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Author(s)	Yonei, Shuji
Citation	大阪大学, 1971, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/2153
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MECHANISM OF POST-IRRADIATION DEGRADATION OF DNA IN A RADIOSENSITIVE *ESCHERICHIA COLI* NG30 IRRADIATED WITH ULTRAVIOLET LIGHT

(Doctoral Thesis)

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MECHANISM OF POST-IRRADIATION DEGRADATION OF DNA IN A RADIOSENSITIVE *ESCHERICHIA COLI* NG30 IRRADIATED WITH ULTRAVIOLET LIGHT

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INTRODUCTION

An x-ray sensitive mutant, *Escherichia coli* NG30, isolated by Kato and Kondo[1] from strain H/r30 is highly sensitive to ultraviolet light, too,[1] and the phenomenon of extensive degradation of cellular DNA after ultraviolet irradiation has been reported.[2,3] Kato and Kondo[3] have also found that the *E. coli* NG30 has the following two genetic characters ; A very low genetic recombination frequency in the crosses with Hfr strain Bl of *E. coli* B and a very low rate of prophage (ϕ80) induction by ultraviolet irradiation, as compared with that of the wild-type strain, *E. coli* H/r30-R. From the above biochemical and genetic analyses, the radiosensitive *E. coli*

NG30 was classified phenotypically as a mutant belonging to the RecA$^-$ type in the three types of recombination-deficient mutants of E. coli K12.[4,5]

RecA$^-$ mutants of E. coli K12, as well as NG30, are also highly sensitive to ultraviolet light and shows an extensive degradation of cellular DNA by ultraviolet irradiation.[4-8] It is sure that there is a close relationship between the marked ultraviolet sensitivity and the distinctive nature with extensive DNA degradation by ultraviolet irradiation in the RecA$^-$ mutants.[5,7,8-12]

Clark, Chamberlin, Boyce and Howard-Flanders,[9] and Howard-Flanders and Boyce[13] postulated that the post-irradiation degradation of DNA in RecA$^-$ mutants would be induced by the single-strand gaps in DNA molecules which were produced by incomplete dark repair events.

Horii and Suzuki[10,11] showed recently that the ultraviolet-induced DNA degradation of a RecA$^-$ mutant of E. coli K12, JC 1569b, occurs on a special DNA portion involving DNA replicating point, because the pulse-labeled DNA region begins to degradate prior to the degradation of pulse-labeled and chased DNA region in growing cells. They suggested that the replicating point is a sensitive site for irradiation, because the dark repair of pyrimidine dimers or the

replication of DNA must be controled by certain mechanism in order that the replicating point does not run across the site under the repair.

The experimental results and speculations by the above authors[10,11] might mean that the DNA portion far from the replicating point would not begin to degradate until the replicating point reaches to the pyrimidine dimers on the DNA portion, or until the degradation initially occured on the replicating point comes along the DNA strand to the damage of the DNA portion.

However, we have observed preliminary that E. coli NG30 under the conditions considered to be absent of DNA replication is also very sensitive to ultraviolet irradiation and a marked degradation of cellular DNA occurs, too, after ultraviolet irradiation.

Although the mechanism of degradation of the chromosome involving replicating point must not be the same to that of the chromosome without DNA replication, it is very interesting to investigate the degradation of DNA by ultraviolet irradiation of the cells without DNA replication, especially to make clear the correlation between the fragmentation or degradation of DNA molecules and the early events operating to the pyrimidine dimers on DNA molecules.

The present experimental results will make evident that, there is an extensive post-irradiation degradation of DNA in the chromosome, too, not involving the replicating point and the initial cause for the degradation is the single strand gap resulted from the excision of pyrimidine dimers which is the first step of dark repair process for ultraviolet damage on DNA molecules.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in the present experiments were *E. coli* NG30 (*recA*uvr^+), NG30*uvrC* (*recAuvrC*, double mutant) and the wild-type strain, H/r30-R (rec^+uvr^+). All of them were arginine-requiring mutants.[3] They were kindly provided by Drs. T. Kato and S. Kondo in the Department of Fundamental Radiology, School of Medicine, Osaka University. The cells were grown at 37°C with aeration by shaking in M9 medium supplemented with 2.5 mg/ml of Difco casamino acids (denoted as EM9). The M9 medium contained the following ingredients per 1,000 ml of water (pH 7.2) : 1.1 %

Na_2HPO_4, 0.3 % KH_2PO_4, 0.5 % NaCl, 0.1 % NH_4Cl, 0.4 % glucose, 10 ml 0.01 M $CaCl_2$ and 10 ml 0.1 M $MgSO_4$. One generation time of E. coli NG30 was about 60 min in EM9 medium at 37°C.

Ultraviolet irradiation

The suspension of bacteria was spread in a Petri dish to a depth of 1 mm and irradiated with a germicidal lamp (Mitsubishi GKL-10, 10 W) with gentle shaking at room temperature. The concentration of bacteria for irradiation was usually about 6 X 10^7 cells/ml. The dose rates used were 2 ergs/mm^2/sec and 20 ergs/mm^2/sec. The intensity of ultraviolet light was determined with a Toshiba UV photometer (Toshiba Elect. Co., GI-1, Japan), which was corrected by Dr. Laterjet's UV-photometer.

Analysis of DNA synthesis of bacteria under various conditions

Cells were grown in EM9 medium to approximately 3 X 10^8 cells/ml, washed twice and resuspended in five times the volume of M9 medium. The cultures in 5 ml were then supplemented with 3 µC/3µg/ml of [H^3]-thymidine and 200 µg/ml of deoxyadenosine. In order to provide two kinds of cell conditions, growth condition and amino acid-

starved condition, they were divided into two portions and then incubated at 37°C with or without 2.5 mg/ml of casamino acids. At regular intervals, 0.2 ml of the cultures was taken into the ice-cold 5 % trichloroacetic acid (TCA). The acid-insoluble fractions were collected on glass fiber filters (Toyo GB 100, Japan), washed and dried. The radioactivity of the fraction was counted using a Liquid Scintillation Counter (Nuclear Chicago Mark I, U.S.A.) in a toluene-DPO-POPOP mixture. In the experiments of DNA synthesis in the presence of chloramphenicol (CM), the bacterial cells were incubated with the [H^3]-thymidine at 37°C in the presence of CM.

Measurement of DNA degradation

1. Analysis by acid-insoluble materials

The measurement of post-irradiation degradation of DNA by the change of amount of acid-insoluble materials in the cells under a growth condition was carried out as follows ; Bacterial cells cultured overnight were diluted forty fold with the fresh EM9 medium supplemented with 3.3 μC/3 μg/ml of [H^3]-thymidine and 200 μg/ml of deoxyadenosine. The cell suspension was incubated aerobically for 2 hr at 37°C. The cells were harvested, washed three times, resuspended

in five times volume of the EM9 medium and irradiated with different doses of ultraviolet light at room temperature. The non-labeled thymidine was added to the irradiated cultures in a concentration of 100 μg/ml, and 0.2 ml was withdrawn from the cultures into the ice-cold 5 % TCA at regular intervals through the post-irradiation incubation at 37°C. The acid-insoluble fractions were collected on glass-fiber filters, dried and counted using a Liquid Scintillation Counter. The degree of DNA degradation in the cells through the post-irradiation incubation was indicated as per cent of radioactive counts in the acid-insoluble fractions of the cells withdrawn immediately after irradiation without incubation.

The measurement of DNA degradation in the amino acid-starved cells was carried out as follows ; [H^3]-thymidine-labeled cells in exponential growth phase were washed twice with M9 medium containing no amino acid, resuspended with five times volume of the M9 and incubated at 37°C for 90 min to starve the cells from amino acids. The amino acid-starved cells were irradiated with different doses of ultraviolet light. The experimental procedures after the irradiation were the same as described above.

In the experiments for DNA degradation of CM-treated cells, the

washed cells labeled with [H^3]-thymidine were incubated at 37°C for 90 min in EM9 with 50 μg/ml of CM and then irradiated with ultraviolet light.

The cells with DNA molecules labeled with [H^3]-thymidine on their different DNA region were prepared. The cells having DNA strands labeled only near the initiation point of DNA replication including the point were called as the cells containing labeled " early region " of DNA. The cells having DNA labeled only far region from the initiation point of DNA replication were called as the cells containing labeled " late region " of DNA. The former cells were prepared as follows ; The cells starved amino acids for 90 min were supplemented with 2.5 mg/ml of casamino acids, 10 μC/ml of [H^3]-thymidine and 200 μg/ml of deoxyadenosine to allow to initiate the DNA replication. After incubation at 37°C for 20 min, the label was chased with a large amount of non-labeled thymidine and the cells were incubated for more 80 min with 50 μg/ml. In order to prepare the latter, the amino acid-starved cells were supplemented first with casamino acids alone and incubated at 37°C for 20 min, and then [H^3]-thymidine, deoxyadenosine and CM were added to the cell suspension in the concentration of 10 μC/ml, 200 μg/ml and

50 μg/ml, respectively. Then the cells were incubated for 80 min at 37°C. The experimental methods for the measurement of post-irradiation degradation of DNA in the cells labeled in different DNA region were essentially the same as described above.

2. Sedimentation analysis by alkaline sucrose-density gradient

The post-irradiation degradation of DNA was also analyzed by sedimentation techniques. The sedimentation analysis of DNA molecules in alkaline sucrose gradient was carried out essentially in the same methods as described by McGrath and Williams.[14] The cells were harvested by a centrifugation at 6,000 rev./min for 5 min and resuspended in 0.01 M Tris-HCl buffer (pH 8.1) containing 14 % sucrose and 0.01 M EDTA. The cell suspension was incubated at 37°C for 5 min with 1 mg/ml of lysozyme to convert the cells into the spheroplasts. The spheroplasts in 0.1 ml of suspension were placed on the layer of 0.5 N NaOH (0.2 ml) which had been layered on a linear gradient of sucrose (5 - 20 % in 4.4 ml) dissolved in 0.2 N NaOH, 0.7 M NaCl and 0.001 M EDTA. Usually, about 3×10^6 spheroplasts were placed on the gradient. The samples were centrifuged for 90 min at 30,000 rev./min at 4°C in

a swinging bucket rotor (RPS-40) of Hitachi Preparative Ultra-centrifuge (Japan). After the centrifugation, two-drop fractions were collected from the bottom of the centrifuge tube pierced with a thin needle on pieces of filter paper (Toyo No. 7). The pieces were suscessively washed with 5 % TCA, 95 % ethanol and ether, and then dried. Radioactivity on the paper was counted using a Liquid Scintillation Counter in a toluene-DPO-POPOP mixture.

Measurement of pyrimidine dimers

Measurement of pyrimidine dimers in DNA molecules was carried out as follows ; [H^3]-thymidine-labeled cells were irradiated with ultraviolet light. The cells were precipitated and washed twice with 5 % TCA. The precipitates were then washed with 10 ml each of 80 % ethanol, 99 % ethanol and a mixture of ethanol and ether (1 : 1), successively by centrifugation. The washed precipitates were dispersed in 1 ml of 5 % TCA and incubated in a water bath at 95°C for 30 min. The extracts containing labeled DNA were treated with ether repeatedly three times to remove the TCA from the extracts and then concentrated to about 0.1 ml by evaporation. The DNA solution was hydrolyzed with 99 % formic acid for 15 min at 175 -

180°C in a sealed glass tube saturated with nitrogen gas. The hydrolysates were applied on a paper strip (Toyo No. 51, 2 X 40 cm) and developed with a mixture of n-butanol-acetic acid-water (80 : 12 : 30)[15] at room temperature by ascending chromatography. In the present system for chromatography, the R_f values of thymine, thymine dimer and thymine-uracil dimer were 0.6, 0.3 and 0.2, respectively.

The measurement of pyrimidine dimers released from the irradiated cells into acid-soluble fraction during post-irradiation incubation was also carried out by essentially the same procedures as described above. Materials in the acid-soluble fractions were hydrolyzed with formic acid and chromatographed by paper.

The amounts of labeled nucleotides and oligonucleotides including pyrimidine dimers in the acid-soluble fractions extracted from irradiated cells were measured. The acid-extracted materials were charged into Dowex-1 (Cl^- type, X 8, 200 - 400 mesh) column (1 X 2.5 cm) and eluted stepwise with water and then with 0.5 N HCl. Labeled nucleosides are eluted by the former step and all of the labeled nucleotides are eluted by the latter step.

Other remarks

Lysozyme (E. C. 3. 2. 1. 17) was purchased from Sigma Chemical Co. Ltd. (U.S.A.). $[H^3]$-thymidine was obtaind from Japan Radioactive Isotope Corporation (Tokyo).

RESULTS

Dose-survival curves of E. coli H/r30-R and NG30 irradiated with ultraviolet light

Cells of E. coli H/r30-R and NG30 in growth phase, stationary phase, starved condition for amino acids or a condition pre-incubated with CM were irradiated with ultraviolet light, respectively, and the colony forming abilities were examined. The numbers of colony were plotted against ultraviolet dose used in Fig. 1.

(Fig. 1)

It was shown that E. coli NG30, even if the cells were in different physiological conditions, was still highly sensitive to ultra-

violet light as compared with the wild-type strain H/r30-R, although the E. coli NG30 in stationary phase, amino acid-starvation condition or pre-incubated condition with CM was a little less sensitive in every case than the cells in growing phase.

Degradation of DNA of E. coli NG30 in growing phase after ultraviolet irradiation

The mode of DNA degradation in growing phase of E. coli NG30 after ultraviolet irradiation was investigated. The cells, of which DNA was uniformally labeled with [H^3]-thymidine, were washed and resuspended in EM9 medium. The cultures were incubated at 37°C in the same medium after ultraviolet irradiation. The doses used were 20, 100 and 400 ergs/mm^2. The remaining radioactivity in the acid-insoluble fractions was determined as described in Materials and Methods. The results are shown in Fig. 2.

(Fig. 2)

It was shown that the radioactivity in the acid-insoluble fractions was rapidly decreased during the post-irradiation incubation

in each case. However, it was also known that there was no linear relationship between the rate of degradation and the ultraviolet dose, and when the cells in growing phase were irradiated, even only by a small dose of 20 ergs/mm^2 resulted an extensive degradation of DNA. A marked spontaneous degradation of DNA of unirradiated cells was also observed.

Stoppage of DNA replication by amino acid-starvation and by chloramphenicol

The DNA replication during amino acid-starvation was examined through the incorporation of [H^3]-thymidine into acid-insoluble fractions. Fig. 3 shows that the rate of incorporation of the label

(Fig. 3 a, b)

in the amino acid-starved conditions are slowly decreased initially with the time of incubation and finally completely stopped by about 90 min incubation in both strains.

The restoration of DNA replication was also shown in Fig. 3b. When the starved cells of NG30 were transfered from the M9 medium

to the EM9 medium, the rate of incorporation of label rose quickly to nearly the same rate of DNA replication of the cells supplemented with amino acids.

Fig. 4 shows that the chloramphenicol stops completely the DNA replication of the cells, E. coli NG30, even if the amino acids were abundantly present in the medium, by the incubation with the drug for about 60 - 70 min as well as the case of amino acid-starvation.

(Fig. 4)

Degradation of DNA of E. coli NG30 after ultraviolet irradiation in the absence of DNA replication

a. Correlation between degradation rate and ultraviolet dose

The degradation of DNA of E. coli NG30 after ultraviolet irradiation in the absence of DNA replication was examined. The cells were starved amino acids by the incubation for 90 min in M9 medium and then irradiated with different doses of ultraviolet light. The mode of the loss of radioactivity from the acid-insoluble fractions in the course of time incubation at 37°C in M9 medium are shown in Fig. 5.

(Fig. 5 a, b)

It is obvious in Fig. 5a that the DNA of the cells, E. coli NG30 in which any DNA replication has been ceased is also extensively degradated as well as the case of cells in growing phase, although the degradation rate, especially at the low doses, is smaller than that in growing cells having replicating point (Reffer Fig. 2). The spontaneous degradation of DNA is also less in the starved cells than the growing cells.

However, the rate of DNA degradation in this physiological state of NG30 seemed to correspond more well to the ultraviolet dose than that in growing cells.

The results in Fig. 5b were shown as reference data. The post-irradiation degradation of DNA in amino acid-starved E. coli H/r30-R and E. coli B_{s-1} were shown. Only a limitted degradation was observed in E. coli H/r30-R irradiated with 1,000 ergs/mm^2. It is sure that the phenomena are related to the ability of excision-repair systems functioning on the ultraviolet damage to DNA molecules, since E. coli B_{s-1} which lacks the ability to excise pyri-

midine dimers[16] exhibits no DNA degradation.

The DNA degradation during the post-irradiation incubation of E. coli NG30 of which DNA replication had been stopped by the pre-incubation for 90 min in the presence of CM is shown in Fig. 6.

(Fig. 6)

It was also shown that the DNA of E. coli NG30 broke rapidly down in course of time of incubation as the case of amino acid-starved cells. The rates of degradation correlated well to the ultraviolet doses.

b. Comparison of post-irradiation degradation of DNA in E. coli NG30 labeled in different DNA regions

The experiments were designed to study whether or not the post-irradiation degradation of DNA might begin at a some specific point even if the DNA had no replicating point. Two kinds of cells of E. coli NG30 labeled in different DNA region (" early region " and " late region " ; see Materials and Methods) were prepared. The loss of radioactivity from the acid-insoluble fractions was

respectively determined in these two kinds of cells, and the rates of post-irradiation degradation of labeled DNA region were compared each other. The results were shown in Fig. 7.

(Fig. 7 a, b)

The quite analogous results were found in the both experiments. The rates of post-irradiation of DNA by the same ultraviolet dose were nearly equal to each other in both DNA regions. These results indicate that the post-irradiation degradation of DNA of E. coli NG30 in the absence of DNA replication does not be induced at a specific site in DNA molecules but can begin at many sites of DNA at the same time.

c. Degradation of DNA of E. coli NG30uvrC after ultraviolet irradiation

In order to know the contribution of dimer-excising phenomena to the post-irradiation degradation of cellular DNA, the degradation of DNA of a double mutant in recA and uvrC was examined. E. coli NG30uvrC which is lacking or leaky in the ability of dimer excision

was used. The experimental results of DNA degradation during post-irradiation incubation of the cells irradiated with various ultraviolet doses were shown in Fig. 8.

(Fig. 8)

It was found that the post-irradiation DNA degradation of E. coli NG30 was markedly suppressed by the additional mutation of uvrC. However, it was also known that a significant degradation of DNA was still occured in the cells of E. coli NG30uvrC.

The amounts of dimers in the DNA of the cells incubated for 60 and 120 min after ultraviolet irradiation of 400 ergs/mm^2 were measured, respevtively. Fig. 9a shows the paperchromatograms indicating the time-dependent decrease of the amount of pyrimidine dimers during post-irradiation incubation of E. coli NG30. The results by E. coli NG30uvrC were shown in Fig. 9b.

(Fig. 9 a, b)

A decrease of pyrimidine dimers from the DNA in accordance with

the time of post-irradiation incubation was apparent in E. coli NG30, but no removal of pyrimidine dimers from the DNA of E. coli NG30uvrC were significantly detected.

d. Effect of caffeine on the DNA degradation in E. coli NG30

The effect of caffeine on the DNA degradation in the cells of E. coli NG30 following ultraviolet irradiation was studied. Caffeine was added to the cultures immediately before irradiation of 400 ergs/mm^2. The results are shown in Fig. 10.

(Fig. 10)

The degradation of DNA of E. coli NG30 during post-irradiation incubation was inhibited in accordance with the increased concentration of caffeine.

It was confirmed from the above all experimental results that the radiosensitive bacteria, E. coli NG30, show a marked post-irradiation degradation of DNA even if the cells were not in growing phase but in a resting condition in which there is no DNA replica-

tion. In addition, the above experimental results allowed also us to assume that the post-irradiation degradation of DNA must be caused by the excision of pyrimidine dimers produced in ultraviolet-irradiated DNA.

If the degradation of DNA of E. coli NG30 was directly related to the excision of pyrimidine dimers, fragmentation of DNA should be accompanied with the release of dimers from the DNA, which produces single-strand gaps in DNA strands, followed by progressive degradation of DNA molecules.

Analysis of degradation by sedimentation profiles of DNA in alkaline sucrose-density gradient

The patterns of DNA in the cells of E. coli NG30 in a condition without DNA replication were analyzed in an alkaline sucrose gradient in relation to the post-irradiation degradation of DNA depending to the ultraviolet doses and/or to the time of post-irradiation incubation.

a. Initial fragmentation of DNA by excision of pyrimidine dimers

The amino acid-starved cells labeled with $[H^3]$-thymidine were

prepared. The cells irradiated with 200 and 1,000 ergs/mm^2 respectively, and allowed to incubate only for 5 min in M9 medium at 37°C were harvested in ice-cold temperature, lysed by lysozyme and centrifuged in alkaline sucrose-density gradient. The sedimentation profiles were shown in Fig. 11.

(Fig. 11 a, b)

It was seen that the main peak of DNA distribution was shifted more to the smaller size of DNA in correspondence to the increase of ultraviolet dose even in the radioresistant cells of H/r30-R. The shifting rates of the DNA peak from the peak of unirradiated cells were nearly the same each other in both strains.

The facts indicate strongly that the initial events followed by extensive degradation of DNA in E. coli NG30 during the post-irradiation incubation is the removal of pyrimidine dimers from the irradiated DNA resulting in single strand gaps in the DNA molecules.

b. Post-irradiation degradation of DNA in E. coli NG30 in correspondence to ultraviolet doses

The amino acid-starved E. coli NG30 cells were irradiated with different doses of ultraviolet light. The cells were then incubated for 20 min at 37°C in the M9 medium. The patterns of the DNA of the cells were compared in relation to the ultraviolet dose. The results were shown in Fig. 12.

(Fig. 12)

It is known that the main peak of DNA distribution was shifted more to the small size of DNA in correspodence to the increase of ultraviolet dose, and that the distribution of DNA becomes broad as compared with that of non-irradiated cells.

The mean size of each DNA distribution shown in Fig. 12 was compared with that of non-irradiated cells. The relative values of number average molecular weight of DNA of the each distribution were calculated using Kapp and Smith's formula[17] assuming the molecular weight of DNA from non-irradiated cells as 1. The values were 0.72, 0.57, 0.37 and 0.26, for 100, 200, 400 and 1,000 ergs/mm^2, respectively.

c. Degradation of DNA in E. coli NG30 during post-irradiation incubation

E. coli NG30 and H/r30-R were used. The cells starved amino acids were irradiated with 200 or 1,000 ergs/mm^2 and then incubated at 37°C for 20 and 120 min in the M9 medium. The lysed cells were centrifuged as before. The sedimentation profiles are shown in Fig. 13.

(Fig. 13 a, b, c)

It was found that the degradation of DNA in the irradiated E. coli NG30 progressed only into shorter fragment of DNA in correspondnece to the time course of post-irradiation incubation. (Fig. 13 b and c) Both the molecular size of DNA strand and the acid-precipitable amounts of DNA at the post-irradiation incubation of 120 min are smaller than those at the time of 20 min. This phenomenon is more distinct in the cells irradiated with 1,000 ergs/mm^2 than the cells with 200 ergs/mm^2.

On the contrary, in E. coli H/r30-R (Fig. 13 c), it was known that, although the irradiation with 1,000 ergs/mm^2 resulted in a

small size of DNA strands in alkaline condition at 5 min incubation, the DNA molecules did not continue to break down into small pieces but recovered almost to the molecular size of DNA in non-irradiated cells during the post-irradiation incubation for 20 min.

The change in molecular size of DNA in E. coli NG30 in course of time of post-irradiation incubation was summarized in Table I. The molecular size of the main peak in DNA distribution was represented as a distance from the meniscus in alkaline sucrose gradient.

(Table I)

Release of pyrimidine dimers from irradiated E. coli NG30 into acid-soluble fraction

In order to make sure the hypothesis that the initial event for the induction of DNA degradation in ultraviolet-irradiated E. coli NG30 is the fragmentation of DNA caused by removal of pyrimidine dimers from DNA molecules, the cells were irradiated with various doses of ultraviolet light and the labeled nucleotides in the acid-soluble fraction extracted from the cells incubated for 5 min after ultraviolet irradiation were measured by column chromatographic tech-

niques. The amounts of label were plotted against ultraviolet dose in Fig. 14.

(Fig. 14)

It is clear that the removal of pyrimidine dimers from the ultraviolet-irradiated DNA occurs as an early event for the post-irradiation degradation of DNA of E. coli NG30, and that the release of dimers is more in accordance with the increase of ultraviolet dose.

The released pyrimidine dimers were detected by paper-chromatography in the acid-soluble fractions extracted from the cells incubated for 5 min after ultraviolet irradiation by a dose of 2,220 ergs/mm^2. The results were shown in Fig. 15.

(Fig. 15)

It is apparent that the acid-soluble fractions of E. coli NG30 contains pyrimidine dimers excised from the DNA, even when the fractions were prepared from the cells incubated for only 5 min

after ultraviolet irradiation.

From the above all experimental results, it can be concluded that the extensive post-irradiation degradation of DNA in E. coli NG30 is induced by a progressive DNA degradation expanding indipendently from completing the repair of single-strand gaps in DNA molecules produced by removal of pyrimidine dimers.

DISCUSSION

The present experiments cleared that a recombination-deficient mutant, E. coli NG30, is very sensitive to ultraviolet irradiation and a degradation of cellular DNA occurs extensively after ultraviolet irradiation, even when there is no DNA replication in the cells.

Maaløe and Hanawalt[18] have reported that, although amino acid-starvation allows to complete the present cycle of DNA replication to the starved cells, it never allows to initiate the next cycle of DNA replication. It is apparent, from the experimental results

about the incorporation of [H^3]-thymidine into acid-insoluble materials, that the present experimental conditions, that is, incubating the cells in M9 medium or incubation with chloramphenicol, block completely to reinitiate the new cycle of DNA replication in E. coli NG30.

The phenomenon that the DNA molecules of E. coli NG30 in which there is no DNA replication were also extensively broken down by post-irradiation incubation as in the growing cells in which the DNA continues to replicate was confirmed by the rapid decrease in the amount of acid-precipitable labels and by the conversion of initial cellular DNA to small fragments of DNA, although the rates of degradation of DNA in the growing cells were much greater than that in the resting cells when the cells were irradiated with relatively low doses of ultraviolet light.

Moreover, it is also evident that the degradation of DNA in the resting cells of E. coli NG30 does not begin at a specific site of DNA, but can occur from many sites of DNA at the same time and at the same rate in irradiated cells.

The assumption that the first events for the induction of post-irradiation degradation of DNA having no replicating points must be the excision of pyrimidine dimers from the DNA is reasonably accept-

able by the following two facts, (1) the DNA molecules in the irradiated cells of E. coli NG30 incubated for only 5 min after ultraviolet irradiation has been cut into smaller fragments as well as that observed in the wild-type strain H/r30-R ; (2) there is actually the release of pyrimidine dimers into acid-soluble fractions from the cells incubated for the short time after ultraviolet irradiation. The assumption was further supported by the facts that the post-irradiation degradation of DNA was markedly suppressed in a double mutant, E. coli NG30uvrC, and inhibited by the presence of caffeine. It is known that caffeine inhibits the excision of pyrimidine dimers[19,20] by forming a complex with ultraviolet-irradiated DNA.[19] The residual degradation of DNA in irradiated cells of E. coli NG30uvrC may be due to a low activity of dimer-excision detectable in irradiated uvrC mutants.[21]

It is sure that the DNA molecules in growing cells of E. coli NG30 were broken extensively down even when the cells were irradiated with only a small dose of 20 ergs/mm^2, as the observations by Horii et al.[10] on a RecA$^-$ mutant of E. coli K12, JC 1569b, although E. coli NG30 in resting state shows no so great post-irradiation degradation of DNA by such small doses.

It may be true that a different mechanism must operate on the degradation process of DNA in growing cells from that in the resting cells.

The author would like to speculate about the distinct phenomena in growing cells as that, the enhanced post-irradiation degradation of DNA may be resulted from such a synergistic effect as that the function of DNA replication disturbs furthermore the process of DNA degradation induced by a predominant degradation of DNA uncoupled well to the effective repair of ultraviolet lesions, through running of the replicating points across the degradation sites in DNA strands.

The above speculation means that the initial cause for the post-irradiation degradation of DNA in E. coli NG30 is dimer-excision, and the produced gaps would be enlarged by a predominant degradation event not co-ordinated well with the process of repairing the gaps. The arrival of DNA replicating points to such the disordered degradation site would make the events more complex occuring to the degradation sites, resulting in drastic DNA degradation.

There is a report[22] that a RecA$^-$ mutant of E. coli K12 can recover significantly from ultraviolet-inactivation by incubating the irradiated cells in buffer solutions. The phenomana of liquid holding

recovery would be related to the present experimental facts that the post-irradiation degradation of DNA in amino acid-starved E. coli NG30 was greatly suppressed by omitting glucose from the M9 medium (See, Fig. 16).

(Fig. 16)

Anyhow, a phenotypical character of E. coli NG30, high sensitivity to ultraviolet irradiation, must be essentially related to the phenomena observed at the irradiated cellular DNA which breaks extensively down by post-irradiation incubation.

SUMMARY

The post-irradiation degradation of DNA in a radiosensitive mutant, E. coli NG30, was investigated. An extensive degradation of DNA corresponding to the ultraviolet dose was observed in the cells which replicate no DNA.

The post-irradiation degradation of DNA having no replicating

points does not begin to occur from a specific point of DNA, but do occur to many sites of irradiated DNA at the same time and develops at the same rate.

The fact that the initial cause for the DNA degradation is the excision of pyrimidine dimers from the DNA molecules was supported by the following experimental results that, (1) the immediate fragmentation of cellular DNA was observed in the cells shortly after the ultraviolet irradiation by the alkaline sucrose dradient analysis, (2) the pyrimidine dimers were detected in acid-soluble fractions extracted from the cells incubated for only 5 min after ultraviolet irradiation, (3) the post-irradiation degradation was markedly suppressed in a double mutant, E. coli NG30uvrC, (4) caffeine inhibited significantly the post-irradiation degradation of DNA.

The mechanism by which the followed extensive DNA degradation is resulted from the gaps in DNA strands produced by excision of pyrimidine dimers was discussed.

ACKNOWLEDGEMENT

The author would like to acknowledge the helpful guidance and critical discussions by Professor Ichijiro Honjo and Assistant Professor, Dr. Keiichi Nozu during the present experiments.

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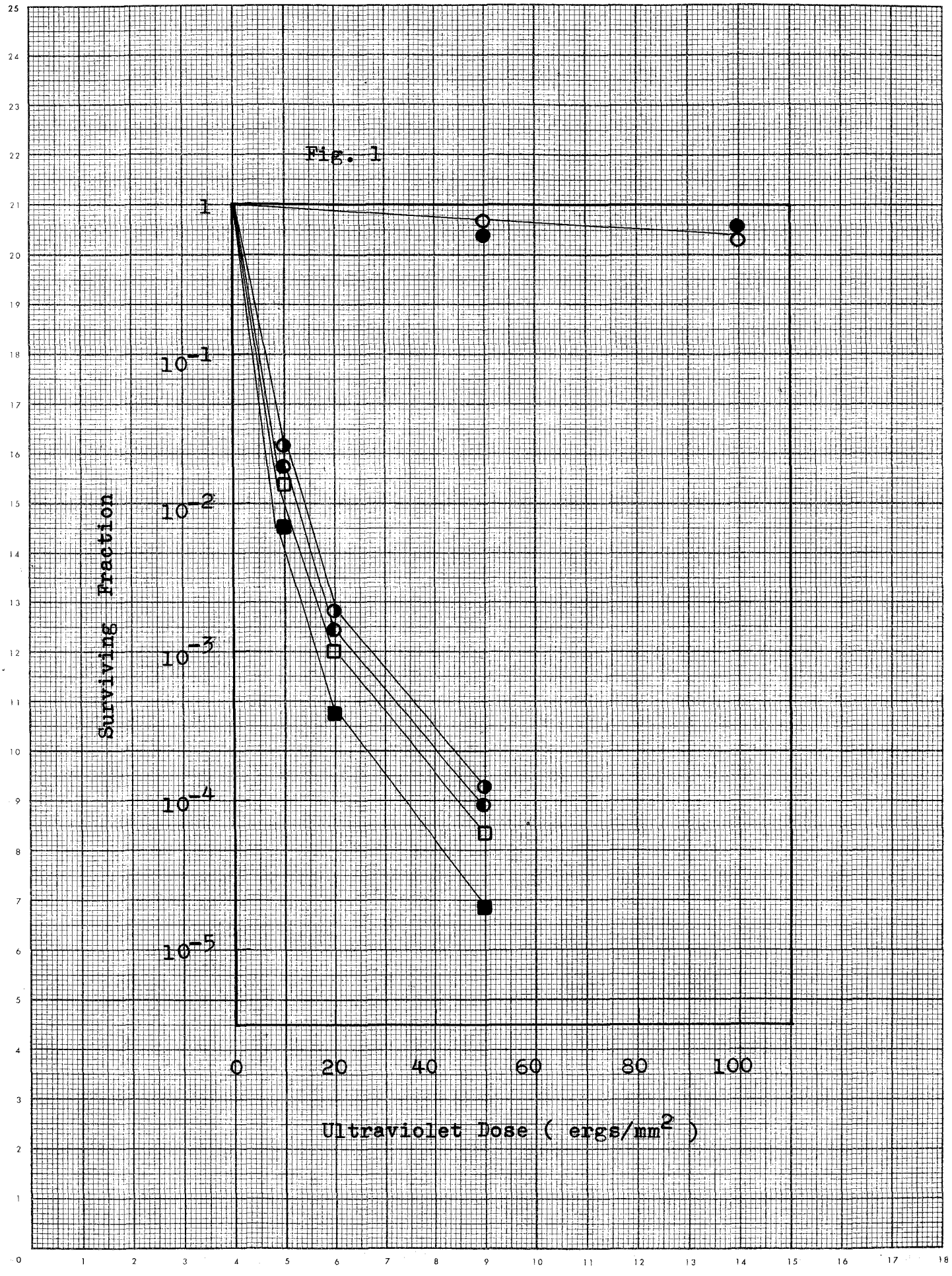

A4 1mm (250×180) コクヨ

Fig. 2

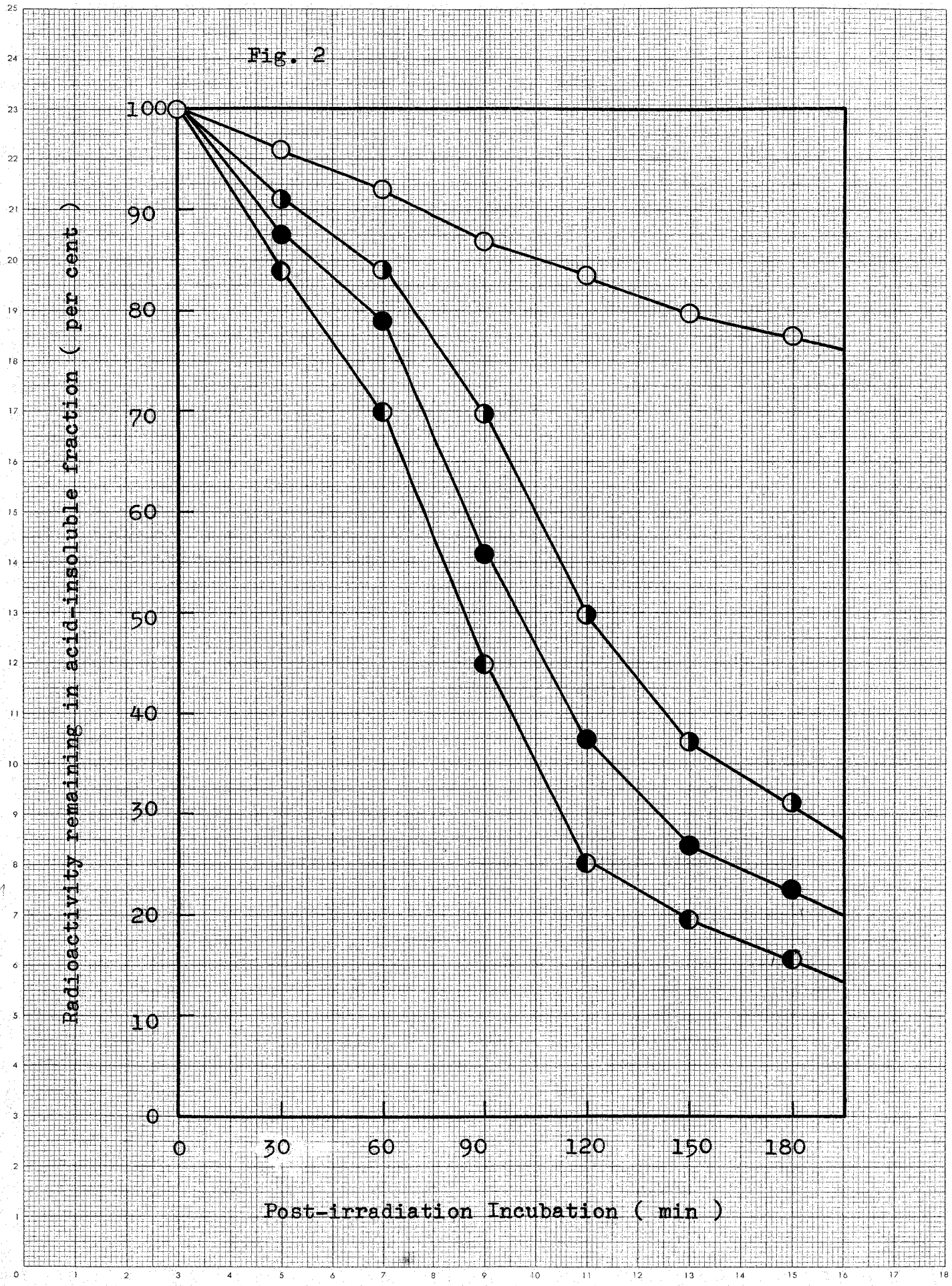

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Fig. 3

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Fig. 4

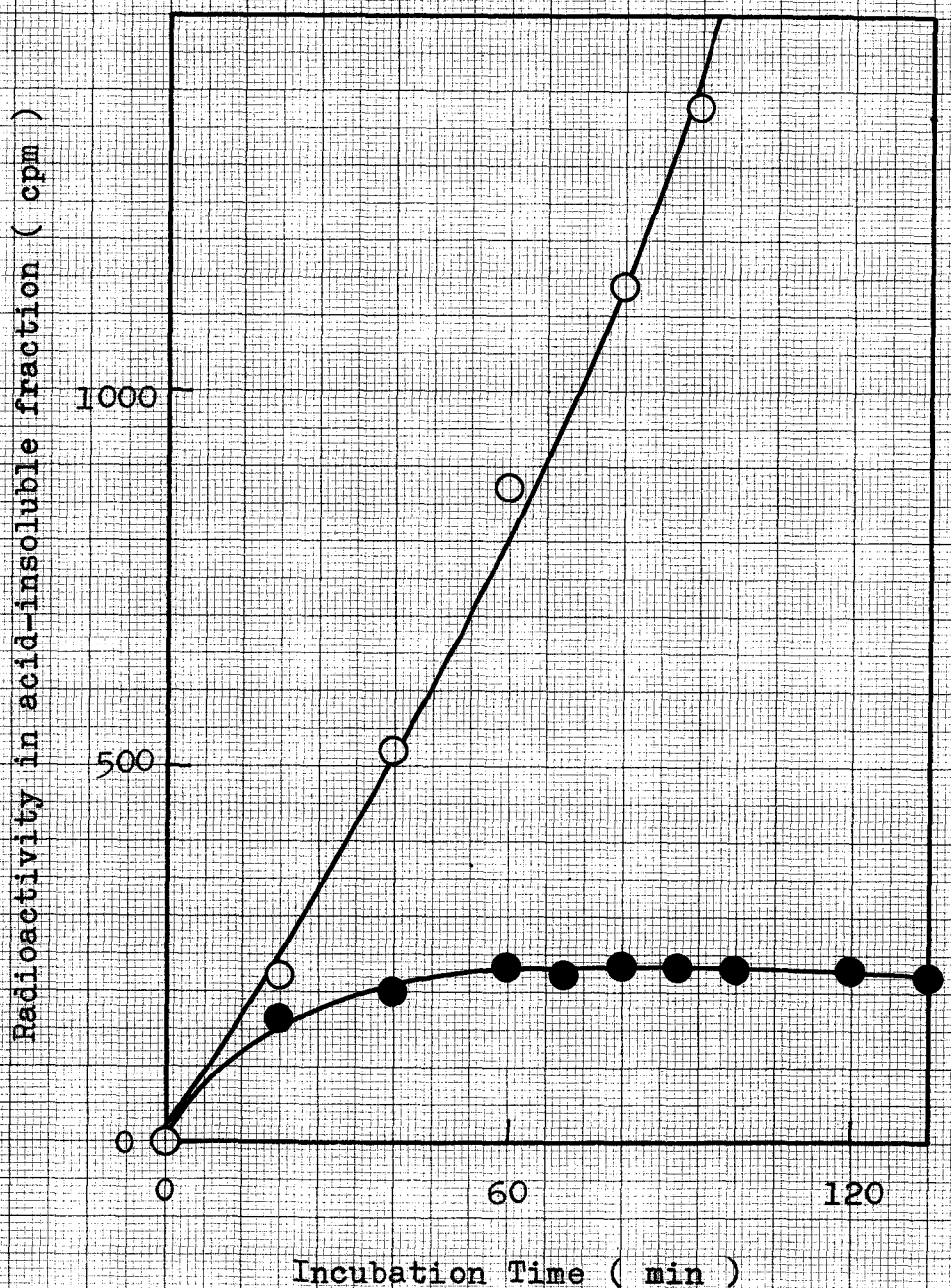

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Fig. 5

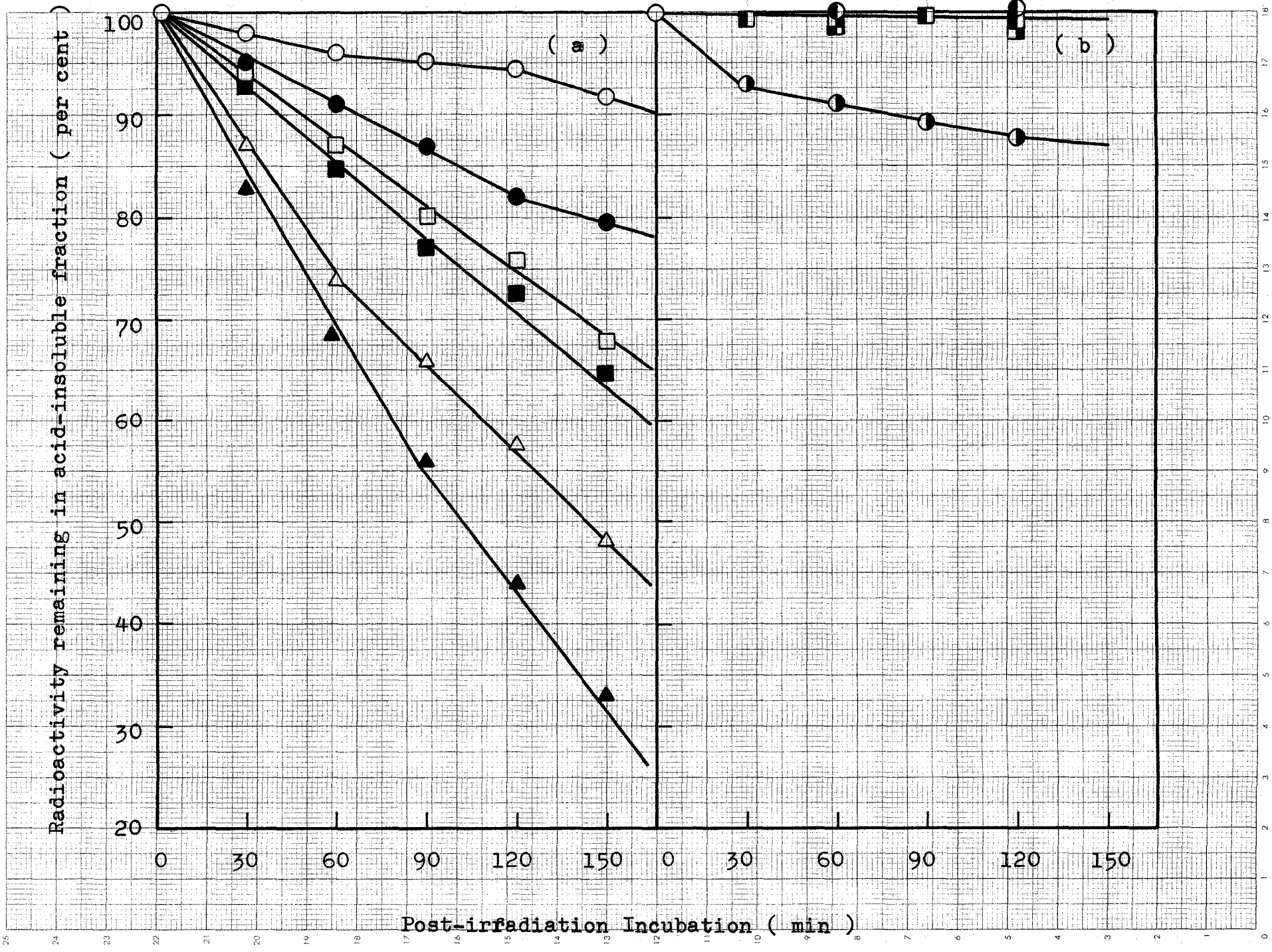
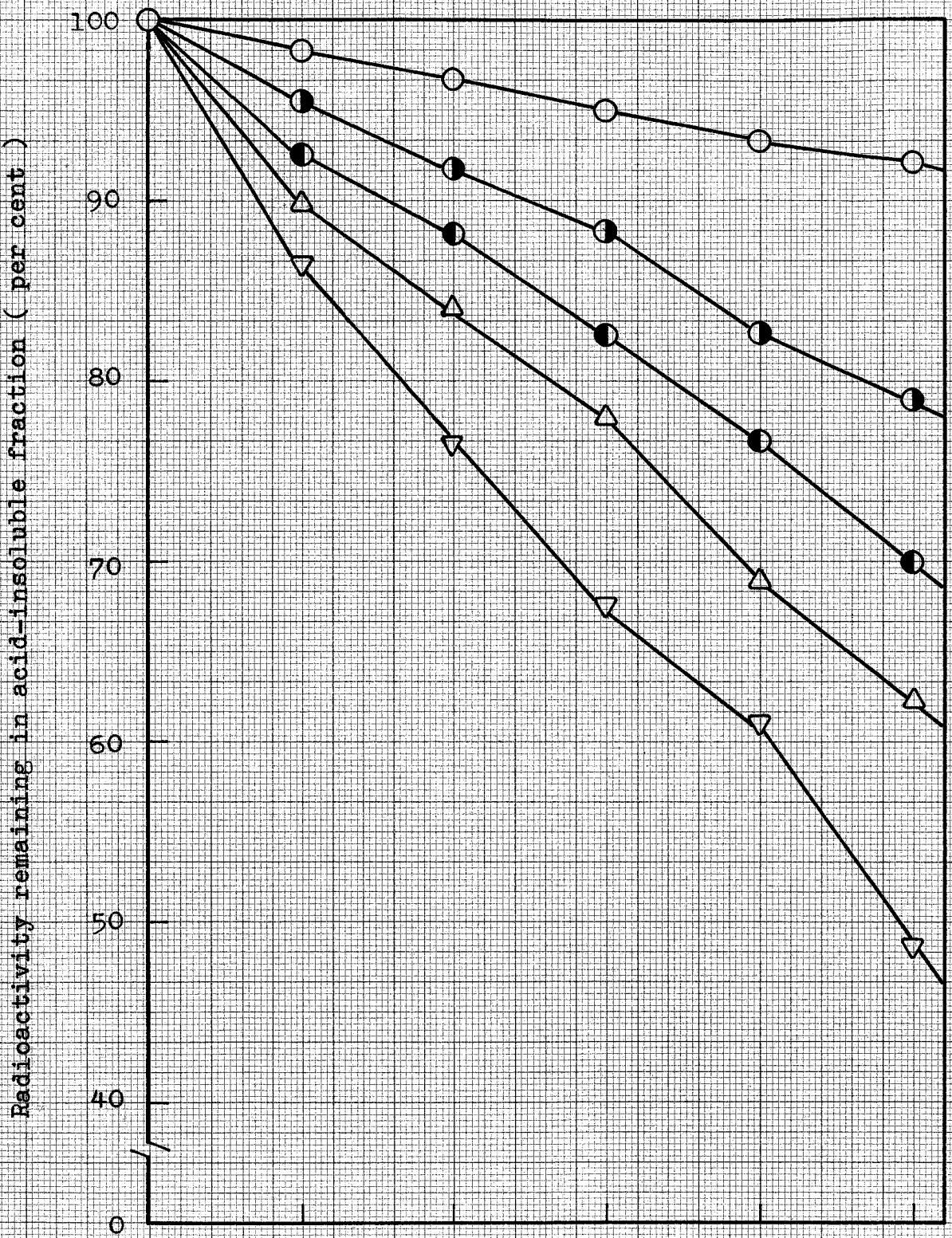

Radioactivity remaining in acid-insoluble fraction (per cent)
100
90
80
70
60
50
40
30
20
(a)
(b)
0
30
60
90
120
150
Post-irradiation Incubation (min)

Fig. 6



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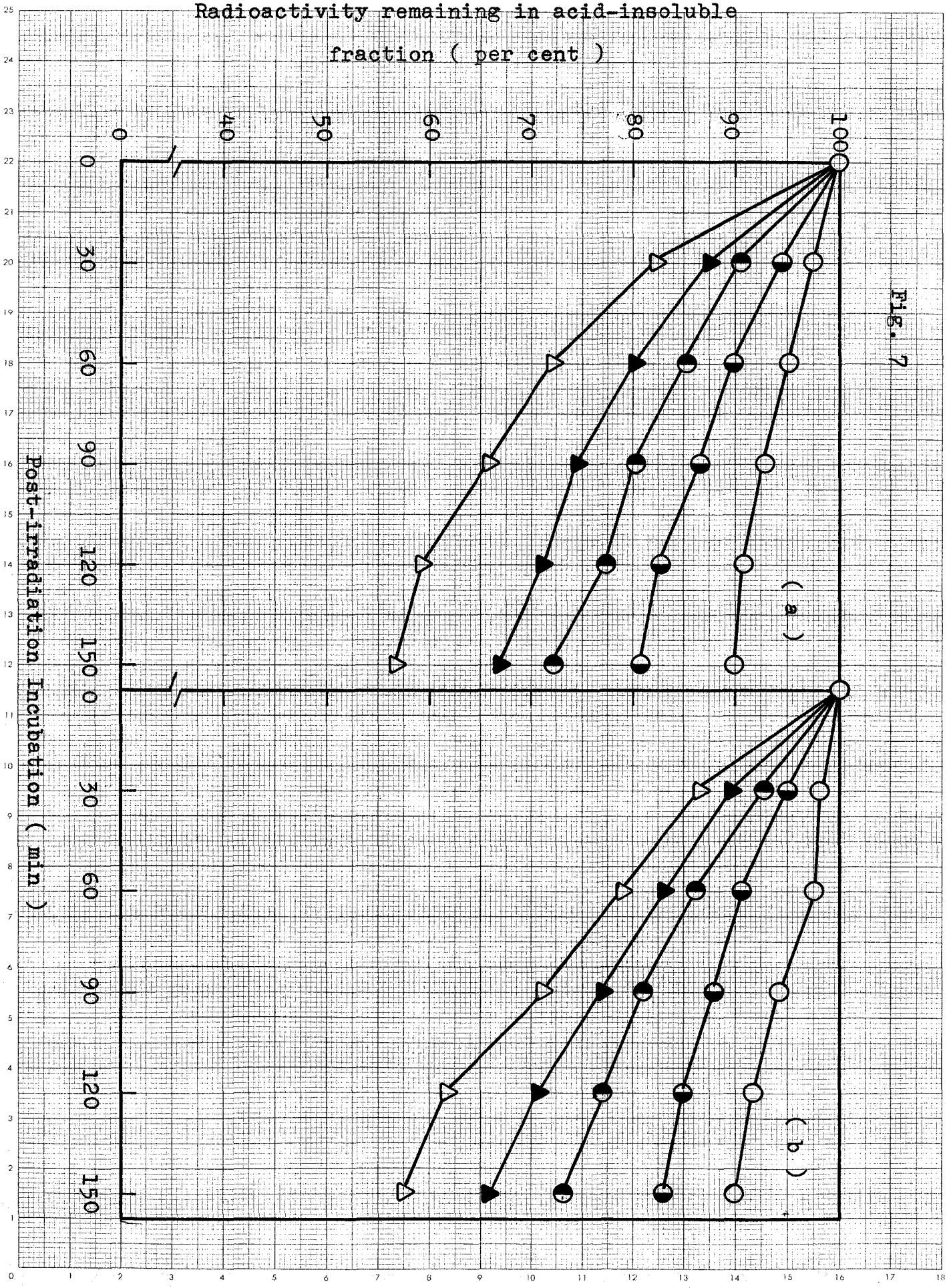

Fig. 7

A4 1 mm (250×180) コクヨ

Fig. 8

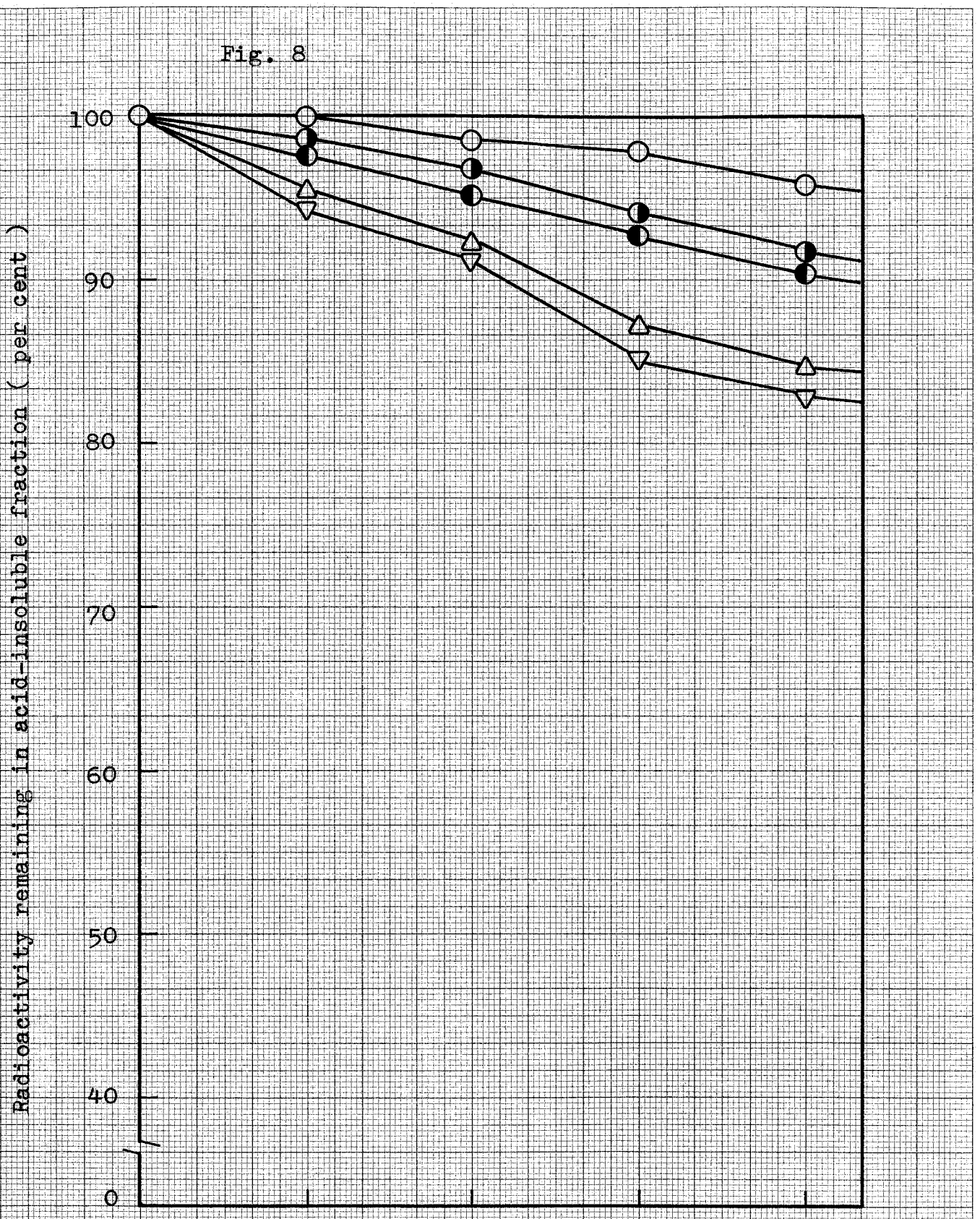

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Fig. 9

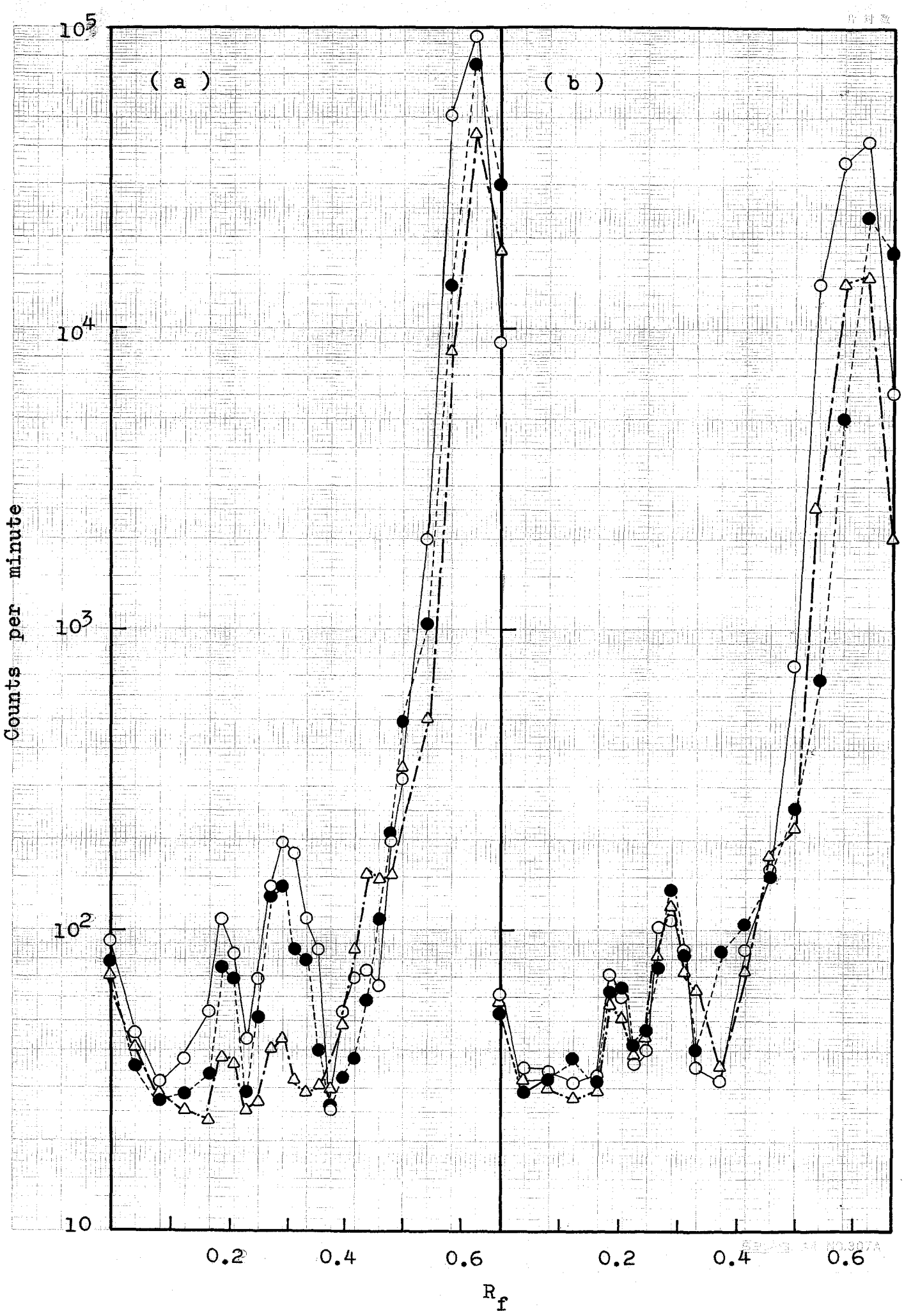

Fig. 10

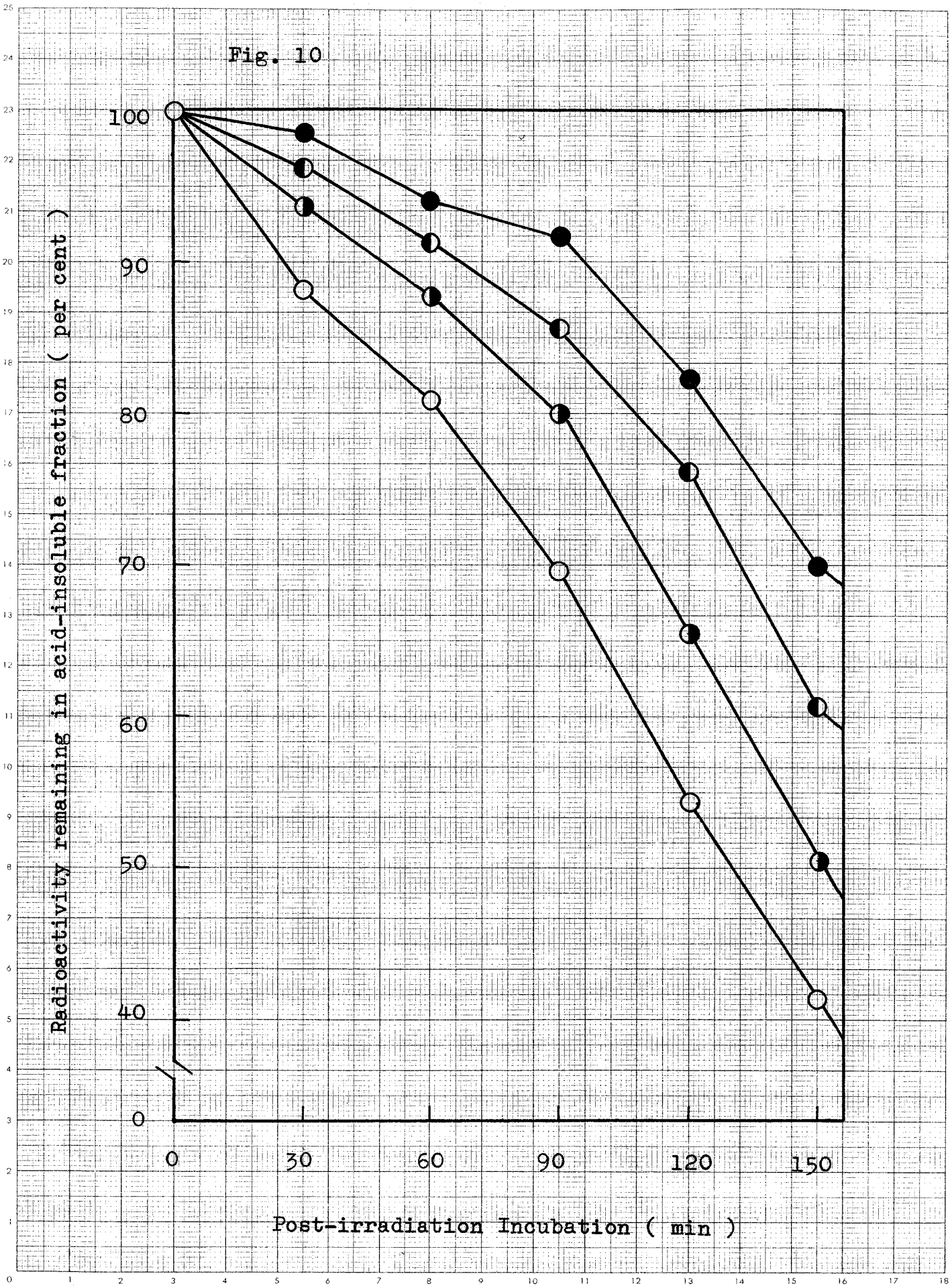

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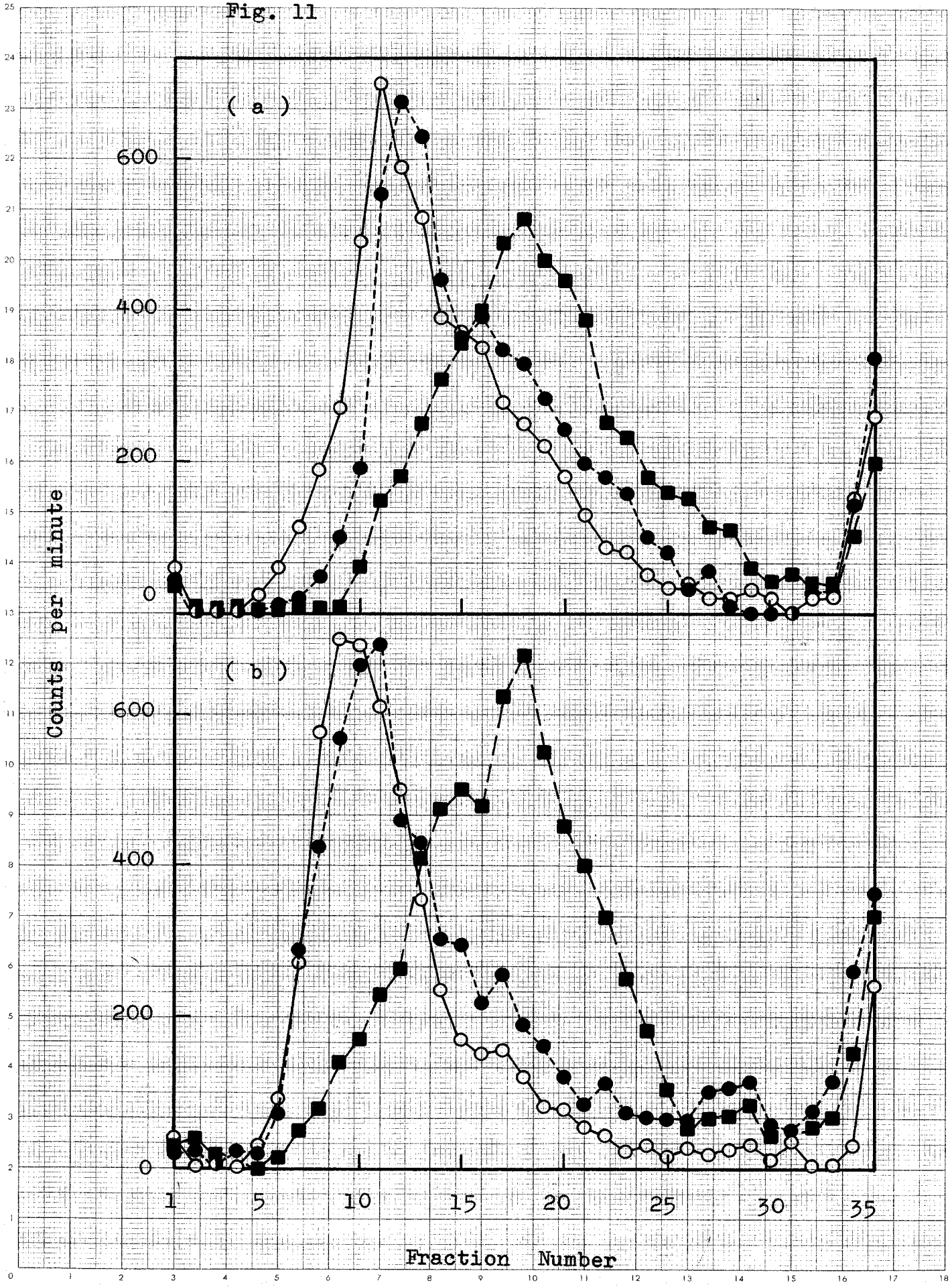
Fig. 11



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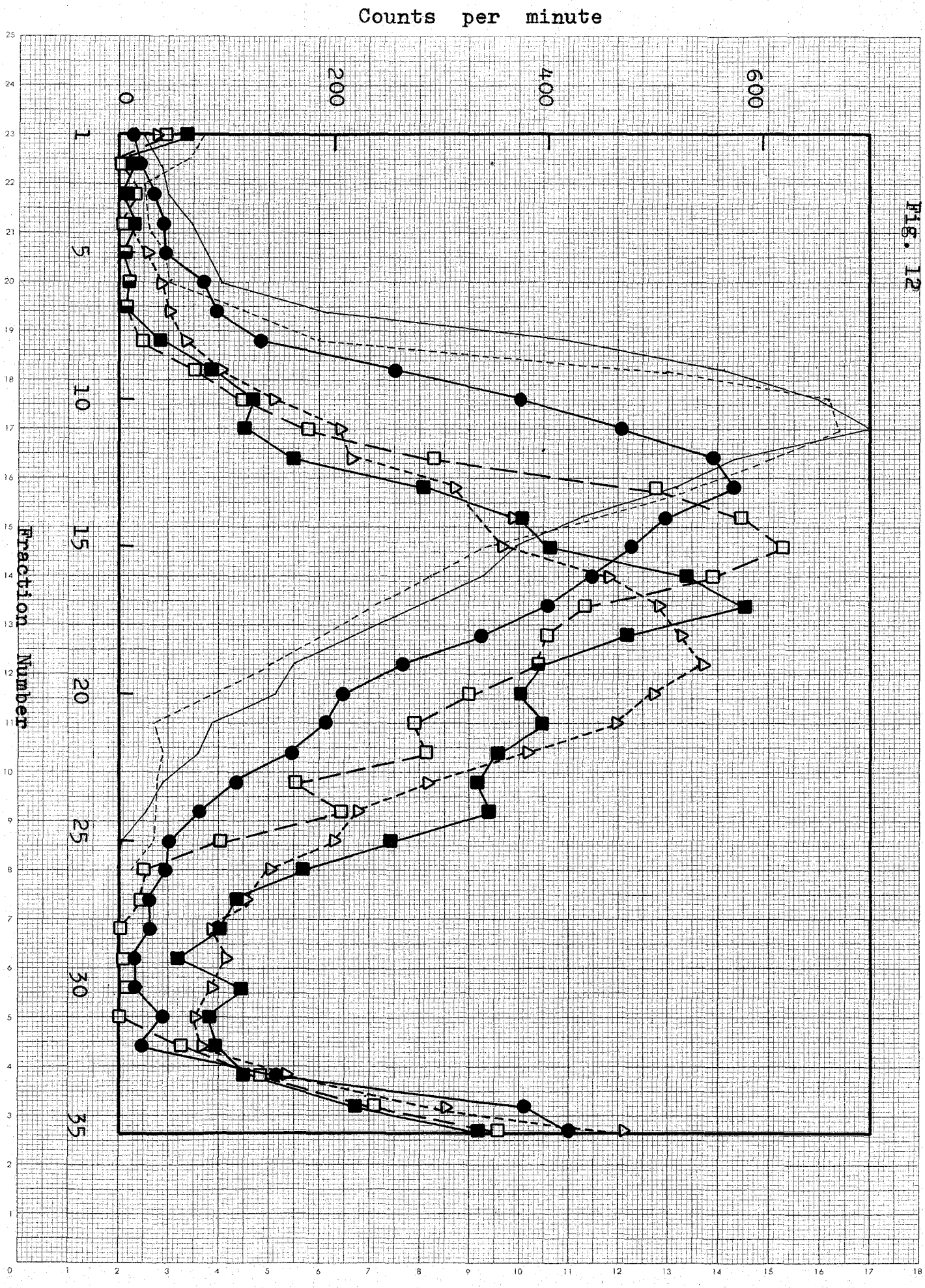

Fig. 12

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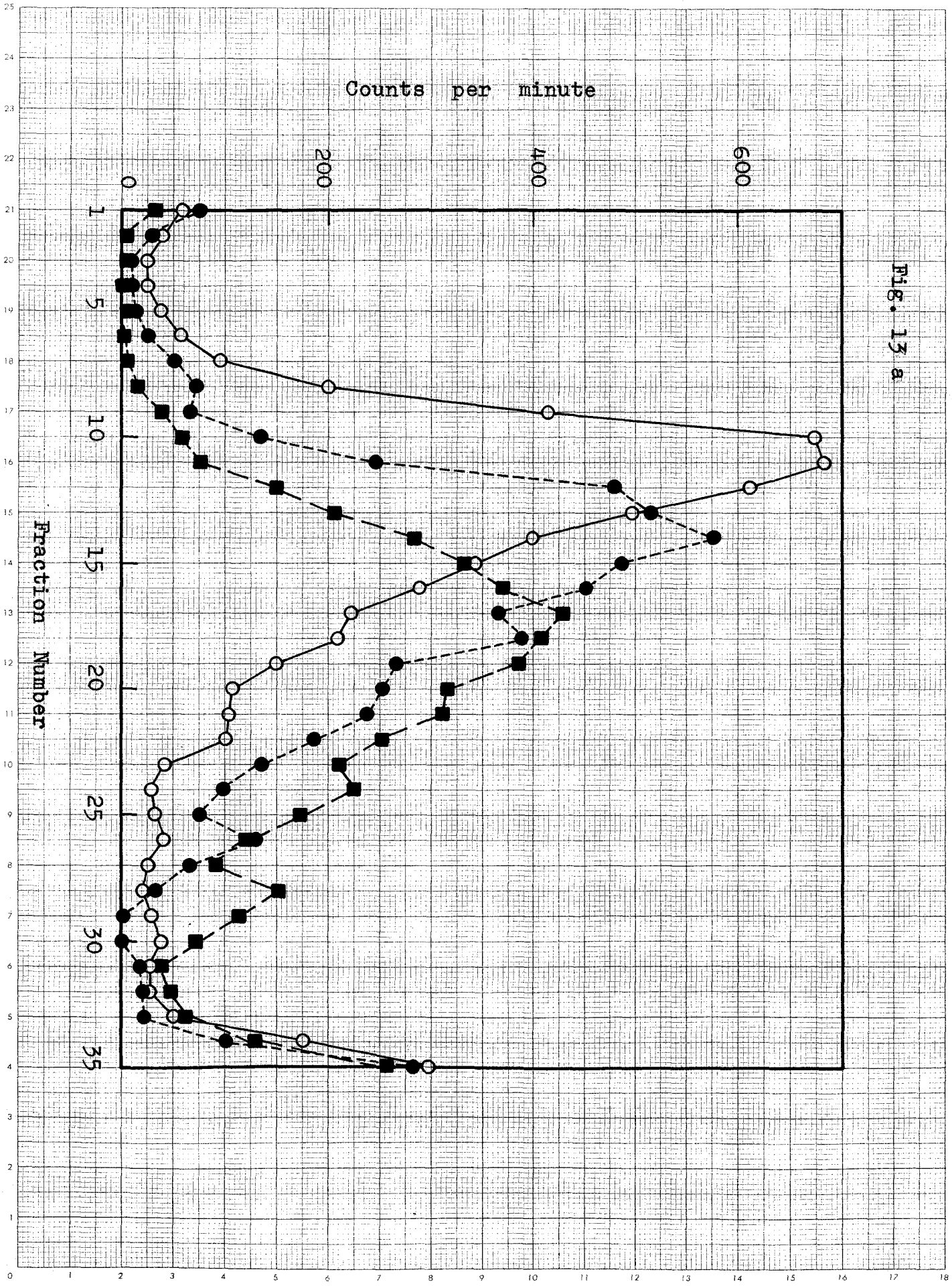

Fig. 13 a

A4 1mm (250×180) コクヨ

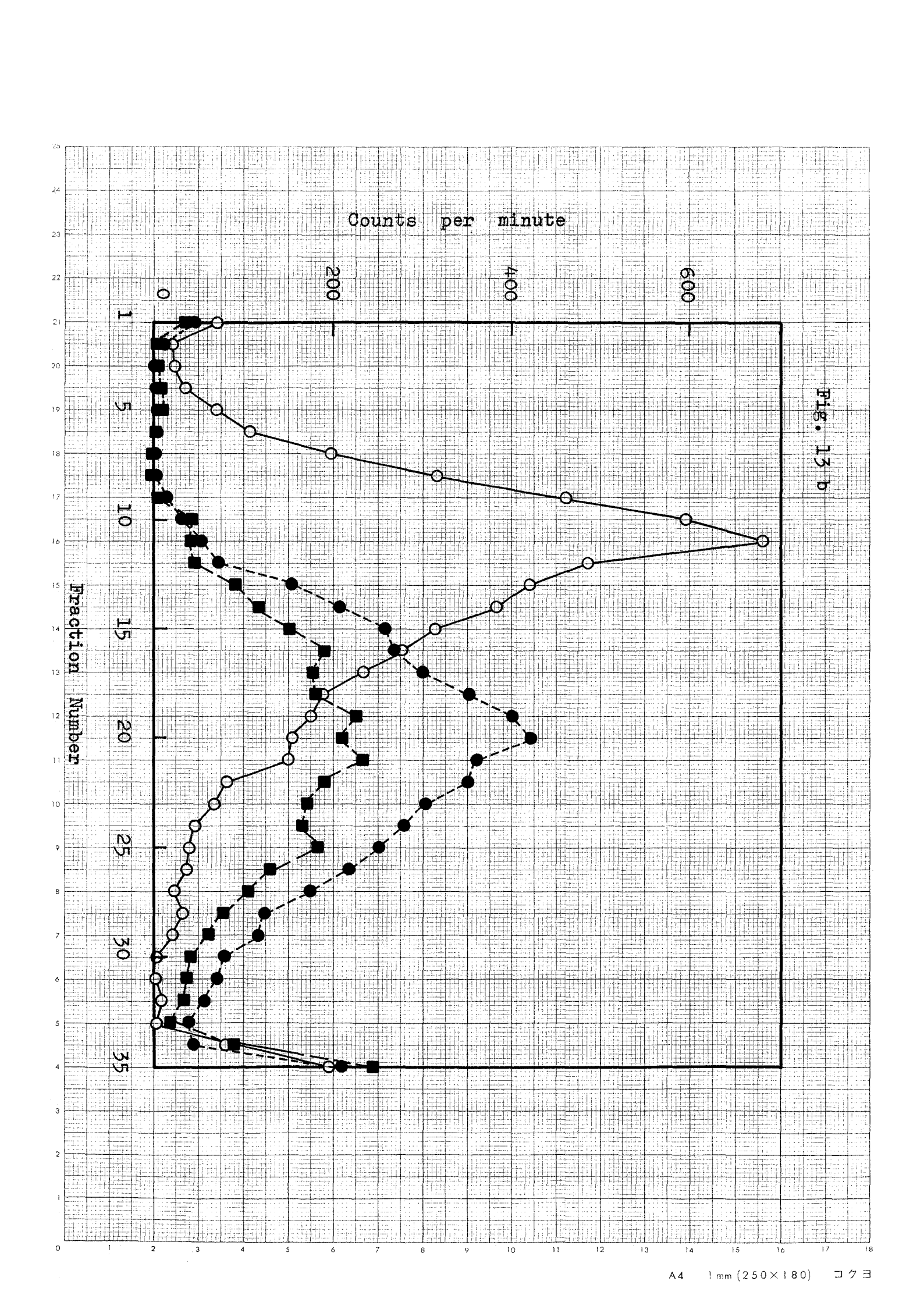

Fig. 13 b

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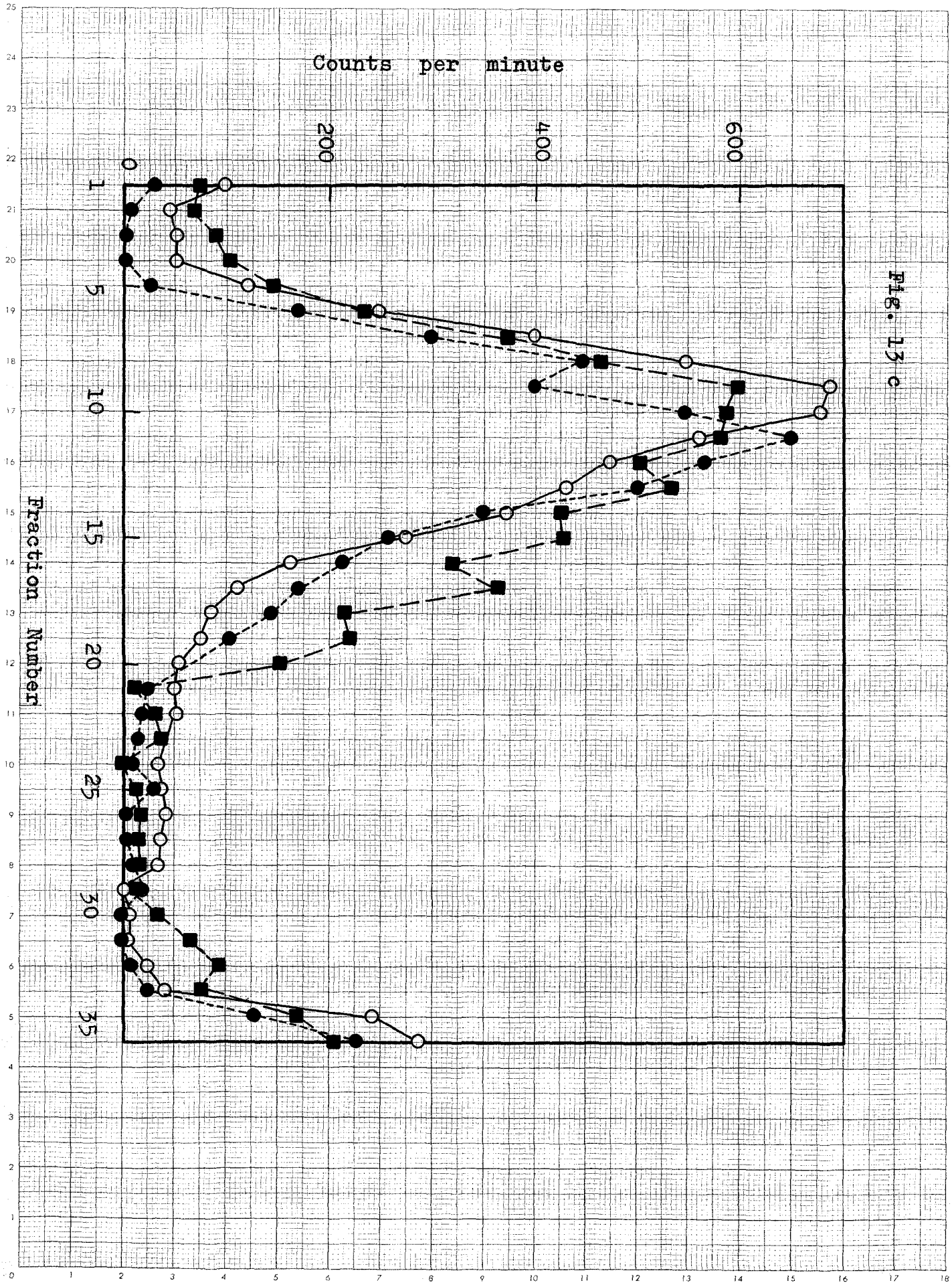

Fig. 13 c

A4 1mm (250×180) コクヨ

Fig. 14

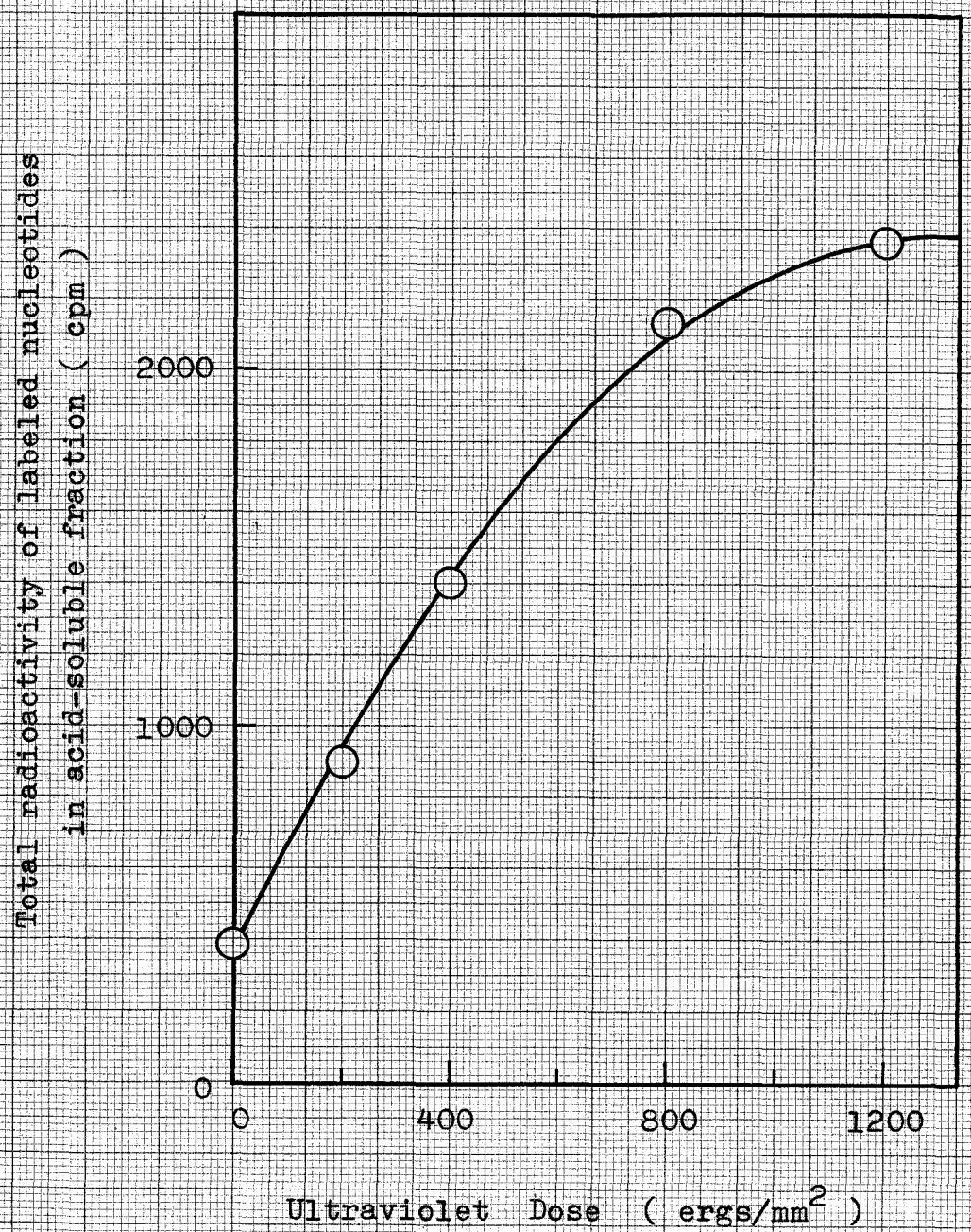

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Fig. 15

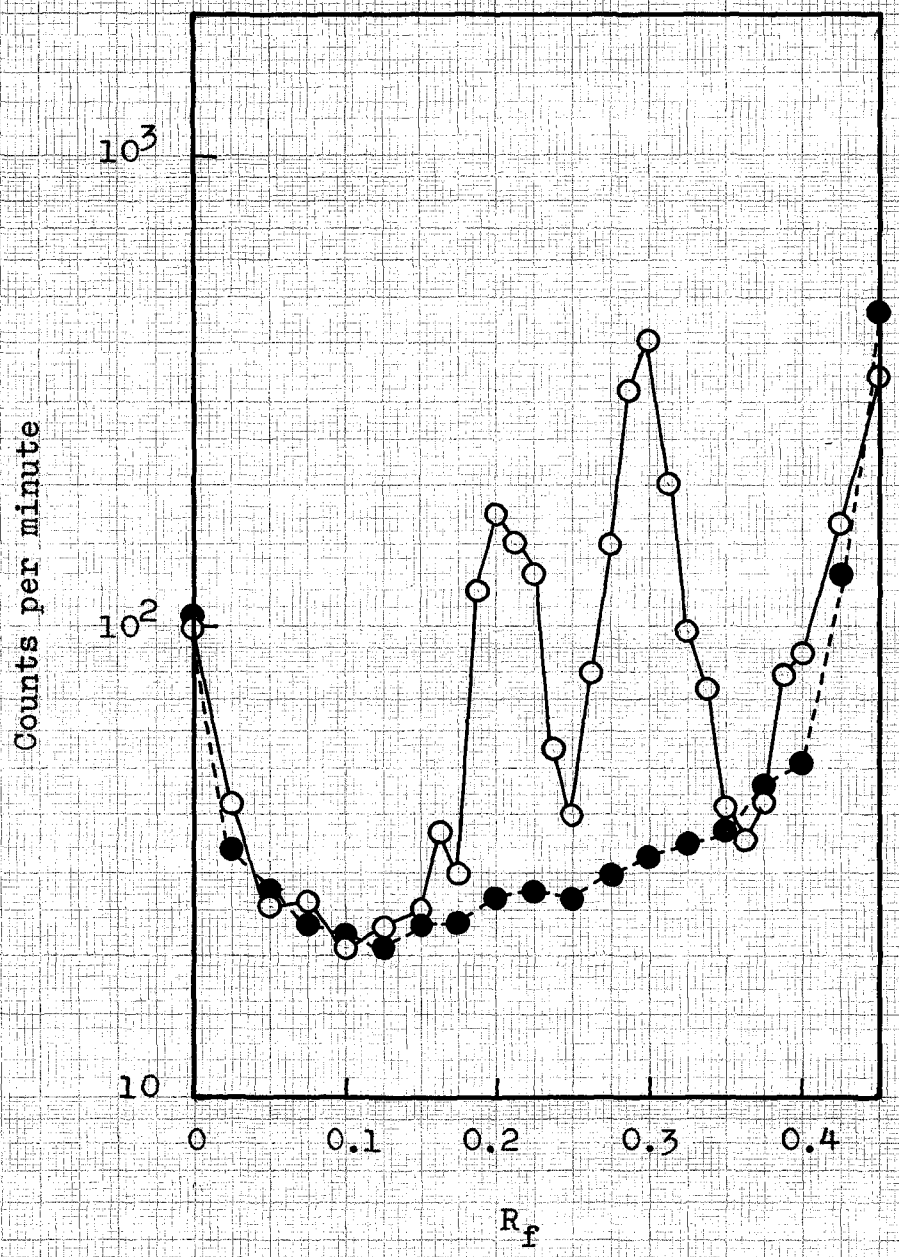
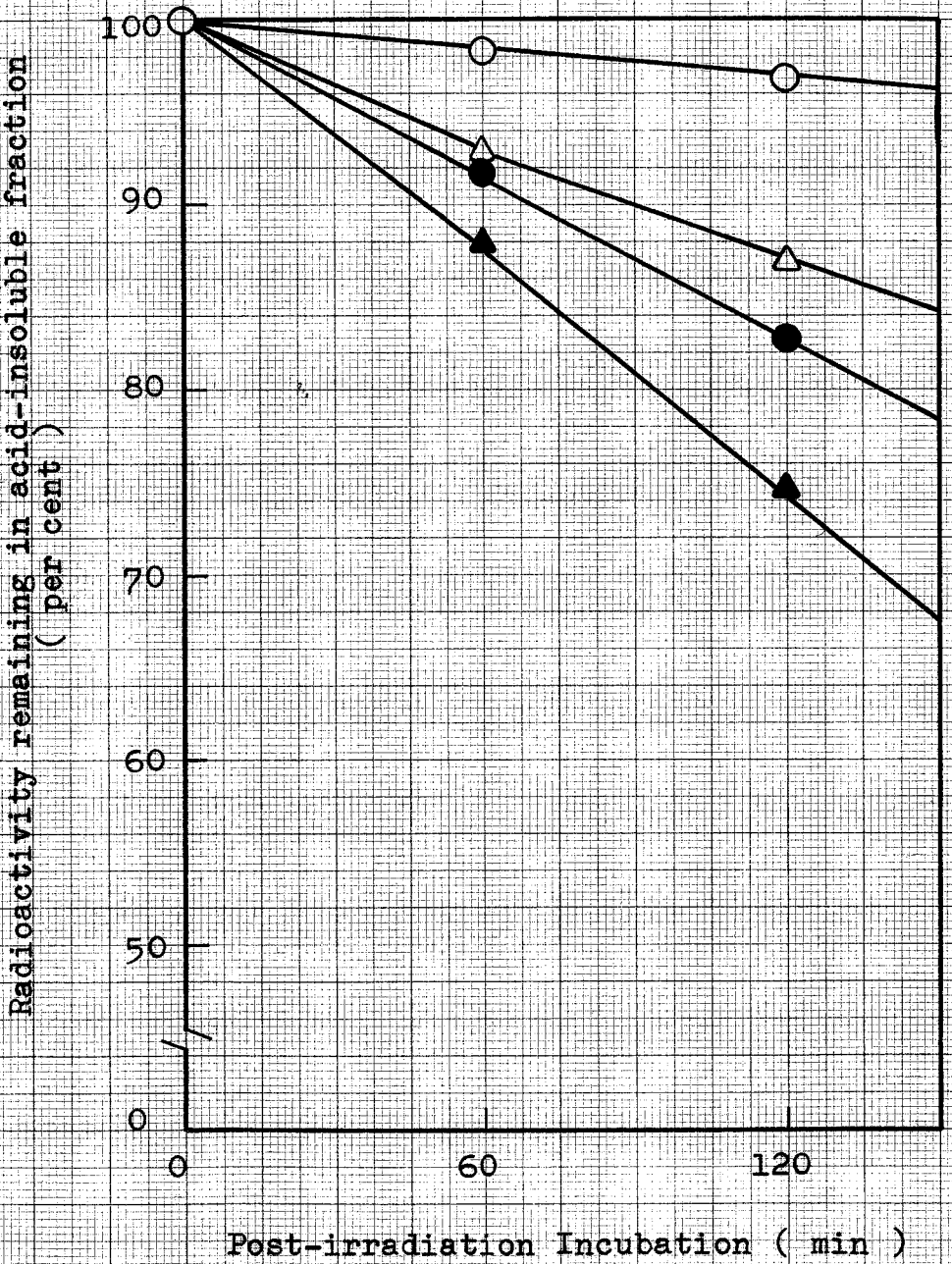


Fig. 16



A4 1mm (250×180) コクヨ

LEGENDS FOR FIGURES

Fig. 1. Ultraviolet survival curves for E. coli H/r30-R and NG30 in different cell conditions.

The ultraviolet-sensitivity of E. coli NG30 in different physiological conditions were examined. The examined cell conditions were as follows ; growth phase, stationary phase, starved condition for amino acids and a condition pre-incubated at 37°C for 90 min with chloramphenical. E. coli H/r30-R was used as control. H/r30-R : stationary, —○— ; growing, —●— . NG30 : growing, —■— ; stationary, —□— ; amino acid-starvation, —◑— ; incubation with chloramphenicol, —◐— .

Fig. 2. Degradation of the DNA in growing cells of E. coli NG30 after ultraviolet irradiation.

DNA was labeled with [H^3]-thymidine by incubating the cells in EM9 medium supplemented with 3.3 μC/3 μg/ml of [H^3]-thymidine and 200 μg/ml deoxyadenosine. The cells were washed, resuspended in EM9 medium and irradiated with various doses of ultraviolet light. The loss of the radioactivity from acid-insoluble fractions of the

cultures during post-irradiation incubation was detected. The 100 per cent values at the zero incubation time were about 2,000 cpm/0.2 ml culture. Ultraviolet dose : 0 erg/mm^2, —○— ; 20 ergs/mm^2, —◑— ; 100 ergs/mm^2, —●— ; 400 ergs/mm^2, —◐— .

Fig. 3. [H^3]-thymidine incorporation into acid-insoluble fractions during incubation in EM9 medium and M9 medium.

The cells grown in EM9 medium were washed, and resuspended in five fold volumn of M9 medium. The cell suspension was divided into two portions and 2.5 mg/ml casamino acids were added to the one portion of the two. [H^3]-thymidine and deoxyadenosine were added to all the cultures at zero incubation time and the cultures were incubated at 37°C. The experimental details for the measurement of radioactivity incorporated into acid-insoluble fractions were described under Materials and Methods.

—○— , in EM9 meium ; —●— , in M9 medium ; —◑— , cells were transfered to EM9 medium after 70 min starvation-incubation in M9 medium.

(a) E. coli H/r30-R, (b) E. coli NG30.

Fig. 4. [H^3]-thymidine incorporation into acid-insoluble fractions during incubation in EM9 medium with or without chloramphenicol.

E. coli NG30 was used. The growing cultures in EM9 medium were supplemented with [H^3]-thymidine and deoxyadenosine, and incubated at 37°C with or without chloramphenicol. The details for measurement of [H^3]-thymidine incorporation were described under Materials and Methods. —●— , with chloramphenicol ; —◐— , without chloramphenicol.

Fig. 5. Post-irradiation degradation of DNA in the cells without DNA replication.

(a) The DNA replication of E. coli NG30 was stopped by amino acid-starvation. The cells which have no DNA replication were irradiated with various doses of ultraviolet light. The other experimental details were described under Materials and Methods. 100 per cent values at zero incubation time were about 1,500 cpm.

—○— , non-irradiated ; —●— , irradiated with 50 ergs/mm^2 ; —□— , irradiated with 100 ergs/mm^2 ; —■— , irradiated with 200 ergs/mm^2 ; —△— , irradiated with 400 ergs/mm^2 ; —▲— , irradiated with 1,000 ergs/mm^2.

(b) The DNA replication of E. coli H/r30-R was stopped by amino acid-starvation and of E. coli B_{s-1} by chloramphenicol. The cells were irradiated with 1,000 ergs/mm^2. The 100 per cent values at zero incubation time were about 2000 and 1200 cpm in H/r30-R and B_{s-1}, respectively. —●— , H/r30-R non-irradiated ; —◐— , H/r30-R irradiated with 1,000 ergs/mm^2 ; —■— , B_{s-1} non-irradiated ; —◧— , B_{s-1} irradiated with 1,000 ergs/mm^2.

Fig. 6. Post-irradiation degradation of DNA in E. coli NG30 ceased the DNA replication by chloramphenicol.

DNA replication had been stopped through incubating the cells with 50 μg/ml of chloramphenicol for 90 min. The cultures were then irradiated with various doses of ultraviolet light and continued to incubate at 37°C. The methods for the measurement of radioactivity in acid-insoluble fraction were described under Methods. The 100 per cent values at zero incubation time were about 1,800 cpm. —○— , 0 erg/mm^2 ; —◐— , 50 ergs/mm^2 ; —◐— , 100 ergs/mm^2 ; —△— , 200 ergs/mm^2 ; —▽— , 400 ergs/mm^2.

Fig. 7. Comparison of the rate of post-irradiation DNA degradation in E. coli NG30 labeled in different DNA regions.

Two kinds of cells labeled with [H^3]-thymidine in different DNA region within the DNA molecules having no replicating points were prepared. One is labeled in " early region " and the other " late region ". The preparation of the cells were described in details under Materials and Methods. The cultures were irradiated with various doses of ultraviolet light and incubated at 37°C in amino acid-free M9 medium. The 100 per cent values at zero incubation time were about 900 cpm in the experiments of " early region " and 1,100 cpm in that of " late region ", respectively. (a) experiment of " early region ", (b) experiment of " late region ".

—○— , non-irradiated ; —◑— , irradiated with 50 ergs/mm^2 ; —●— , irradiated with 100 ergs/mm^2 ; —▲— , irradiated with 200 ergs/mm^2 ; —△— , irradiated with 400 ergs/mm^2.

Fig. 8. Degradation of DNA in amino acid-starved cells of E. coli NG30uvrC after ultraviolet irradiation.

E. coli NG30uvrC was used. The DNA replication was stopped through amino acid-starvation. The cells were irradiated with

various doses of ultraviolet light and incubated at 37°C in M9 medium. The experimental procedures were the same as in Fig. 5a. The 100 per cent values at zero incubation time were about 2,200 cpm. —○— , non-irradiated ; —◐— , irradiated with 50 ergs/mm^2 ; —●— , irradiated with 100 ergs/mm^2 ; —△— , irradiated with 200 ergs/mm^2 ; —▽— , irradiated with 400 ergs/mm^2.

Fig. 9. Change in the amount of pyrimidine dimers in the acid-insoluble fractions of the cells of E. coli NG30 and NG30uvrC during post-irradiation incubation.

E. coli NG30 and NG30uvrC were used. [H^3]-thymidine-labeled cells were starved amino acids, irradiated with 400 ergs/mm^2 and then incubated for 60 and 120 min in M9 medium. The acid-insoluble fractions of the cells were hydrolyzed and pyrimidine dimers were detected by paperchromatography. The experimental details were described under Methods. (a) E. coli NG30, (b) E. coli NG30uvrC. —○— , no incubation ; —●— , incubated for 60 min ; - - —△— - - , incubated for 120 min.

Fig. 10. Effect of caffeine on DNA degradation in E. coli NG30 irradiated with ultraviolet light.

DNA replication of the cells was stopped through amino acid-starvation. Caffeine was added to the cultures and the cells were irradiated with 400 ergs/mm^2 of ultraviolet light. The degradation of DNA during the post-irradiation incubation was measured. The details were the same as in Fig. 5a except for the addition of caffeine. 100 per cent values at zero incubation time were about 1,300 cpm. Caffeine concentration : —○— , 0 per cent ; —◐— , 0.1 per cent ; —◐— , 0.2 per cent ; —●— , 0.5 per cent.

Fig. 11. Initial fragmentation of DNA in the cells soon after the ultraviolet irradiation.

[H^3]-thymidine -labeled E. coli NG30 and H/r30-R cells were incubated at 37°C for 90 min in amino acid-free medium (M9 medium). The cells were irradiated with 200 and 1,000 ergs/mm^2 and incubated for 5 min in M9 medium at 37°C. Immediately after the incubation, the cells were cooled, converted into spheroplasts and centrifuged in alkaline sucrose gradient. The other details were described

in Materials and Methods. (a) E. coli NG30, (b) E. coli H/r30-R. —○— , non-irradiated ; --●-- , irradiated with 200 ergs/mm^2 ; —■— , irradiated with 1,000 ergs/mm^2.

Fig. 12. The sedimentation profiles of DNA of E. coli NG30 incubated for 20 min after ultraviolet irradiation.

[H^3]-thymidine-labeled cells were starved amino acids for 90 min, and then irradiated with various doses of ultraviolet light. The irradiated cultures were incubated at 37°C for 20 min in the amino acid-free medium. The cells were lysed by lysozyme, layered on a alkaline sucrose gradient (5 - 20 %), and then centrifuged for 90 min at 30,000 rev./min. Measurement of radioactivity and the other details were described under Materials and Methods. Two sets of experimental results were piled up in the same figure.

——— and ------ , non-irradiated ; —●— , irradiated with 100 ergs/mm^2 ;— —□— — , irradiated with 200 ergs/mm^2 ; —■— , irradiated with 400 ergs/mm^2 ; ---△--- , irradiated with 1,000 ergs/mm^2.

Fig. 13. Degradation of DNA of E. coli NG30 in the course of time of post-irradiation incubation.

Both strains, E. coli NG30 and H/r30-R were used. The cells labeled with [H^3]-thymidine were starved amino acids for 90 min and then irradiated with 200 or 1,000 ergs/mm^2. The irradiated cells were incubated at 37°C in M9 medium for 20 and 120 min. The cells converted into spheroplasts were lysed and layered on the top of alkaline sucrose gradient. The other details for experiments were the same as before experiments.

(a) E. coli NG30, —○— , non-irradiated ; ---●--- , irradiated with 200 ergs/mm^2 and incubated for 20 min ; —■— , irradiated with 200 ergs/mm^2 and incubated for 120 min.

(b) E. coli NG30, —○— , non-irradiated ; ---●--- , irradiated with 1,000 ergs/mm^2 and incubated for 20 min ; —■— , irradiated with 1,000 ergs/mm^2 and incubated for 120 min.

(c) E. coli H/r30-R, —○— , non-irradiated ; ---●--- , irradiated with 1,000 ergs/mm^2 and incubated for 20 min ; —■— , irradiated with 1,000 ergs/mm^2 and incubated for 120 min.

Fig. 14. Dose-dependent increase in the amount of radioactivity of acid-soluble fractions containing pyrimidine dimers of E. coli NG30.

[H^3]-thymidine-labeled cells were starved for amino acids and then irradiated with various doses. The cultures were incubated for 5 min at 37°C. The acid-soluble fractions were neutralized and charged to the column of Dowex-1 (200 - 400 mesh, X8, Cl^- type). After washing the resin with excess volume of water to release free [H^3]-thymidine, 20 ml of 0.5 N HCl was passed through the column. The radioactivity of the eluates were counted.

Fig. 15. Released pyrimidine dimers into acid-soluble fractions.

E. coli NG30 was used. The cells starved amino acids were irradiated with 2,220 ergs/mm^2 (dose rate : 74 ergs/mm^2/sec), and then incubated at 37°C for 5 min. The acid-soluble fractions were condensed and hydrolyzed with 99 per cent formic acid at 175°C for 15 min. The hydrolysates were then analyzed by paperchromatography. Experimental details are described under Materials and Methods. —○— , irradiated cells ; --●-- , non-irradiated cells.

Fig. 16. Post-irradiation degradation of the DNA in E. coli NG30 in the conditions with or without glucose.

The cells were starved amino acids by incubating them for 90 min in M9 medium. The irradiated cells were incubated in the M9 medium with or without glucose. The ultraviolet doses used were 50 and 100 ergs/mm^2.

The other experimental procedures were the same as in Fig. 5a.

—●— , 50 ergs/mm^2, with glucose ;

—○— , 50 ergs/mm^2, without glucose ;

—▲— , 100 ergs/mm^2, with glucose ;

—△— , 100 ergs/mm^2, without glucose.

Table I. Change in the sedimentation velocity of DNA molecules in course of time of post-irradiation incubation

Strains	Dose (ergs/mm^2)	Post-irradiation Incubation (min) 5	20	120
HG30	0	12.0	12.0	—
	200	11.5	10.5	9.0
	1000	8.5	7.5	7.0
H/r30-R	0	13.0	12.5	—
	200	12.0	—	—
	1000	8.5	12.0	13.0

The sedimentation velocity of DNA molecules in main peaks of DNA distribution was represented as a distance from the meniscus of alkaline sucrose gradient in the figures (in cm)