at which the vitamins were irradiated. Since studies hereafter were to be performed on the concentrations listed in Table I, the latter results were of prime interest. It was reasoned, therefore, that lack of effect on thiamine and thioctate was due to an insufficient radiation dose or a retention of these vitamins in the cells so that enough was present to produce normal growth despite damage to the extracellular vitamins. Therefore, cells which were subsequently to be used for determining thioctate and thiamine damage were passed through two serial transfers in thioctate- and thiamine-deficient media respectively before inoculation into the experimental media containing the irradiated vitamins. By employing this method, damage to these two vitamins was found to occur. All results are recorded in Figs. 3-4.

These dose-response curves were obtained by comparing growth of the cultures at different levels of radiation, at the time when control growth reached its peak. In all cases, the critical radiation range is between 2.5 x 10^5 and 1 x 10^6 rep, except for niacin. This vitamin is affected between 1 x 10^6 and 2 x 10^6 rep, indicating that of the vitamins tested, niacin is the most stable. The plots shown are not calculated curves, but represent the investigators' interpretation of the experimental data.

When single vitamins irradiated by 1 x 10^6 rep supplemented the complete non-irradiated medium, no growth inhibitions were observed, demonstrating that irradiation damage to the vitamins does not result in the formation of substances toxic to *Tetrahymena*.

Irradiation of the amino acids and nucleic acid components. The usual method of preparation was employed and found suitable for these experiments since media deficient in any amino acid would not support growth of *Tetrahymena*. The only nucleic acid component required for the growth of this organism was guanylic acid, which has been shown by Kidder *et al.* (12) to be essential for strain W. Five serial transfers of the ciliate through media deficient in any one of the other three nucleic acid components still yielded normal growth. It was therefore concluded that irradiation effects on these compounds could not be determined with *Tetrahymena* in the basal synthetic medium utilized in this research. Nevertheless, adenylic and cytidylic acids, and uracil were irradiated to determine color and odor changes (Table II), and to test them for toxicity.

The amino acids and guanylic acid were tested by the method of single omissions described previously for the vitamins. All radiations were made upon components at concentrations given in Table I. Neither the amino acids nor guanylic acid was damaged at any radiation level up to 4 x 10^6 rep. However, at 1 x 10^7 rep, serine activity was completely destroyed (Fig. 5). Growth recovery was effected by adding non-irradiated serine to the cultures inhibited by irradiated serine. The other amino acids and guanylic acid were not damaged at this level of radiation.

When the amino acids were irradiated at 2.3 x 10^7 rep, methionine as well as serine was damaged (Fig. 5). Guanylic acid was also almost completely destroyed at this radiation dose (Fig. 5). There were indications that some of the other amino acids were slightly damaged at this high radiation level, but the extent of damage was not conclusive. At this highest radiation dose, only a single experiment was performed and therefore these results remain to be confirmed by further tests.

Lack of toxicity of the irradiated components was demonstrated in the usual manner of supplementing complete non-irradiated medium with irradiation-damaged components and observing that growth was normal.

Organoleptic Observations on Irradiated Materials. Early in this study it became obvious that color and odor changes occurred in irradiated materials. Progressively higher radiation doses caused additional changes. It was of interest to note that although organoleptic tests revealed radical changes in some of the irradiated compounds, the biological activity of most of the substances as determined by growth of *Tetrahymena* remained unimpaired. On the other

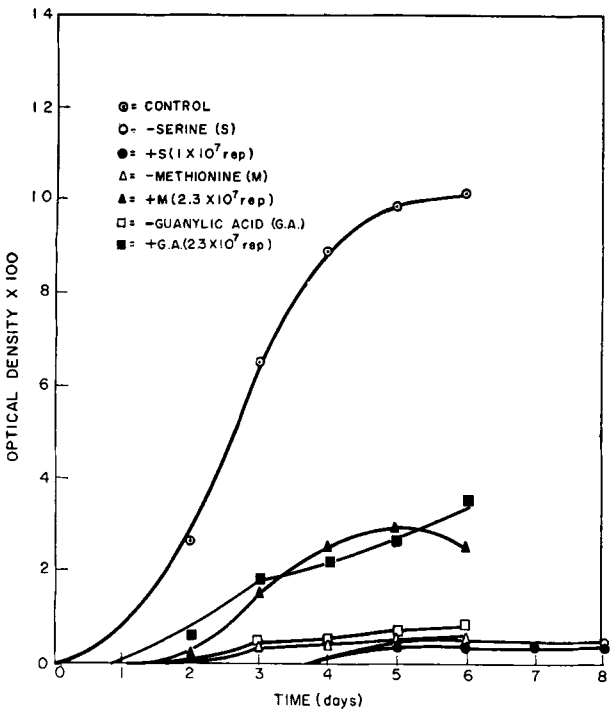

FIG. 5. Inhibition of growth with irradiated Serine, Methionine, and Guanylic Acid.

hand, biological damage was incurred upon some of the vitamins, yet their color and odor remained unaltered.

The organoleptic observations listed in Table II were made by several persons. No records are given for the inorganic salts, glucose, and sodium acetate because no changes of any kind occurred. It should be noted that all non-irradiated solutions were essentially odorless and colorless, except for riboflavin which is yellow, and folic acid which is a light greenish-yellow. Therefore all changes recorded are the results of radiation. It is believed that these observations may afford some insight into the kinds of radiation damage which have been shown to occur by growth experiments.

DISCUSSION

Earlier in this paper it was reported that irradiation of the complete liquid defined medium resulted in a pH drop relative to the radiation dose. At 4 x 10^6 rep, pH was lowered from 7.6 to 6.7. This variation is well within the range of optimal growth of *Tetrahymena* as demonstrated by Elliott(7) and Slater (18). Therefore, the observed pH decline in irradiated medium must be considered as negligible. The acid production was probably caused by the breakdown of glucose. Baumgartner(4) showed that bacteria failed to grow on irradiated carbohydrate media due to the production of acid by irradiation. Addition of base reconditioned the medium so that bacterial growth occurred normally.

The lack of effect of irradiation on dry materials was to be expected since, in general, compounds in the dry state are more stable than substances in solution. Lea(13) states that the enzyme, ribonuclease, in the dry state was inactivated by X-rays only between 1.5 x 10^7 and 8 x 10^7 r (roentgens). The highest radiation dose received by the dry defined medium used in this investigation was about one-tenth of the dose required to damage dry ribonuclease.

Taylor *et al.*(20) demonstrated that X-radiation of yeast medium was subsequently lethal to the ciliate *Colpidium*. The present report also indicates that irradiated aqueous medium is unable to support growth of another ciliate, *Tetrahymena*.

Since the total concentration of the complete liquid medium is of the order of 10^{-2} *M*, it means that the constituent compounds are each present in far greater dilution. The low radiation dose necessary to inactivate the medium in this form can be attributed to the instability of dilute solutions and the radiation-sensitivity of the vitamins. Lea(13) states that dilute solutions are readily inactivated by radiation doses which are so small that they may have little or no effect at all upon dry preparations or more concentrated solutions. Furthermore, in considering the radiation effects on the complete medium, a complex situation arises which involves a protective interplay among the various components of the medium. Such protection has been reported in solutions of two compounds(5). For example, in pure solution ascorbic acid is known to be highly radio-sensitive, but Proctor and Goldblith(14) have reported that niacin at 10 μg./ml. can protect as high as 500 μg./ml. of ascorbic acid against the effects of ionizing radiations. Obviously such facts confuse the situation in a complex medium. For these reasons it was thought advisable to discontinue studies with the complete medium and instead investigate radiation effects on pure solutions of the medium components.

It is well known that vitamins are affected by various types of radiations(1,16,17). It has been demonstrated, for example, that riboflavin irradiated by ultra-violet is changed to lumiflavin, which separates out from the original aqueous solution because of its extremely low solubility(16). On the other hand, cobalt^{60} radiation caused riboflavin to become colorless, but no precipitate was formed. Robinson(16) describes the reduction of riboflavin to a colorless compound when treated with sodium dithionite. It seems likely, therefore, that gamma radiation from cobalt^{60} reduces riboflavin rather than decomposes it to lumiflavin. The one difference between the actions of cobalt-radiations and sodium dithionite is that color

is not restored after aeration of the irradiated solution.

Niacin was shown to be the most radiation-resistant of the vitamins. This observation supports the contention of Proctor and Goldblith(14) that niacin is a relatively radio-stable substance.

The dose-response data for thioctate seems to indicate activation of growth at low radiation levels. This stimulation may be real and, if so, may be strengthened by the report that photo-radiation of enzymes causes an initial enzyme-activity increase followed by decrease(17).

The effects of radiations on proteins and amino acids have been studied for many years. This material has been excellently reviewed by Arnow(3) up to 1936. A more recent review with a comprehensive bibliography is that by Sparrow and Rubin(19). Other reports also emphasize the radiation sensitivity of amino acids(6,22).

In the light of previous studies it was considered likely that cobalt^{60} radiations would decompose amino acids. It was shown in the present research that serine is inactivated and apparently is the most radio-sensitive of all the amino acids that were irradiated in this investigation. The destruction is probably similar to that found by Dale and Davies(6). These studies also showed that methionine appeared as the next most radio-sensitive amino acid. No information was found in the literature regarding radiation studies on methionine solutions.

Several reports emphasize that nucleic acids are depolymerized by various types of radiations both in irradiated living cells(13) as well as *in vitro*(19,21). What the effects of irradiation of nucleotides may be is not known. However, it was shown in this investigation that guanine nucleotide loses its biological value for *Tetrahymena* at high radiation doses.

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