Effects of Gamma Irradiation on the Protein Content of *Euglena* Cells and on the Alanine-^{14}C Incorporation into the Cells

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Effects of 1×10^5 r gamma irradiation on protein content and alanine-^{14}C incorporation into cell protein were compared between *Euglena* cells in two different growth phases, logarithmic and stationary, of culture. The following irradiation effects were common to these two growth phase cells: first, the soluble protein content per cell of the irradiated cells was slightly larger than that of the non-irradiated cells, but it remained almost constant during the incubation period; second, the non-extractive protein content per cell of the non-irradiated cells increased according to incubation time, but that of the irradiated cells remained unchanged during the same incubation period after irradiation ; third, the nucleoprotein in fraction C separated through Sephadex column was more unstable in quantity to gamma rays than the other soluble proteins ; fourth, the incorporation of alanine-^{14}C into the irradiated cells was markedly inhibited by irradiation.

Comparing the irradiation effects between two growth phase cells, the logarithmic phase cells were more susceptible to irradiation with regard to all the points mentioned above than the stationary phase cells. Although the alanine-^{14}C incorporation into protein was recognized slightly in the case of irradiated logarithmic phase cells, but it was hardly recognized in the case of irradiated stationary phase cells.

INTRODUCTION

The growth of a given cell generally includes an increase in cell number and /or the increase in dry weight of single cell. For instance, Epstein *et al.* (1962)[1] have reported that in *Euglena* an increase in cell number is directly proportional, while the cell size is inversely proportional to vitamin B_{12} concentration in culture medium. Kimball and Vogt-Köhne (1962)[2] have reported that in *Paramecium* irradiated with x-rays the growth rate as expressed by dry weight decreases, and the cell division is inhibited. But it has been previously reported[3] by the present author that the growth in cell number of *Euglena* cells is inhibited by gamma irradiation, but no effect of irradiation was recognized on the growth in dry weight. However, this conclusion must require a further investigation, because the degree of irradiation effect may vary according to the criterion to estimate the effect. In *Euglena* cells, the intracellular proteins occupy about 70% of the dry weight,[4] and it may play an important role in cell growth. Accordingly, it seems interesting to investigate the cell growth from the following two viewpoints; protein content and amino

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Abbreviations: RNA ; ribouncleic acid, DNA ; deoxyribonucleic acid.

acid incorporation into protein. This paper deals with the effect of gamma irradiation on the protein content and the alanin-^{14}C incorporation into protein of *Euglena* cells in two growth phases, logarithmic and stationary.

MATERIAL AND METHODS

Cells, Media, and Conditions for Culture and Irradiation

The material employed in the present study was cells of *Euglena gracilis var. bacillaris*, Camb. Coll. No. 1224/7 in two different growth phases, logarithmic and stationary, of culture. The cells cultured in the culture medium[3] under artificial light (about 2500 lux) at 30°C for 5 days served as the logarithmic phase cells, and those cultured for 20 days as the stationary phase cells. The cells were gathered from the culture and washed with distilled water. A known number of the washed cells was suspended in 200 ml of distilled water. This cell suspension was divided into equal halves and each of which was enclosed into a polyethylen capsule. One capsule containing either growth phase cells was exposed to 1×10^5 r gamma rays, while the other capsule was kept as the control. The gamma irradiation was carried out with the ^{60}Co gamma-ray irradiation facility of Institute for Chemical Research of Kyoto University. The dose rate of the irradiation facility was 0.95×10^5 r/hr. Immediately after the irradiation, the irradiated and the non-irradiated cell suspensions were transferred into each 1500 ml of fresh culture medium from the capsule and incubated in the same manner as above. The culture medium used for alanine-^{14}C incorporation was 1/10 strength of the above described culture medium. To this diluted medium alanine-^{14}C was added to give the final concentration of about 15 $\mu\mu$c/ml. No effect of the dilution of culture medium and of the addition of alanine-^{14}C on cell growth was recognized at all in the present work.

Cell Number

The estimation of cell number was made as described previously[3].

Soluble and Non-extractive Proteins

About 1×10^7 washed cells mentioned above were homogenized in 25 ml of 5 mM phosphate buffer (pH 7.4) and 10 ml of n-hexan with a Kubota KMS-100 sonicator. The homogenate obtained was centrifugalized at 13000 g for 20 min. at 5°C. The supernatant obtained was taken as "soluble fraction" of protein in the present work. From the centrifugal pellet, lipids, acid soluble substances, and nucleic acids were eliminated according to the procedure of Tyner *et al.*[5] Thus, the residue obtained was also dissolved in 20 ml of 1N sodium hydroxide. This solution was taken as "non-extractive fraction" of protein in the present work. The protein amount of these two fractions was determined by means of Folin's color reaction.[6]

Sephadex Gel Filtration

The material used in this experiment was the above mentioned "soluble fraction" of protein. For gel filtration, Sephadex G-50 particles, 20-80 μ in diameter,

were converted into gel with 0.1M aq. sodium chloride solution of about 20 fold volume of the particles. A bed 1.5 cm in diameter and 80 cm in height was used. The material was charged to the column and then eluted with 250 ml of a buffered saline (pH 7.4) in a cold room. Every 3.5 ml effluent was collected with constant flow of 20 ml/hr. The absorbancy of effluents was read at 260 mμ and 280 mμ. Thus, the main fractions obtained were examined spectrophotometrically. The relative amount of protein in a given fraction was determined by absorbancy at 280 mμ and by Folin's color reaction.

Radioactivity

The radioactivity of alanine-^{14}C of all the fractions was measured with a Kobe-Kogyo PC-26 gasflow counter.

RESULTS

1) Growth in Cell Number

When the non-irradiated cells of both growth phases were transferred into fresh culture medium and incubated for 24 hours at 30°C, the non-irradiated logarithmic phase cells increased in number by about 1.7 fold of the original cells number, and the non-irradiated stationary phase cells by about 1.2 fold. But the irradiated cells of both growth phases hardly increased in number during this period under the same culture condition (Fig. 1, A and A').

2) Distribution of Intracellular Protein

The non-irradiated logarithmic phase cells incubated for 12 hours in fresh culture medium were homogenated in n-hexan and phosphate buffer (pH 7.4), and separated centrifugally into seven fractions as follows; (A) n-hexan extract, (B) the soluble fraction mentioned above, (C) cold 3% sodium chloride extract, (D) cold 10% sodium chloride extract, (E) 0.4N perchloric acid extract, (F) 10% sodium chloride extract at 100°C, and (G) the non-extractive fraction mentioned above. Among these fractions, the protein distribution was about 62% of total protein in fraction B, and about 28% in fraction G. All the remaining fractions contained about 10% of total protein. In the irradiated logarithmic phase cells, the protein distribution was similar to that of the non-irradiated logarithmic phase cells. But the protein amount of the fraction B increases slightly in percentage (about 4%) than that of the non-irradiated cells, while the protein amount of the fraction G decrease slightly (about 4%). The other five fractions remained almost unchanged in percentage of protein content after irradiation. The protein distribution among these seven fractions, and the irradiation effect on protein distribution of the stationary phase cells were also similar to those of the logarithmic phase cells.

3) Soluble and Non-extractive Protein Centents per Cell

When the non-irradiated logarithmic phase cells were cultured for 24 hours, the soluble protein content per cell of these cells remained unchanged during this period (Fig. 1, B). But when the irradiated logarithmic phase cells were cultur-

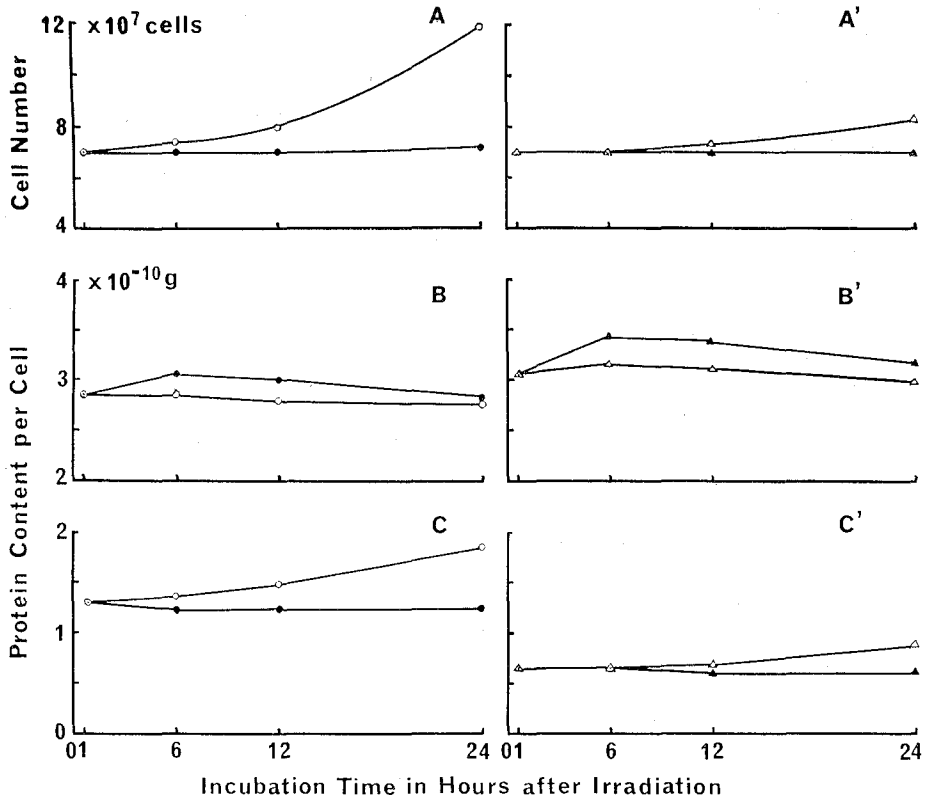

Fig. 1. Growth response and protein content of the non-irradiated and the irradiated cells.

Logarithmic phase cells; A to C, Stationary phase cells; A′ to C′, Non-irradiated; (—○—, ···△···), Irradiated; (—●—, ···▲···), A and A′; cell growth, B and B′; soluble protein, C and C′; non-extractive protein.

ed, the soluble protein content per cell increased slightly in quantity as compared with that of the non-irradiated cells at 6 hours after irradiation, and then gradually decreased according to incubation time. A tendency similar to this was also recognized in the soluble protein of the non-irradiated and the irradiated stationary phase cells (Fig. 1, B′).

The non-extractive protein content per cell of the non-irradiated cells of both growth phases increased about 1.4 fold of the original content during the incubation period of 24 hours. This may show a net increase of protein content per cell by protein synthesis (Fig. 1, C). Contrary to this, in the irradiated cells of both growth phases, the non-extractive protein content per cell remained unchanged during the incubation period (Fig. 1, C′). The non-extractive protein content per cell of the logarithmic phase cells was generally larger than that of the stationary phase cells.

4) Soluble Protein Filtrated through Sephadex Gel

When the soluble protein fraction was further fractionated by gel filtration through Sephadex G-50 gel, various kinds of protein fractions were obtained. For

convenience's sake, those fractions which showed different u.v. spectrum, and different color reactions with indole,[7] orcinol[8] and Folin's reagents were denoted by different letters A, B, C, D and E. While these fractions which gave similar color reactions with these reagents and showed different eluting patterns were denoted by C, C′ and C″ etc..

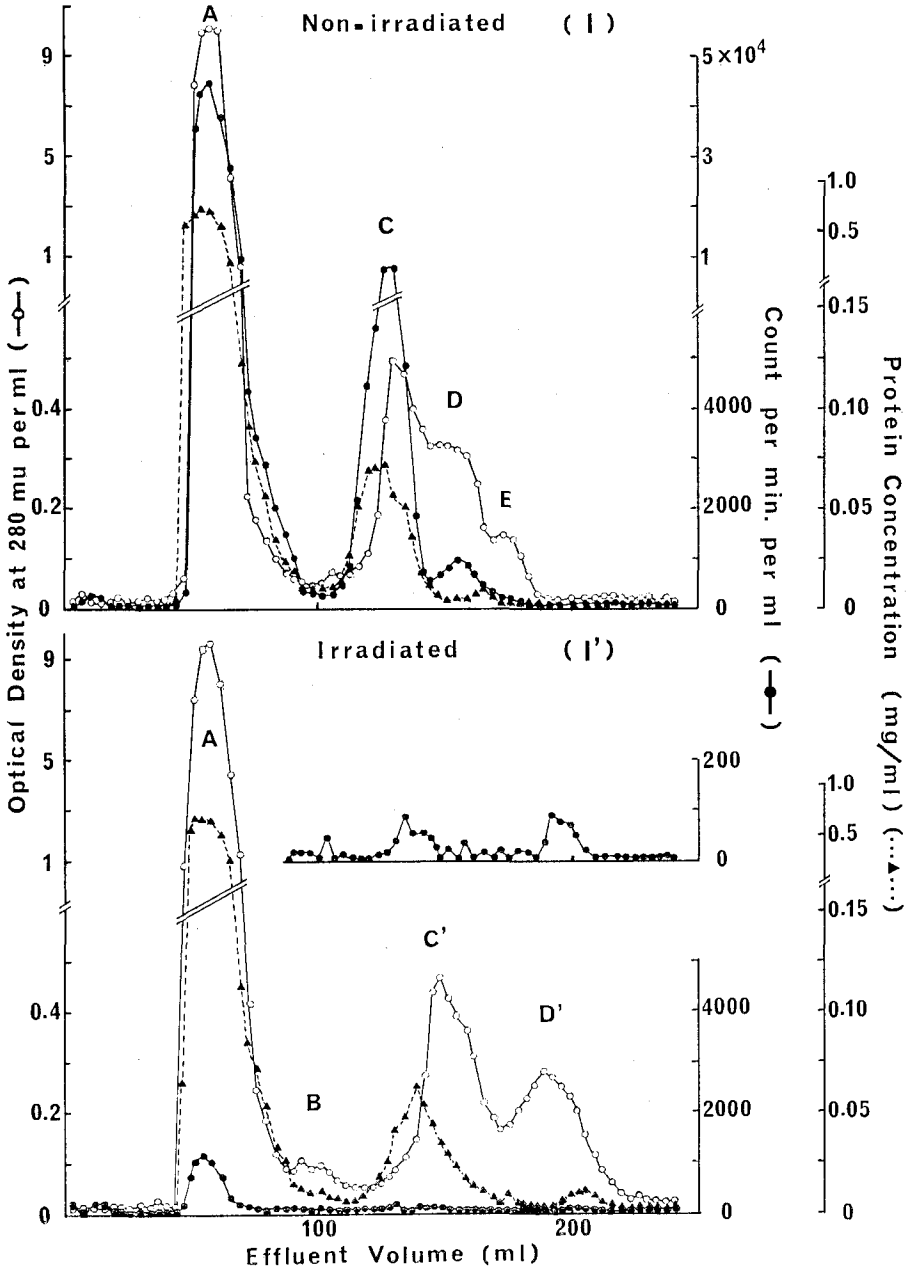

Fig. 2. Sephadex column chromatography of soluble protein of the logarithmic phase cells.

As show in Fig. 2, the soluble protein fraction mentioned above of the non-irradiated logarithmic phase cells was separated into four fractions of A, C, D and E by gel filtration. The fraction A was a green and clear solution which had at least five absorption maxima at 260, 335, 385, 440 and 684 mμ (Fig. 3, A-1, A -2 and A-3). It is well known that nucleic acids, nucleoprotein, adenine, uridine

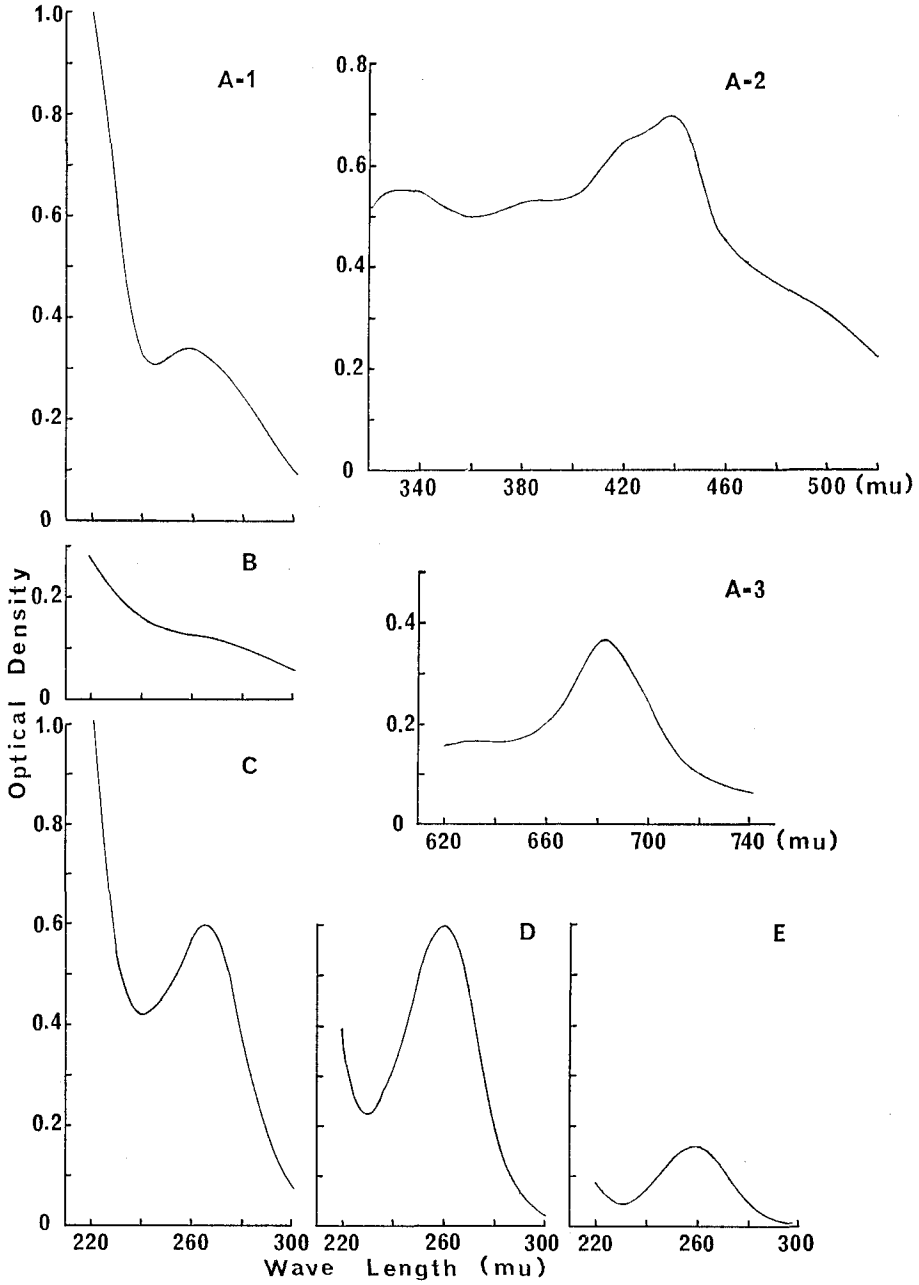

Fig. 3. Absorption spectra of the effluents in the soluble protein fractions separated through Sephadex.

and others have an absorption maximum at about 260 mμ. Carotenoids generally absorb the light in the range from about 350 to 550 mμ. Chlorophyll-a has two main absorption maxima at about 430 and 660 mμ. This fraction showed a strong color development with Folin's reagent. Accordingly, this fraction seems to contain at least all the substances mentioned above in addition to protein. The fraction C may contain nucleic acids and nucleprotein, because the effluent of this fraction showed an absorption maximum at 265 mμ, and was positive in indole, orcinol and Folin's color reactions. The fractions D and E may contain a large amount of RNA and a small amount of DNA and protein, because the effluents of these fractions have an absorption maximum at 260 mμ respectively, and were positive in orcinol color reaction, slightly positive in indole and Folin's color reactions, the fraction E was recognized only in the non-irradiated cells. In Sephadex gel filtration, the higher the molecular weight of a substance is, the more rapidly the substance be filtrated. Accordingly, the fraction A must contain the substance of highest molecular weight among the present four fractions obtained, and the fraction E that of lowest molecular weight.

When the soluble protein fraction of the irradiated logarithmic phase cells were separated through Sephadex gel in the same manner as above, four fractions of A, B, C′ and D′ were obtained. The fraction A in this case shows a little decrease in total u.v. absorbancy at 280 mμ, and by a considerable decrease in Folin's color development as compared with the fraction A of the non-irradiated cells (Figs. 2 and 4). The fraction B appeared only in the case of the irradiated cells. The absorption maximum of this fraction could not be determined owing to low optical density, but at least there was no strong absorption at 260 mμ. The fraction C′ showed a similar absorption spectrum and color reaction to fraction C of the non-irradiated cells. But the former fraction may contain somewhat smaller molecule than the latter one as judged by the difference of eluting sequence, that is, the size of molecule in the fraction C′ seems to be close to that in fraction D of the non-irradiated cells. The fraction C′ remained almost unchanged in total u.v. absorbancy, but decreased by about 15% in Folin's color development as compared with the fraction C of the non-irradiated cells. The fraction D′ showed a similar absorption spectrum and color reaction to those of the fraction D of the non-irradiated cells. But the former fraction was also obtained in more delayed eluting sequence from the column than the latter one. The fraction D′ decreased by about 20% in total u.v. absorbancy, but remained unchanged in Folin's color development as compared with the fraction D.

When the soluble protein fraction of the non-irradiated stationary phase cells was separated by gel filtration in the same manner as above, four fractions of A, C″, D″ and E′ were obtained. The absorption spectra and the color reaction of these fractions were similar to the fractions of the non-irradiated logarithmic phase cells; C″ corresponded to C, D″ to D, and E′ to E respectively. But the chromatographic pattern of the non-irradiated stationary phase cell protein was rather similar to that of the irradiated logarithmic phase cell protein than to that of the non-irradiated logarithmic phase cell one, especially in eluting sequence, (Fig. 4).

When soluble protein fraction of the irradiated stationary phase cells were separated by gel filtration in the same manner as above, four fractions A, B, C′ and D‴ were also obtained. The chromatographic pattern of the irradiated stationary phase cell protein showed a little change as compared with that of the non-irradiated cell protein, that is, the fraction A increased by about 9% in u.v. absorbancy, and remained almost unchanged in Folin's color development as

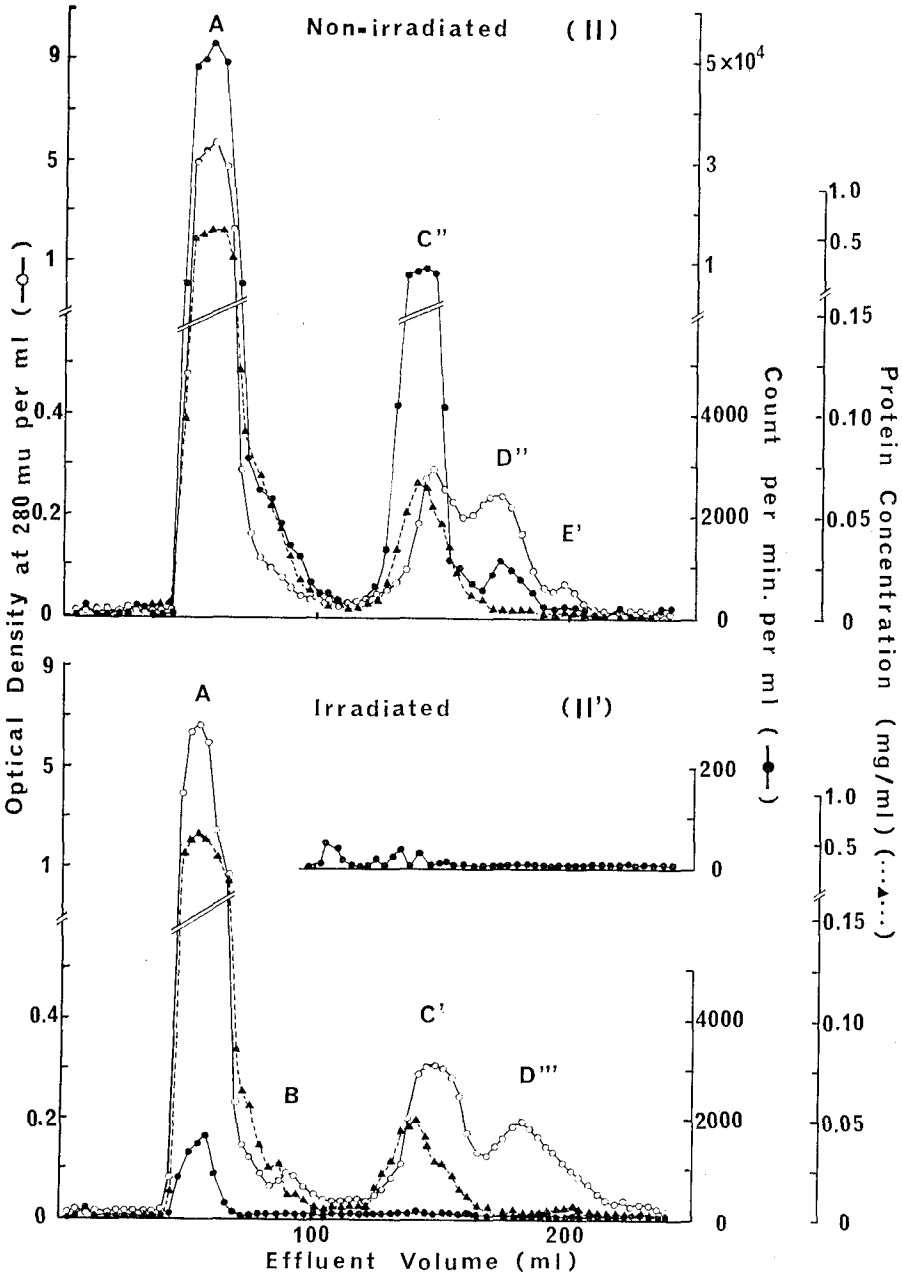

Fig. 4. Sephadex column chromatography of soluble protein of the stationary phase cells.

compared with the fraction A of the non-irradiated cells. The fraction B was recognized with a small peak in the irradiated cells. The absorption spectrum of this fraction was similar to that of the fraction B of the irradiated logarithmic phase cells. The fraction C′ increased by about 10% in u.v. absorbancy, but decreased by about 7% in Folin's color development as compared with the fraction C″. The above increase in u.v. absorbancy may due to the depolymerization of nucleic acids or nucleoprotein in fraction A and C′. It is known that depolymerized DNA has a higher absorption at 260 mμ by about 30% than highly polymerized one[9]. Though the fraction D‴ differed in eluting position from fraction D″ of the non-irradiated celld, the u.v. absorbancy remained almost unchanged

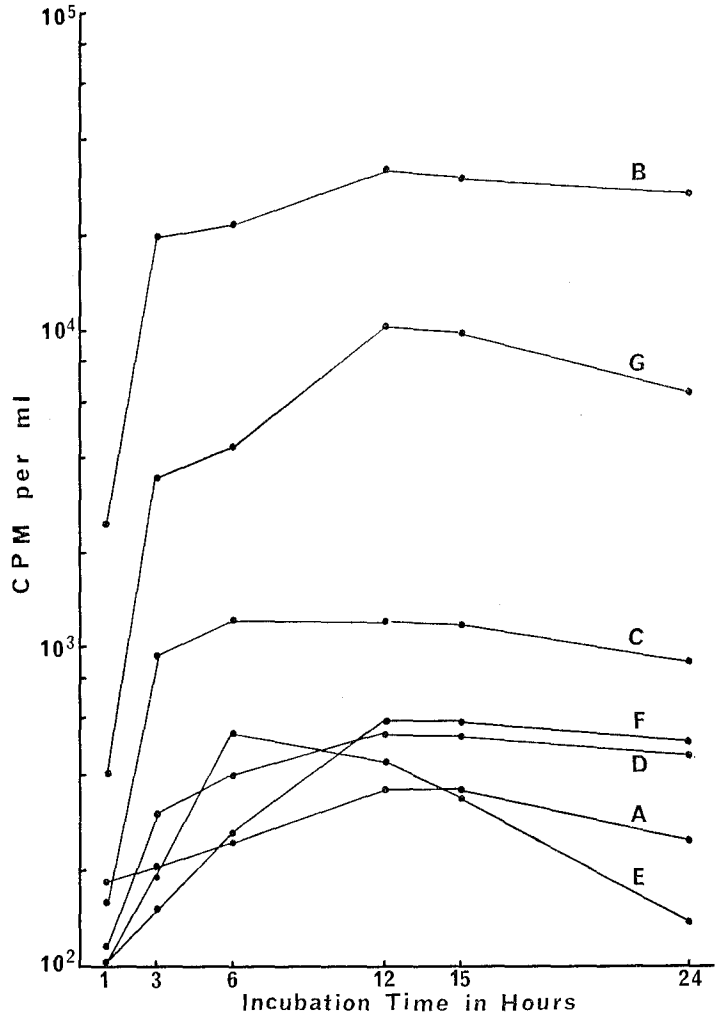

Fig. 5. Incorporation rate of alanine-^{14}C into the non-irradiated logarithmic phase cells.

(A) ; n-hexane extract, (B) ; 5 mM phosphate buffer (pH 7.4) extract, (C) ; 3% sodium chloride extract at 5°C, (D) ; 10% sodium chloride extract at 5°C, (E) ; 0.4N perchloric acid extract at 2°C, (F) ; 10% sodium chloride extract at 100°C, and (G) ; the residue dissolved in 1N sodium hydroxide.

in the case of the irradiated cells.

5) Incorporation Rate of Alanine-^{14}C into Non-irradiated and Irradiated Cells

The non-irradiated logarithmic phase cells were incubated for 24 hours in fresh culture medium containing alanine-^{14}C, as mentioned above, and a known volume was removed from the culture at 1, 3, 6, 12, 15 and 24 hours respectively. The cells were harvested and were washed thoroughly with distilled water and fractionated into seven fractions as described above, that is, (A) n-hexane extract, (B) 5 mM phosphate buffer (pH 7.4) extract; soluble protein fraction, (C) 3% sodium chloride extract at 5°C, (D) 10% sodium chloride extract at 5°C, (E) 0.4N perchloric acid extract at 2°C, (F) 10% sodium chloride extract at 100°C, and and (G) the residue dissolved in 1N sodium hydroxide; non-extractive fraction. Among these fractions, the radioactivity of alanine-^{14}C reached the maximum at about 6 hours of culture in the fractions C and E, and at about 12 hours in all the remaining fractions. After the maximum, the radioactivity decreased gradually according to incubation time (Fig. 5).

The non-irradiated and the irradiated cells incubated for 12 hours were fractionated in the same manner as above, the distribution of radioativity was shown in Table 1. The alanine-^{14}C incorporation into the irradiated logarithmic phase cells was inhibited by about 91% as compared with that of the non-irradiated cells, and the incorporation into the irradiated stationary phase cells by about 63%. Consequently all the fractions of the irradiated logarithmic phase cells showed somewhat lower radiactivity than those of the irradiated stationary phase cells.

Table 1. Radioactivity of Alanine-^{14}C Incorporated into Non-Irradiated and Irradiated Cells.

Fraction	Logarithmic Phase Cells			Stationary Phase Cells		
	Non-Irradiated Count/min	Irradiated Count/min	Decreased (%)	Non-Irradiated Count/min	Irradiated Count/min	Decreased (%)
A	3,915	631	83.9	4,238	1,376	67.5
B	1,350,275	103,119	92.4	879,825	284,346	67.7
C	138,502	24,419	82.4	150,382	84,313	43.9
D	28,658	4,441	84.5	2,166	11,839	46.6
E	4,249	800	81.2	5,798	1,963	66.1
F	9,366	642	93,2	14,750	2,171	85.3
G	86,520	10,658	87,7	29,193	26,385	9.6
Total	1,621,485	144,710	91.1	1,106,353	412,393	62.7

A; n-hexane extract at 5°C, B; 5 mM phosphate buffer extract at 5°C, C; 3% NaCl extract at 5°C, E; 0.4N perchloric acid extract at 2°C, F; 10% NaCl extract at 100°C, G; the residue.

6) Alanine-^{14}C Incorporation into Soluble Protein Separated by Sephadex Gel Filtration

The soluble protein fraction of the non-irradiated logarithmic phase cells incubated for 12 hours was fractionated through Sephadex G-50 gel (Figs. 2 and 4), and then the radioactivity of alanine-^{14}C incorporated was measured with all the

fractions obtained. The alanine-^{14}C incorporation into protein was recognized in large quantity in the fractions A and C. Further, a little incorporation of alanine-^{14}C was also recognized in the fractions D and E (Fig. 2). When the soluble protein fraction of the irradiated cells was examined in the same manner as above, it was recognized that the alanine-^{14}C incorporation into protein was inhibited to a large extent in all the fractions obtained. In other words, the radiactivity of this fraction A decreased by about 92% as compared with the fraction A of the non-irradiated cells. The fraction B showed a little radioactivity. Although a little, the radioactivity was recognized in the fractions C′ and D′. These results suggest that the protein synthesis is carried out in the irradiated logarithmic phase cells.

When the soluble protein fraction of the non-irradiated stationary phase cells was examined in the same manner as above, the alanine-^{14}C incorporation into protein was also recognized in almost the same degree as in the case of the non-irradiated logarithmic phase cells (Fig. 4, Table 1). When the soluble protein fraction of the irradiated cells was examined in the same manner as above, it was recognized that the alanine-^{14}C incorporation into protein was inhibited to a large extent in all the fractions obtained. Only a little radioactivity of alanine-^{14}C was recognized in the fraction A. But the radioactivity was hardly recognized in the fractions C′ and D‴ (Fig. 4). These results suggest that the metabolism of most soluble proteins is found to some extent in the irradiated stationary phase cells, but the nucleoprotein metabolism is hardly recognizable at least during the present incubation period.

DISCUSSION AND CONCLUSION

The effect of irradiation on cell growth is one of the most fundamental problem in radiation biology. The cell growth generally means an increase in cell number, and/or an increase in dry weight of a single cell. The increase in cell number or the cell division is generally inhibited by irradiation. This phenomenon has been studied by numerous investigators,[2,10,11] especially there are many investigations as to the effect of irradiation on dividing cells.[12,13] But little is known as to the irradiation effect on dry weight of a single cell.

It is well known that unicellular organisms are more resistant to irradiation than multicellular organisms.[2] Accordingly, an unicellular organism generally requires a considerably high lethal dose of irradiation.[14] When the normally growing unicellular organisms are irradiated by such lethal dose of radiation, some cells are damaged to death, while others survive.[3] These survivals play an important role in revealing the irradiation effect on cell growth. Some of the survivals may die soon, but others may gradually recover from damage. The latter survivals on the way of recovering, are inhibited in all the cell functions relating to growth. This may result in the division delay or the growth inhibition in cell number.

Intracellular nucleic acids and protein may be regarded as the important substances relating to cell growth. It is well known that an increase in cell number,

especially a cell division is related to DNA duplication[15]. But an irradiation-induced sluggish cell does not show any cell division at least until it recovers perfectly from damage. It took from 4 to 5 hours for *Tetrahymena* irradiated with 6×10^4 r x-rays,[16] and about 7 days for logarithmic growing *Euglena* cells irradiated with 1×10^5 r gamma rays.[3] Accordingly, the growth response of the irradiated cells during this period may be taken as a clew to analyse the irradiation effect on cell growth. Lea *et al.*[17] have reported that the cells of *E. coli* irradiated with gamma rays enlarge in size during the period of division delay. Kimball and Vögt-Köhne[2] have reported that the growth rate as expressed by dry weight of x-irradiated *Paramecium* cell is decrease immediately, but recovers from inhibition later dur ing the interval between cell divisions. In *Euglena*, although the gamma-irradiated cells increased in volume during the incubation period after irradiation, no change in dry weight of single cell was recognized.[3] This fact was also supported by the result obtained in the present study, that is, the intracellular protein content of *Euglena* cells was not markedly affected by gamma irradiation.

It is well known that the protein synthesis is hardly inhibited or sometimes rather expedited by irradiation dose, by which the DNA synthesis is inhibited to a large extent.[18,19] However, by a considerably high dose of irradiation, the protein synthesis is also inhibited. This fact was also recognized in the present work. Although any change in dry weight or any change in protein content per cell was not recognized in the irradiated *Euglena* cells, a slight alanine-^{14}C incorporation into these cells is recognized. This fact suggests that a slight activity of protein synthesis remains in these irradiated cells, or that partial turnover or exchange of protein moiety occurs in these cells. If these cases are true, it seems that the activity does not result in any recognizable increase in dry weight or protein content.

The RNA level of *Euglena* cells was considerably affected by the gamma irradiation dose, by which the protein level is hardly affected.[20] Especially the RNA level of the logarithmic phase cells decreased by about 30% by gamma irradiation. It has been reported that the loss of intracellular nucleotides and sRNA may play an important role in the above mentioned decrease in RNA level[3]. Further, the present author has found that a part of the intracellular RNA components decomposed by irradiation diffuse out of the irradiated cells into outer medium.[21] Indeed, it has been reported that some nucleotides, such as CMP, ATP, etc. diffuse out from the irradiated cells or organelle.[22,23]

There have been reported by several investigators that the radiosensitivity of a cell differs with age of the cell or growth phase of culture. For instance, Snider[24] has reported that the nerve sheath cells in a three-week chick suffer great damage with 800 r total body x-ray irradiation, whereas these same cells are not damaged with this dose when the animal is 11 weeks old. This suggests that the younger the cell, the more susceptible to irradiation damage it will be. Contrary to this, Tang and Gaw,[25] have found that older cultures of *Paramecium* are more susceptible to the immediate lethal effect of ultraviolet light than younger ones.

It has been previously reported that the radiosensitivity of *Euglena* cells varies

with growth phase of culture[3,20]. The RNA level of the logarithmic phase cells was more susceptible to gamma irradiation than that of the stationary phase cells. But the former cells were less in death rate after irradiation than the latter ones. Moreover, a little incorporation of uracil-^{14}C into RNA was recognized in the irradiated logarithmic phase cells, but was hardly recognizable in the irradiated stationary phase cells. In the present study also a phenomenon similar to this was recognized as to the alanine-^{14}C incorporation into nucleoprotein of *Euglena* cells.

From the above mentioned results as to the irradiation effect on cell growth, it is concluded that the growth of *Euglena* cells are inhibited to a large extent by gamma irradiation of 1×10^5 r, but the degree of irradiation damage of the cells varies with growth phases of culture or with the criterion to estimate the irradiation damage.

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