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CHEMICAL REACTIVATION OF *ESCHERICHIA COLI* B FROM RADIATION DAMAGE

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CHEMICAL REACTIVATION OF ESCHERICHIA COLI B FROM RADIATION DAMAGE

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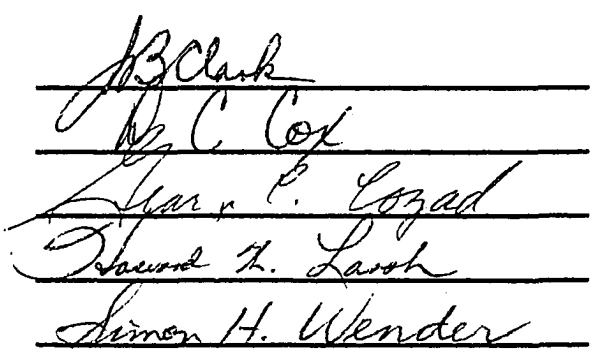

DISSERTATION COMMITTEE

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CHEMICAL REACTIVATION OF ESCHERICHIA COLI B FROM RADIATION DAMAGE

CHAPTER I

INTRODUCTION

Indisputable evidence is now available which supports the view that deoxyribonucleic acid (DNA) is the principal target for inactivation of micro-organisms by ultraviolet (UV), ionizing radiations and radiomimetic agents such as nitrogen mustard (HN2) (19, 20). It is also established that DNA is the essential genetic material of the cell. Radiation and HN2 treatments produce fast physico-chemical reactions which result in structural alterations in the DNA molecule. This is followed by slow biochemical reactions of the cell which can be influenced by the physiological state of the cell or the conditions of growth after treatment.

Structural Damage Produced by Radiation and HN2:

Nucleic acids strongly absorb electro-magnetic radiation in the region 240-280 nm, which is more commonly referred to as far UV. It has been shown that far UV radiation effectively produces several photoproducts in

small organisms like bacteria and viruses (28). Although pyrimidine dimers are the only photoproducts which have been directly implicated in microbial inactivation (4,44, 46,57), pyrimidine hydrates and cross linking of DNA to proteins may also play an important role (50).

Ionizing radiation, such as X-rays, has been shown to produce single strand breaks in the DNA molecule (13,49). Other important effects of X-ray irradiation include chemical changes in sugar residues, cross linking of DNA to proteins, and elimination or alteration of bases (13).

Difunctional alkylating agents such as HN2 have been shown to produce guanyl or diguanyl products (6,31). These are generally produced by reaction with the N-7 atom of guanine, which is sometimes followed by a slower reaction resulting in the cross linking to a second guanine.

Mechanisms of inactivation by radiation and radiomimetic agents have been reviewed by Haynes (19,20). Haynes has suggested that the first few hours after irradiation or chemical treatment are very crucial for survival. During this period normal DNA replication might encounter a block (due to structural defect in DNA molecule) and consequently cell multiplication would be impossible. In addition there might be blocks in transcription of messenger RNA (m-RNA) or an erroneous m-RNA might be transcribed which could also lead to lethality. However, if the repair of the damage following radiation or chemical treatment

was completed before the onset of normal replication or transcription, the cell would survive.

Recovery of Damaged DNA:

The first clear demonstration of the specific role of DNA structural defects in lethality and recovery came from the studies of far UV inactivation and photoreactivation (PR) in Escherichia coli. It was demonstrated that far UV induced pyrimidine dimers blocked DNA synthesis. Photoreactivating enzyme in the presence of visible light cleaved these dimers (4,44,46,57).

The existence of dark recovery systems in modifying the radiation damage has also been known for some time. The role of dark recovery in UV reactivation was first demonstrated by Hollaender and Claus (23). It was shown by these workers that, if far UV irradiated cells were held in liquid medium before plating, the survival of these cells was much higher than those that were plated immediately after far UV irradiation. Roberts and Aldous (43) made more careful studies on this phenomenon. Elaborate studies of this effect have also been made by Castellani et al. (8), Harm (16), Harm and Haefner (17), and Jagger et al. (26). This phenomenon has been termed Liquid Holding Recovery (LHR).

Heat reactivation was demonstrated by Anderson (2), and Stein and Meutzner (51). These workers found that incubation of far UV irradiated E. coli B at a temperature

higher than 37 C resulted in an increased survival. Monod et al. (38) had shown that treatment with catalase also increased the UV survival of E. coli K-12; later this was studied in greater detail by Latarjet and Caldas (34). Chemical reactivation with phenol, glycine and hydrogen sulfide was reported by Lembke et al. (35). Post-irradiation treatment with cysteine, cystine and ascorbic acid showed some reactivation of UV irradiated E. coli K-12 (33).

Many workers have studied the effect of metabolic inhibitors on UV survival after irradiation. Wainwright and Nevill (53) have indicated that post-irradiation treatment with iodoacetate in distilled water for three hours caused an increase in UV survival of E. coli and spores of Streptomyces sp. These workers further investigated the effect of the metabolic inhibitors arsenate, azide, and 2,4-dinitrophenol (DNP) on the survival of irradiated Streptomyces T 12 (54). Presence of azide or arsenate in the holding medium caused a slight increase in survival while DNP was ineffective in causing any change. These authors also mention a private communication with a L. Jacobs, who found that if DNP was added to the plating medium, the UV survival was increased.

Alper and Gillies (1) postulated that sub-optimum growth conditions provide a better chance for survival of irradiated cells by preventing death due to "unbalanced

growth." They discovered that the surviving fractions were smaller if irradiated cells were plated in a medium optimum for growth. Anaerobiosis during post-irradiation growth or post-irradiation contact with iodoacetate for one to three hours resulted in a small increase in survival. However, post-irradiation contact with an inhibitor of protein synthesis, chloramphenicol, was highly effective in causing an increase in UV survival of E. coli B.

Clark et al. (9) found that azide at subinhibitory concentrations increased recombination frequency of E. coli K-12 mutants. Later Berger et al. (3) reported that pretreatment with azide increased the UV survival of E. coli. These workers were unable to show any effect of azide on survival if irradiated cells were held in the presence of this chemical. Pretreatment with cyanide or carbon monoxide protected E. coli from subsequent far UV radiation (36).

Weatherwax (55) and Miki (37) independently discovered that exposure to near UV (310-380) decreased the sensitivity of E. coli to subsequent far UV irradiation. This phenomenon has been termed as photoprotection (PP). PP is greater in magnitude than any other modifying treatment except PR. PP against X-ray inactivation was discovered by Clark and Frady (10). Recently Lakchaura and Clark (32) have reported PP against HN2.

Jagger et al. (26,27) have shown that near UV

radiation induced a growth and division delay. Action spectra for this growth and division delay and PP are the same. Kashket and Brodie (29) have shown that near UV destroys cellular quinone compounds, which are the essential components of oxidative metabolism. Destruction of these compounds leads to a temporary inhibition of respiration which in turn may lead to a growth delay. Jagger and co-workers (26) therefore, postulate that this growth delay may provide more time for the operation of dark repair enzymes.

Dark repair is an enzymatic process. Hill (22) isolated a mutant strain of E. coli which was extremely UV sensitive. It was later established by Ellison, Feins and Hill (12) that this strain lacked the normal ability of the parental strain to reactivate the far UV inactivation. Setlow and Carrier (48) established that this strain was unable to excise the thymine dimers formed by far UV radiation while the parental strain was able to remove these dimers and replace them with normal bases. The importance of excision enzymes which are involved in the removal of the lesion from DNA molecule came from the studies of Boyce and Howard-Flanders (5), Setlow and Carrier (48), and Pettijohn and Hanawalt (41).

The UV resistance of E. coli strain B/r has been attributed to the presence of a reactivation mechanism capable of very efficiently repairing the UV induced

pyrimidine dimers. Overwhelming evidence is now available which shows that dark repair is accomplished by a multi-step "cut and patch" process which involves nuclease excision of single strand oligonucleotide fragments containing pyrimidine dimers, DNA degradation, and nonconservative repolymerization (repair replication) which fills in the resulting gaps.

Excision repair can restore the damage in E. coli B/r by HN2, X-ray, and mitomycin C, as well as far UV (18, 20). Similar results have been obtained by Haynes (20) with Bacillus subtilis and Micrococcus radiodurans.

It has been suggested that repair and normal DNA replication are competitive processes after DNA structural damage (20). If this hypothesis is true, then the conditions (at least for a brief period of time after irradiation or HN2 treatment) which allow repair but inhibit normal DNA replication should increase survival after irradiation or HN2 treatment.

In general terms Jagger's hypothesis seems to be parallel to that of Haynes. Extension of Jagger's hypothesis would suggest that any physical or chemical agent which could induce a growth delay should also have a recovery effect.

This work was initiated to investigate the effect of metabolic inhibitors of oxidative phosphorylation and respiration on the survival of irradiated cells. In

addition, HN2, which is a radiomimetic agent, was used as an inactivating agent. This was used to strengthen evidence that enzymatic repair systems, which are involved in the restoration of radiation damage, can also repair damage caused by HN2. The mode of action of the chemical compounds selected for this investigation, cyanide, and 2,4-dinitrophenol, resembles that of near UV, a physical agent. All interfere with the oxidative metabolism of the cell. Finally, it was necessary to show whether a common mode of action existed among these agents with respect to repair of radiation or HN2 damage.

CHAPTER II

MATERIALS AND METHODS

Escherichia coli strain B, which was obtained from Dr. John Jagger, was used for this study. Cells were grown overnight with continuous shaking at 37 C in M-9 medium containing 0.5% casamino acids. A 1:100 dilution of these cells was made in fresh medium and was incubated for two hours to produce exponential cultures. These cells were then centrifuged, washed twice with, and resuspended in, M/15 sodium potassium phosphate buffer, pH 6.8. This buffer was also used for all dilutions. Cells were starved for at least one hour before any treatment was given. Difco nutrient agar was used for plating. Plates were incubated at 37 C for approximately 20 hours and visible colonies were counted. For anaerobic incubation, the plates were placed in an environment where air was replaced with methane.

Appropriate amounts of DNP or KCN were added to melted nutrient agar after autoclaving to give the desired concentrations. KCN was filter sterilized prior to adding to nutrient agar. Since DNP is relatively water insoluble, this chemical was added in the solid form to boiling

medium. These media have been referred to as DNP agar or cyanide agar.

UV-inactivation: UV inactivation was carried out with one General Electric 15-watt (G15T8) germicidal lamp. The chief emission of this lamp is at 254 mm (28). Only the central 20 cm portion of the lamp was used for radiation. During irradiation one ml of cell suspension containing not more than 10^7 cells was held in a depression spot plate. UV dose rate was measured with a General Electric germicidal ultraviolet intensity meter.

Photoprotection: The photoprotecting technique was the same as described by Lakchaura and Clark (32). PP treatment was applied by two G. E. 15-watt (T8BLB) black light lamps. A 1/4 inch thick sheet of window glass was mounted in front of the lamps to filter out any radiation of wavelength less than 310 nm. The sample was placed in a glass cuvette at approximately 5 cm from the lamps. The cells were continuously stirred at room temperature while being illuminated.

Far UV inactivation and PP treatment were carried out in a room illuminated with Ken-Rad gold fluorescent lights (F40GO/3). Cells, once exposed to far UV, were never again exposed to visible light until visible colonies could be observed.

X-ray inactivation: The source of X-ray was a Machlett OEG-60 X-ray tube with beryllium window operating

at 70 Kv and a ma setting to give the desired dose rate. Dose rate was measured with a Victoreen model R rate meter. The cell suspensions were in phosphate buffer and contained in a 5 ml glass dish. Cells were continuously stirred while being irradiated.

HN2 inactivation: HN2 [methyl bis(beta-chloroethyl) amine hydrochloride] was obtained from Merck and Co. Inc., Rahway, N. J. Cells were treated with HN2 (0.3 mg/ml in phosphate buffer) at 37 C with shaking. Aliquots were removed at different time intervals and suitable dilutions were plated. Comparisons of survival were made between DNP agar and plain nutrient agar. Residual HN2 was not chemically inactivated but was effectively reduced by the dilution procedure.

DNP treatment: 2,4-dinitrophenol (DNP) was obtained from Fisher Scientific Co. In preliminary experiments, cells were exposed at room temperature to different concentrations of DNP for several hours. Then these cells were plated on nutrient agar after making appropriate dilutions. During this dilution procedure DNP concentration was considerably reduced and practically no DNP was transferred to the plating medium. It was found that concentrations 5×10^{-4} M and lower did not cause any reduction in the number of viable cells.

Log phase washed and starved cells were incubated with 4×10^{-4} M DNP and without DNP in phosphate buffer for

45 minutes at 37 C. These cells were then centrifuged and washed with phosphate buffer. After resuspending in buffer, both samples were irradiated at the same time in two depressions of the same spot plate. This will be referred to as pretreatment in later discussion.

Post-treatment consisted of incubating the irradiated cells in the presence of DNP in phosphate buffer before plating. This will be referred to as Liquid Holding Recovery.

Cyanide treatment: Reagent grade potassium cyanide was used essentially in the identical way as DNP. In the plating medium a 6×10^{-4} M concentration was used, while for pretreatment a 1×10^{-3} M concentration was used.

Growth delay and DNA synthesis: Growth delays caused by DNP, cyanide, and PP treatment were compared. The control tube and PP tube contained 9 ml of nutrient broth. DNP and cyanide tubes were supplemented with 2×10^{-4} M DNP and 6×10^{-4} M KCN respectively in 9 ml of nutrient broth. Control, DNP and cyanide tubes were inoculated with one ml (10^7) log phase washed and starved cells, while PP tube received the same number of cells previously exposed to PP treatment for 25 min. Each tube with the exception of the cyanide tube also contained radioactive thymidine (1 ug/ml, 30 mc/Mm). Radioactive thymidine was obtained from New England Nuclear Corp., Boston, Mass. After adjusting the volume of each tube to

a 10 ml mark, all were shaken in a 37 C water bath. At different time intervals optical density of each tube was determined at 660 nm by Bausch and Lomb Spectronic 20.

One ml aliquots were also removed from each tube at different time intervals and filtered through a 25 mm (HA 0.45 u) Millipore filter. The filters were then washed with 5% trichloroacetic acid and 5% acetic acid, and dried overnight in a 50 C oven. These filters were then dropped into a scintillation vial containing 10 ml of 0.5% 2,5-diphenyloxazole in toluene. These vials were counted to 2% efficiency level in a Beckman DPM-100 liquid scintillation counter.

CHAPTER III

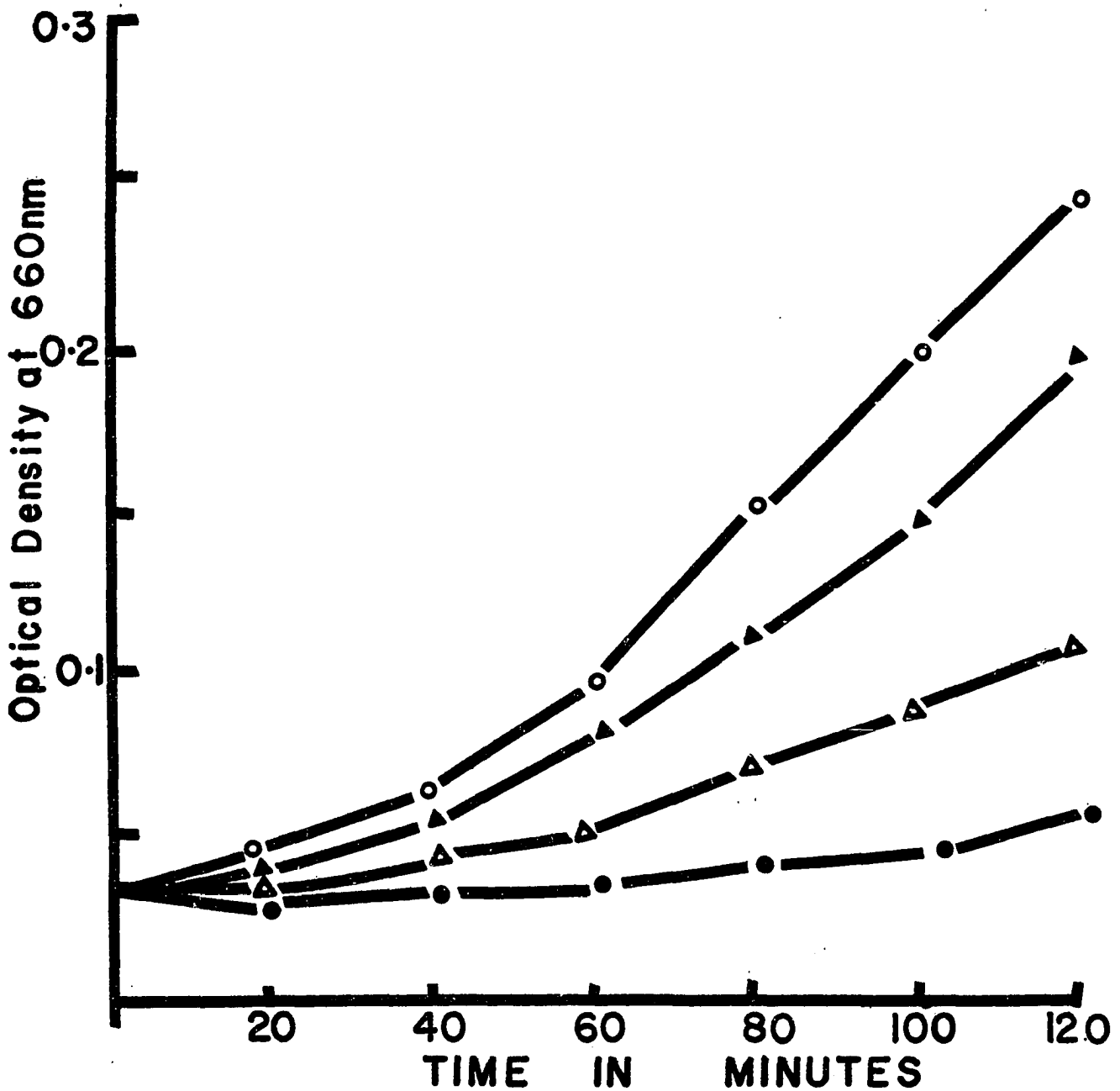
RESULTS

Radiation damage to microorganisms can be modified by treatment both before and after irradiation. Many physical and chemical agents are known to influence the survival of the irradiated cell. One common feature to all these agents appears to be their ability to slow down the rate of growth of the cell by interfering with the metabolism. Several parameters can be employed to detect any change in the rate of growth caused by these agents. The overall rate of growth, which includes both increase in the size of a single cell as well as increase in the population, can be determined by measuring the turbidity of the culture. Determination of the rate of synthesis of such a vital molecule as DNA would also reflect a significant change in the rate of growth.

Figure 1 shows the growth delays caused by DNP (0.0002 M), KCN (0.0006 M) and PP treatment (25 min. near UV exposure). DNP and KCN were present in the growth medium while near UV treatment was applied to the inoculum in phosphate buffer. Neither near UV nor DNP or KCN at the level used was lethal to the cells. Therefore, the

Figure 1. Growth delay in nutrient broth caused by DNP, near UV and KCN. Optical density of the growing culture is plotted against time.

Symbols correspond to: O- control, ▲-presence of 0.0002 M DNP in the medium, ▲-inoculum subjected to 25 min near UV; ● - presence of 0.0006 M KCN.


0·3
0·2
0·1
Optical Density at 660nm
20
40
60
80
100
120
TIME IN MINUTES

growth delay did not result because of a decrease in inoculum due to killing by these agents but was a result of the effect of these agents on cellular metabolism.

Growth delay can be correlated with the decrease in the rate of DNA synthesis as shown in Figure 2. DNP caused a very slight change in the rate of growth and rate of DNA synthesis but KCN on the other hand markedly affected rate of growth. The effect of KCN on DNA synthesis was not measured. However, it has been shown recently by Cairns and Denhardt (7) that 0.01 M KCN inhibited DNA synthesis completely in E. coli in less than one thousandth of a generation time. Therefore it can be expected that KCN, even at the concentration used, would cause a significant decrease in the rate of DNA synthesis. Inhibition of DNA synthesis by near UV and DNP have been mentioned by Setlow (45) and Dalrymple et al. (11), respectively.

It is established that E. coli cells can be photoprotected against far UV damage by pre-exposure to near UV. Pre-exposure to cyanide and DNP remained ineffective in causing any change in the UV survival (Figure 3 and 4). It should be noted that after treatment with these chemicals, the cells were washed and then irradiated. This was necessary in order to eliminate any possibility of interference by these chemicals during irradiation.

Incorporation of DNP and cyanide in the plating medium was effective in increasing the UV survival.

Figure 2. Effect of DNP and near UV on DNA synthesis of E. coli B. Cells grown in the presence of 1 ug/ml of (^{3}H) thymidine (30 mc/Mm).

Symbols correspond to: O- incorporation of (^{3}H) thymidine by control cells; ▲-incorporation of (^{3}H) thymidine by cells growing in the presence of 0.0002 M DNP; ▲- incorporation of (^{3}H) thymidine by cells exposed to near UV for 25 min.

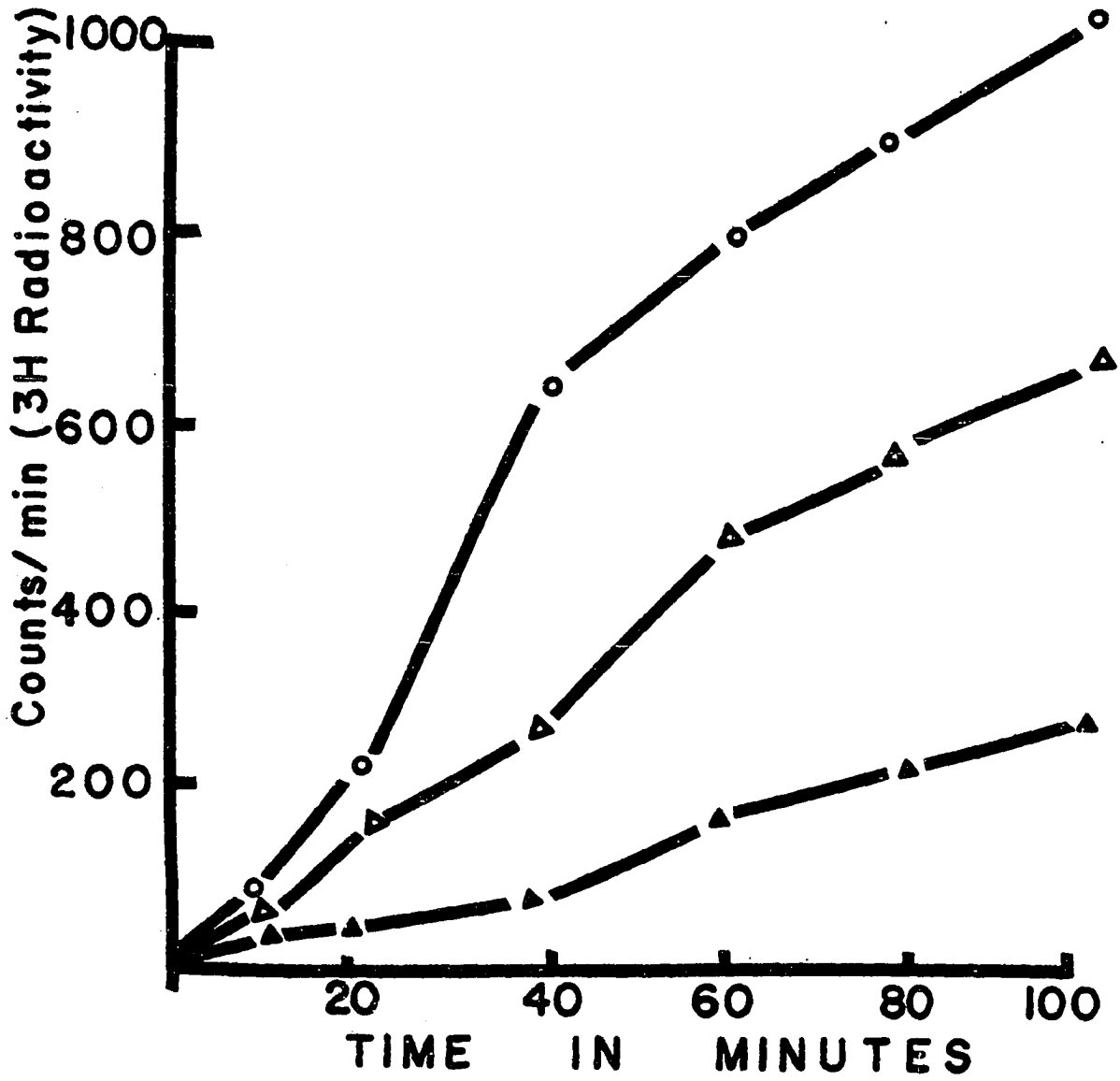

Counts/ min (3H Radioactivity)
1000
800
600
400
200
20
40
60
80
100
TIME IN MINUTES

Figure 3. Effect of pretreatment with DNP on the UV survival of E. coli B.

Symbols correspond to: O-, control; ▲-, cells exposed to 0.0004 M DNP for 45 min prior to far UV irradiation.

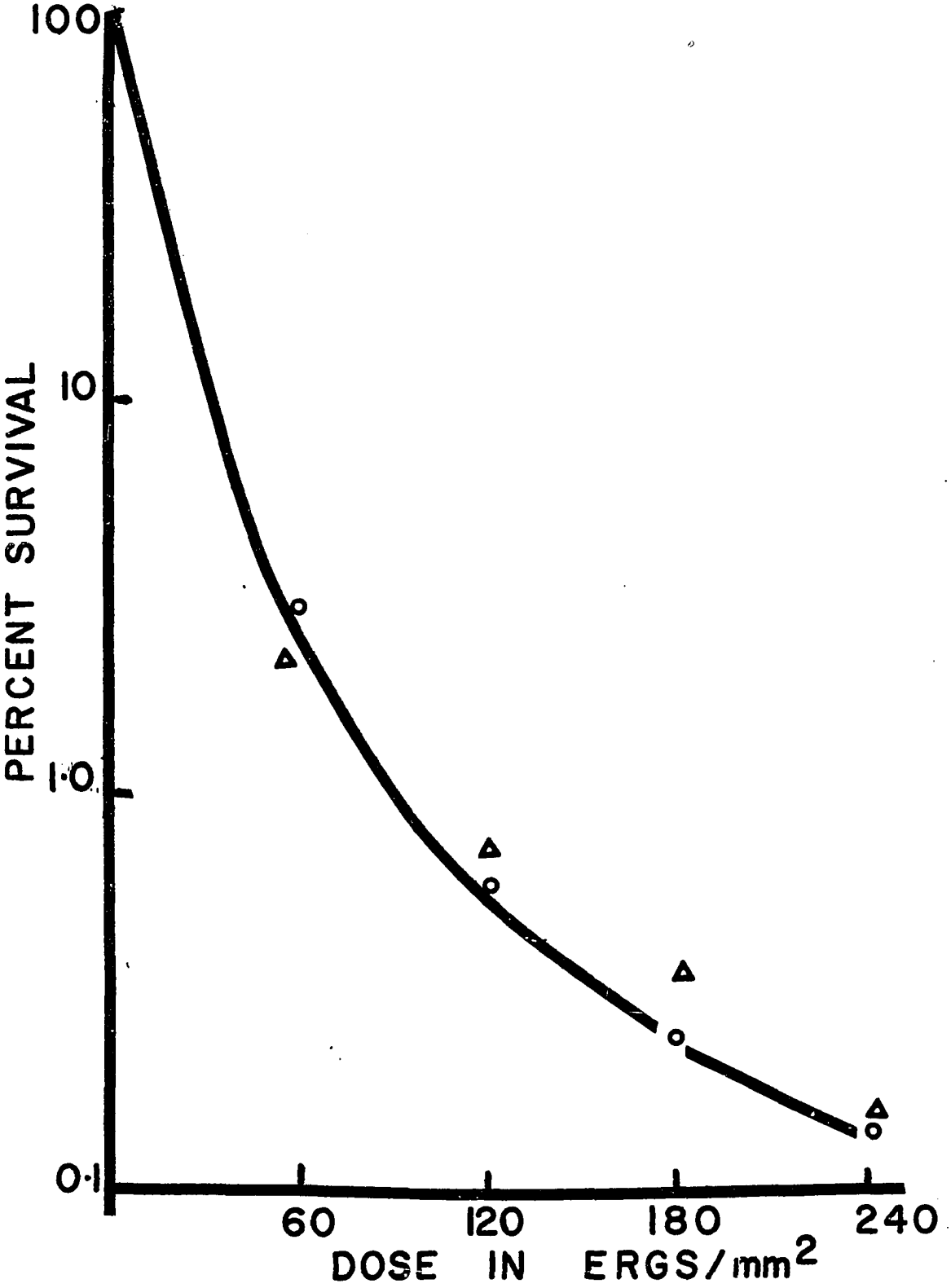

100
10
1·0
0·1
PERCENT SURVIVAL
60
120
180
240
DOSE IN ERGS/mm2

Figure 4. Effect of pretreatment with KCN on UV survival of E. coli B. Percent survival is plotted on a log scale against dose.

Symbols correspond to: O-, control; ▲-, cells exposed to 0.001 M KCN for one hour.

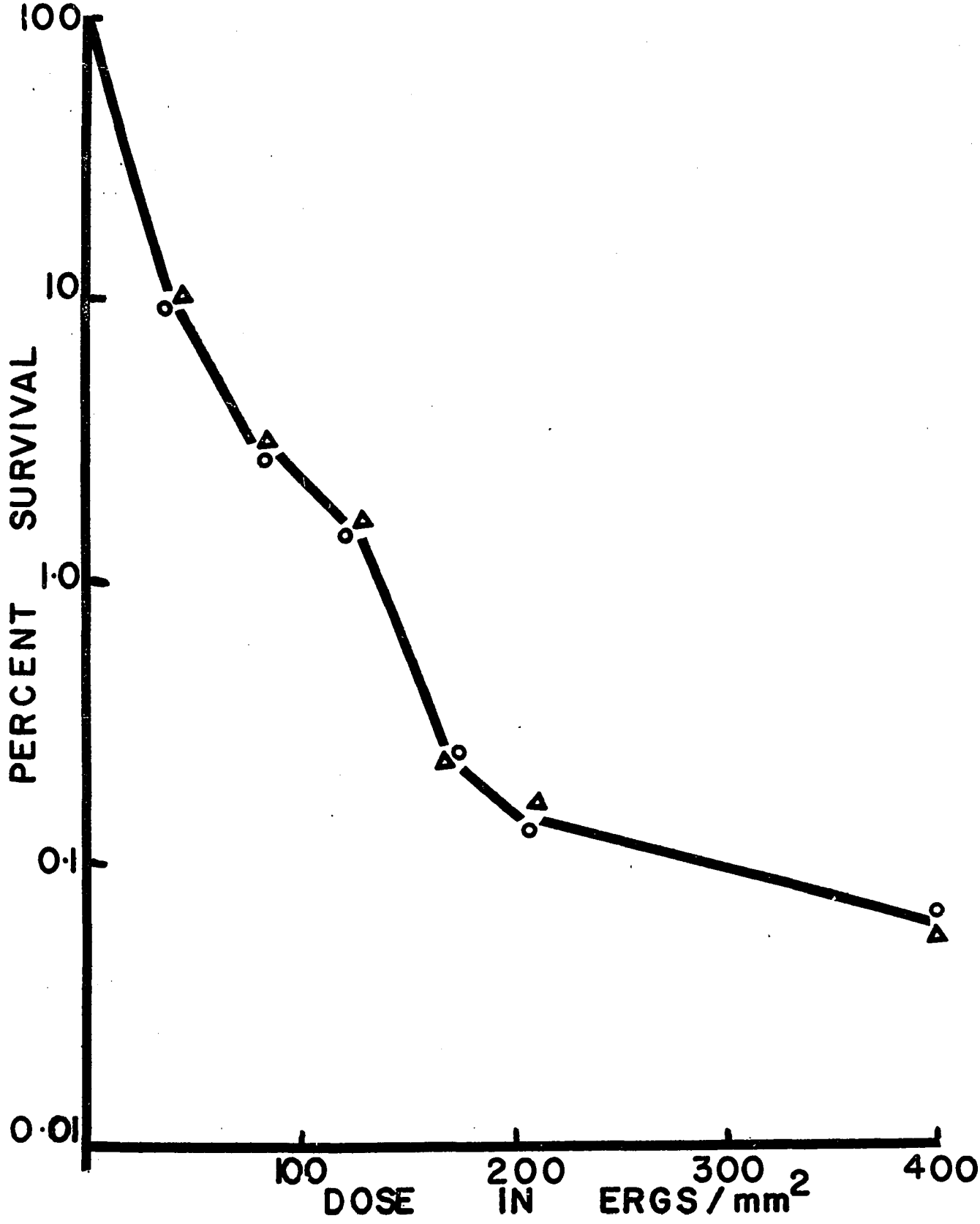

100
10
1·0
0·1
0·01
PERCENT SURVIVAL
100
200
300
400
DOSE IN ERGS/mm2

Figure 5 shows the effect of different concentrations of DNP in the plating medium on irradiated and unirradiated cells. Although 0.0003 M DNP causes maximum increase in the survival of irradiated cells, this concentration resulted in a decrease in the survival of unirradiated cells. Therefore, 0.0002 M concentration was used for all subsequent experiments. When a similar experiment was repeated using different concentrations of KCN, it was found that 0.0006 M concentration gave maximum increase in UV survival without affecting the survival of unirradiated cells.

Since Figure 5 shows the effect of DNP on only one dose of far UV, it was necessary to confirm this by running a survival curve in the presence of DNP in the plating medium. Figure 6 shows these results. 0.0001 M and 0.0002 M DNP in the plating medium significantly increased the survival of far UV irradiated cells. Effect of 0.0006 M KCN on UV survival is shown in Figure 7.

Figure 8 shows the effect of DNP on UV survival under anaerobic growth conditions. Incubation of far UV irradiated cells under anaerobic conditions resulted in a greater survival compared to aerobic incubation. This effect is enlarged if DNP is present in the plating medium. The ratio of percent survivors of cells under anaerobic to aerobic conditions is 2:1, but the ratio became 3.3:1 if DNP plates were used. Therefore, DNP appears to be much more effective under anaerobic growth conditions.

Figure 5. Effect of different concentrations of DNP on the survival of far UV irradiated and unirradiated E. coli B. Percent survival is plotted on a log scale against different concentrations of DNP.

Symbols correspond to: ●-, unirradiated cells; ▲-, irradiated cells (far UV dose 60 ergs/mm^2).

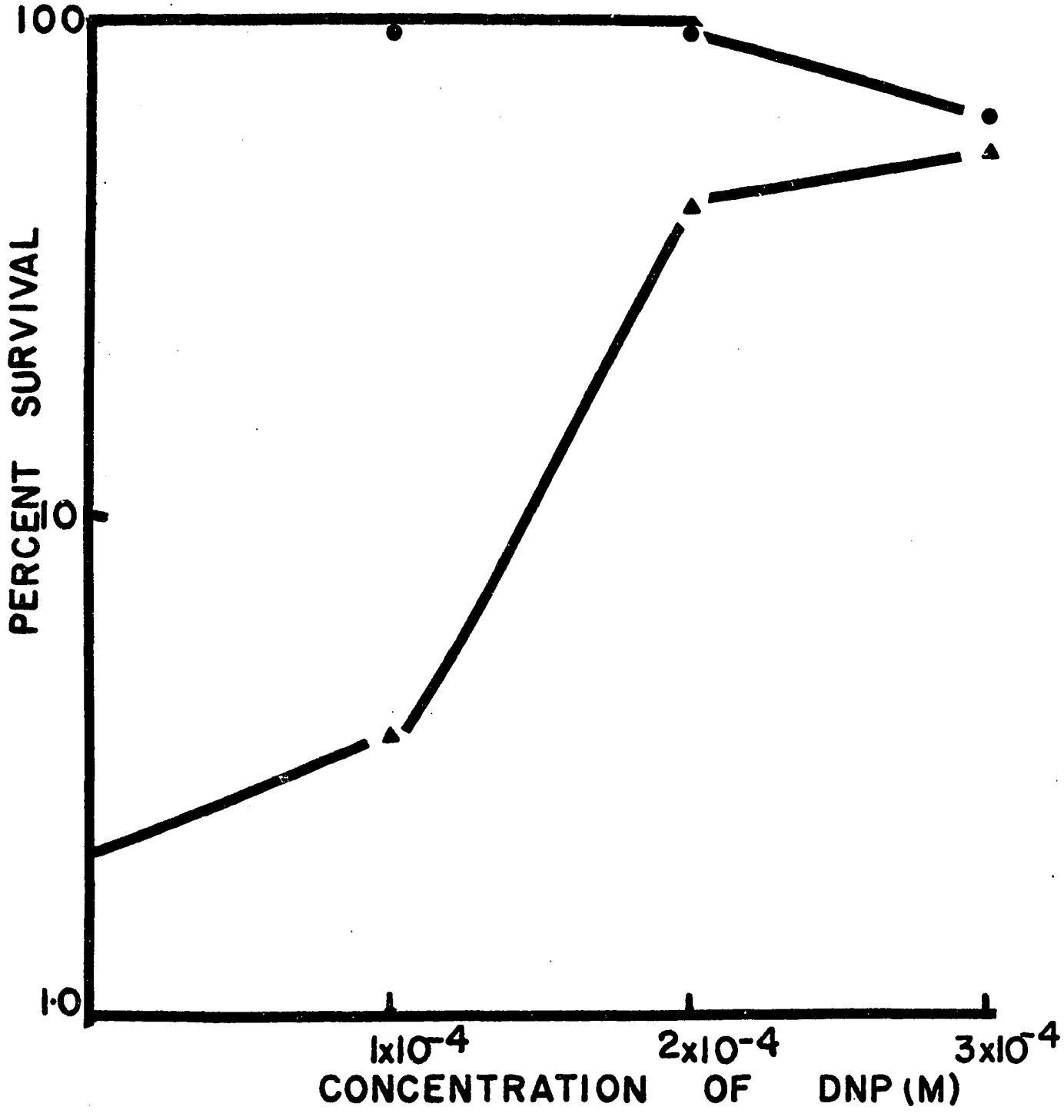

100
10
1·0
PERCENT SURVIVAL
1x10⁻⁴
2x10⁻⁴
3x10⁻⁴
CONCENTRATION OF DNP (M)

Figure 6. Effect of DNP on the survival of *E. coli* B. Percent survival is plotted on a log scale against far UV dose.

Symbols correspond to: O–, cells plated on plain nutrient agar; ▲–, cells plated on nutrient agar plus 0.0001 M DNP; ▲–, cells plated on nutrient agar plus 0.0002 M DNP.

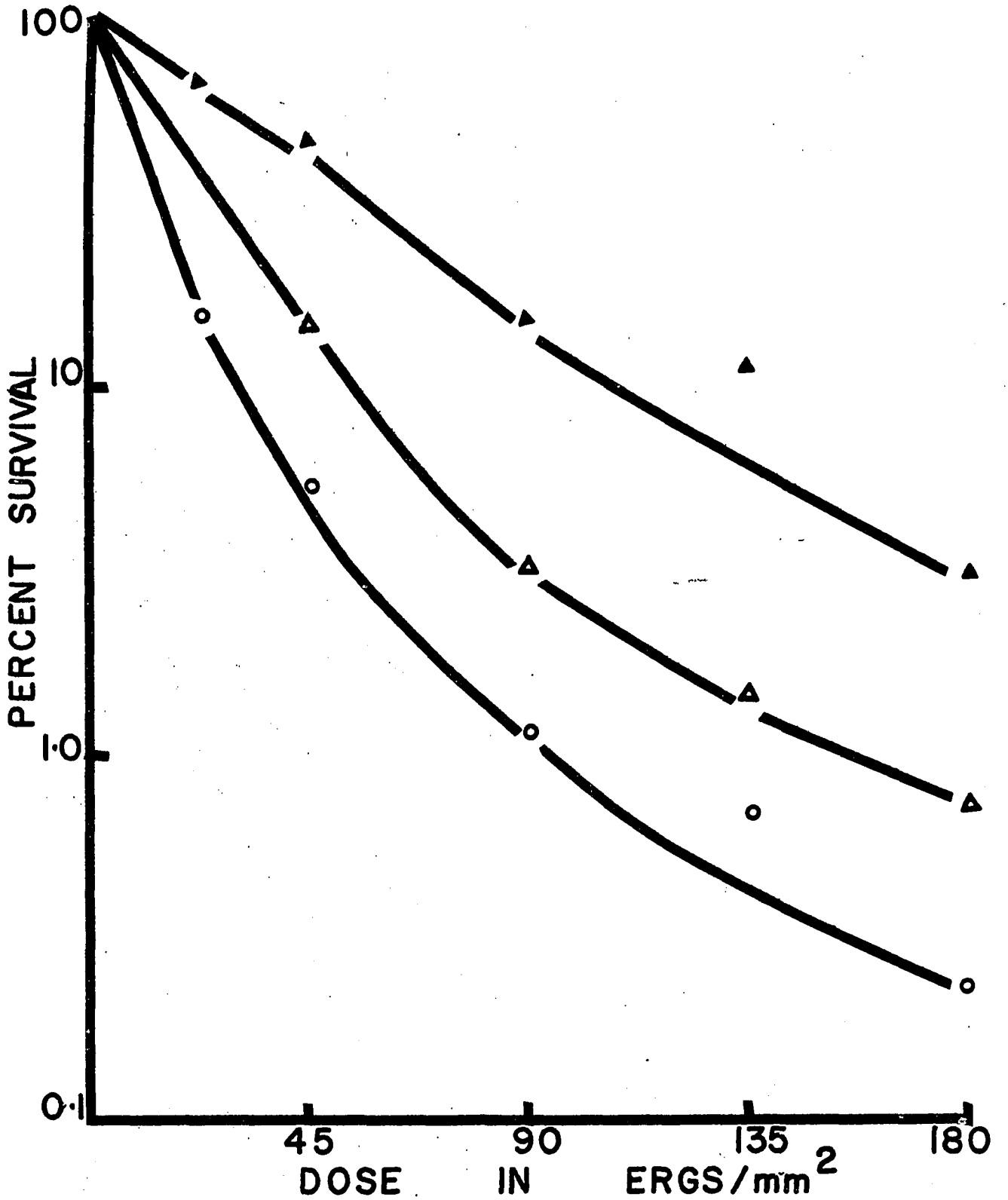

100
10
1·0
0·1
PERCENT SURVIVAL
45
90
135
180
DOSE IN ERGS/mm 2

Figure 7. Effect of KCN on the survival of far UV irradiated E. coli B. Percent survival is plotted on log scale against UV dose.

Symbols correspond to: ●-, cells plated on nutrient agar; ▲-, cells plated on nutrient agar plus 0.0006 M KCN.

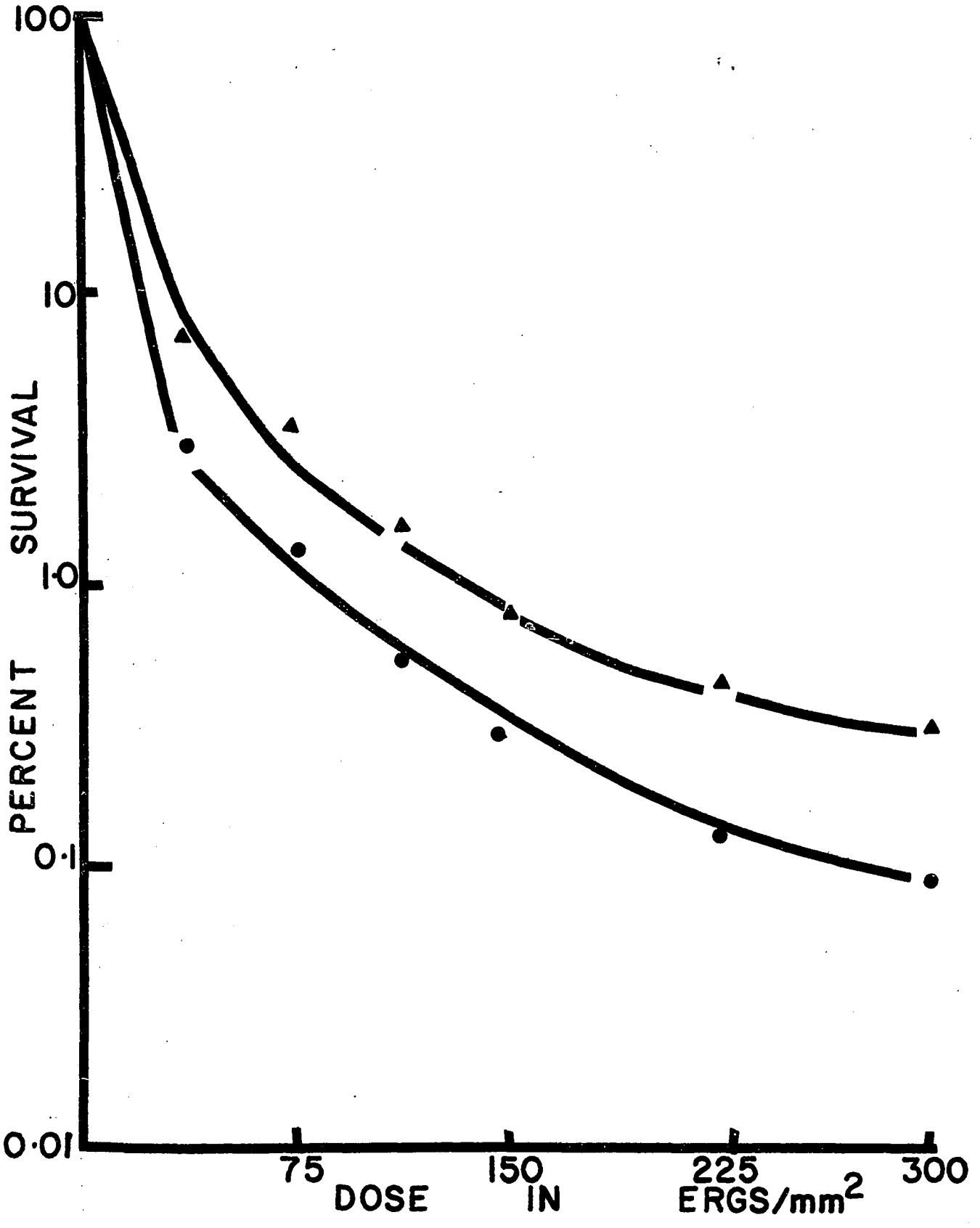

100
10
1·0
0·1
0·01
PERCENT SURVIVAL
75
150
225
300
DOSE IN ERGS/mm2

Figure 8. UV survival of E. coli B under aerobic and anaerobic growth conditions. Percent survival is shown on a log scale.

Solid bars represent the survival of control cells under aerobic and anaerobic conditions. Striped bars show the survival under similar conditions but with DNP agar (nutrient agar plus 0.0002 M DNP).

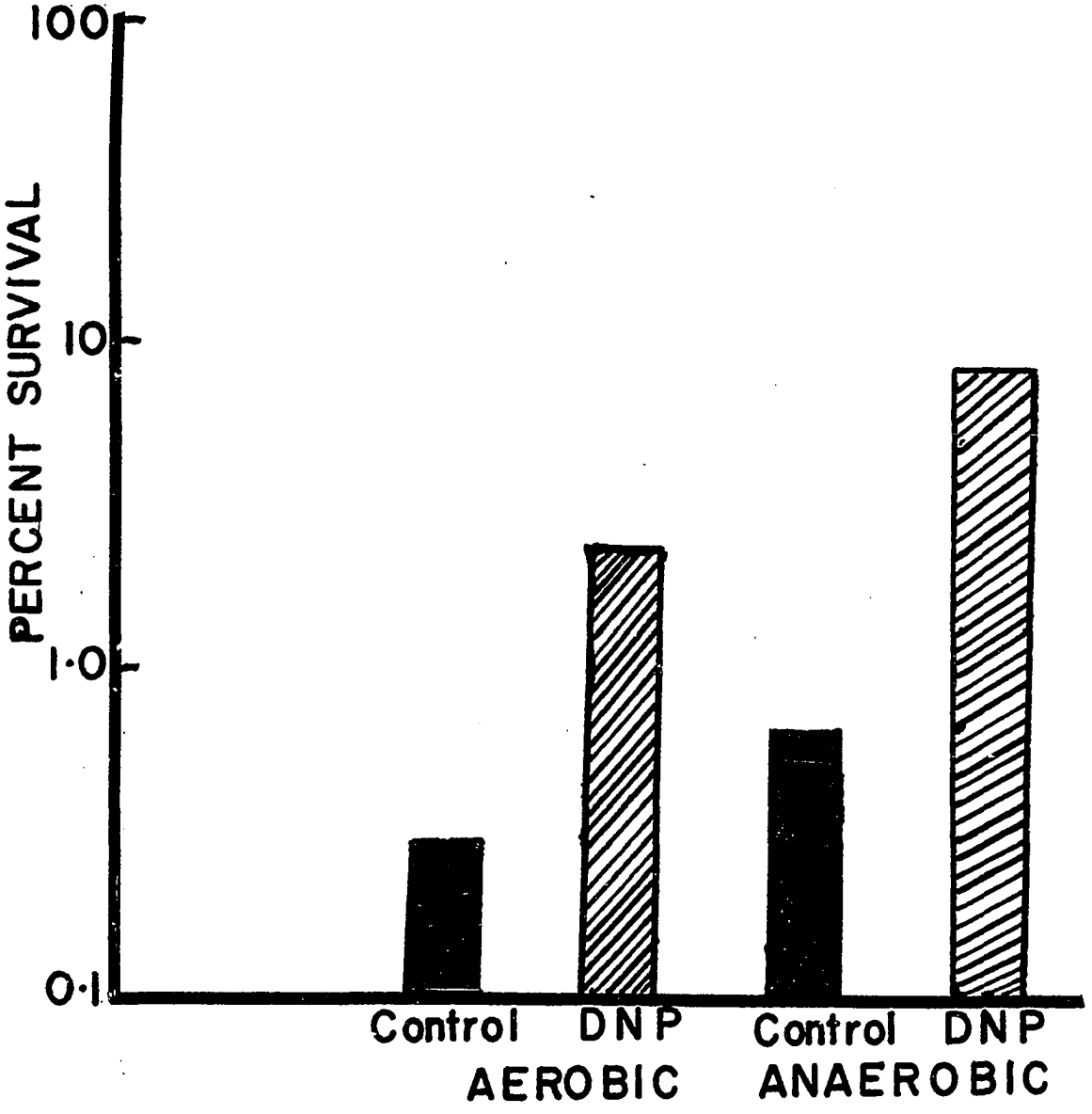

100
10
1·0
0·1
PERCENT SURVIVAL
Control
DNP
Control
DNP
AEROBIC
ANAEROBIC

Figure 9 shows the effect of different doses of near UV on subsequent far UV radiation. Twenty minute exposure to near UV appears to give maximum PP. Figure 10 shows the effect of PP, DNP, and PP plus DNP. PP dose used in this experiment was twenty five minutes exposure to near UV, which is more than sufficient to give maximum PP. Analysis of these results shows that DNP alone is much more effective in helping the recovery of irradiated cells as compared to PP. Above all the most significant aspect seems to be the additive effect of these two treatments. When a fully photoprotected cell sample is irradiated and then plated on DNP agar the survival is approximately the sum of survivals when using each treatment separately.

Figure 11 shows the effect of different PP doses on far UV irradiated cells plated in plain nutrient agar and DNP agar. This observation supports the additive effect of these two agents as earlier stated.

LHR is the dark recovery observed when irradiated cells are held in a non-nutrient liquid medium before plating. Jagger et al. (26) have shown a complete overlap of LHR and PP. They suggest that holding irradiated cells in non-nutrient fluid medium simply provides more time for the operation of dark repair enzymes. However DNP appears to inhibit LHR as shown in Figure 12. It was suspected that this inhibition might be due to a decreased

Figure 9. Effect of different doses of near UV radiation on unirradiated and far UV irradiated E. coli B. Percent survival is plotted on a log scale against time of exposure to near UV.

Symbols correspond to: ●-, survival of cells only exposed to near UV; ▲-, survival of cells exposed to far UV (after near UV).

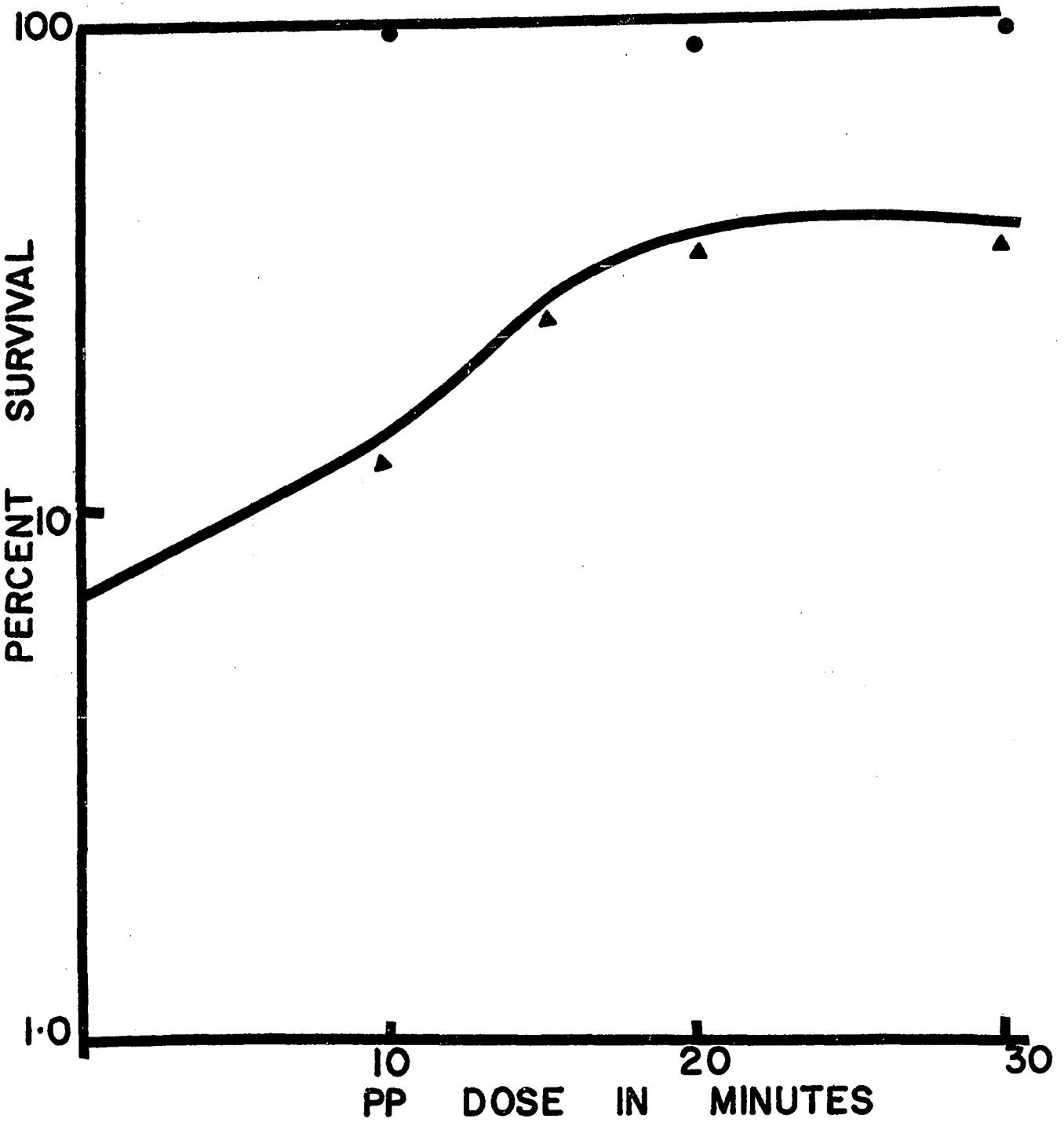

100
10
1·0
PERCENT SURVIVAL
10
20
30
PP DOSE IN MINUTES

Figure 10. Photoprotection, DNP reactivation, and photoprotection plus DNP reactivation of irradiated E. coli B. Percent survival is plotted on log scale against far UV dose.

Symbols correspond to: O-, cells plated on nutrient agar after far UV irradiation; ▲-, cells exposed to near UV (25 min) prior to far UV irradiation and plated on nutrient agar; ● -, cells plated on DNP agar (nutrient agar plus 0.0002 M DNP) after far UV irradiation; ◭-, cells exposed to near UV (25 min) prior to far UV irradiation and plated on DNP agar.

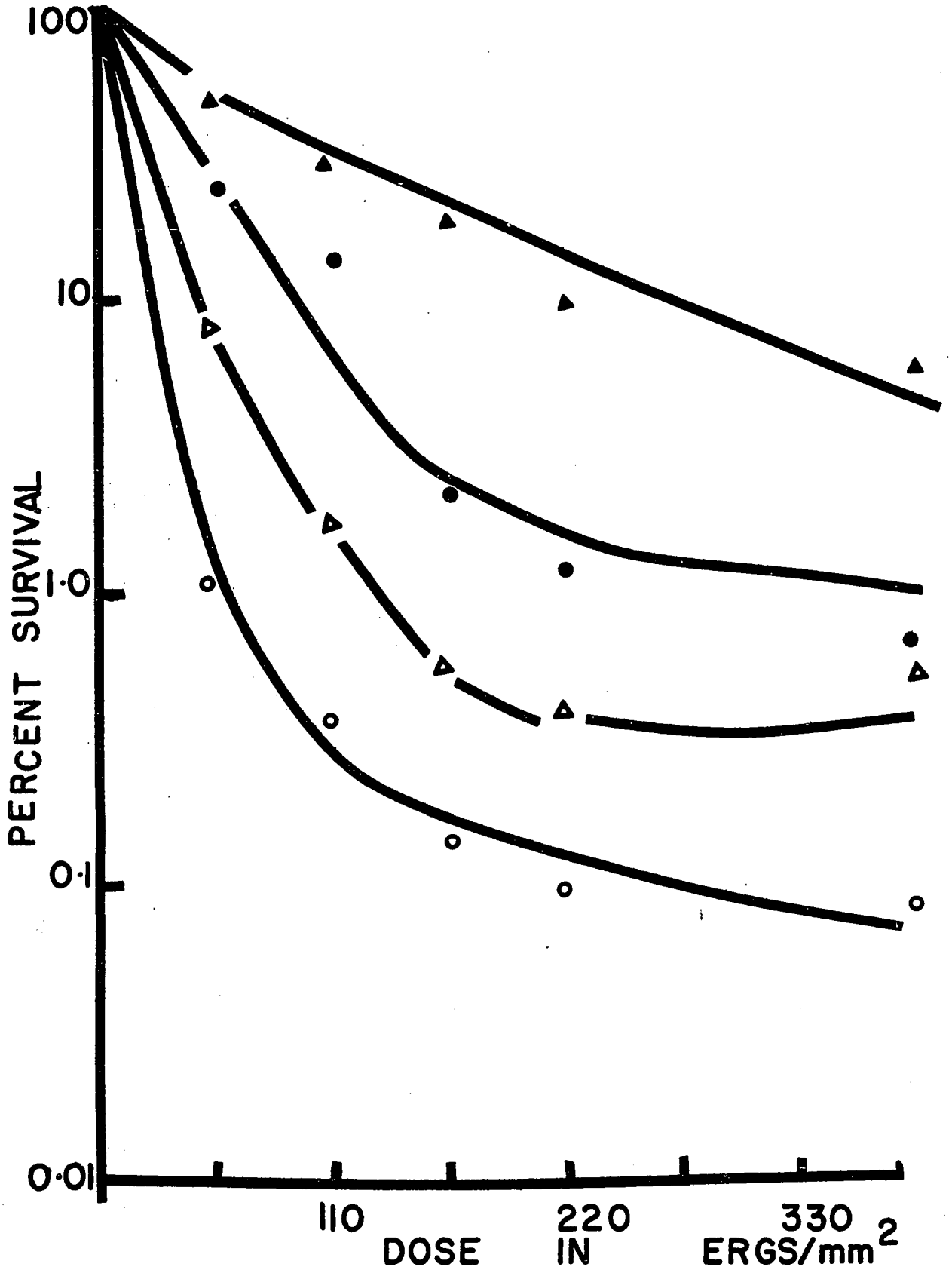

100
10
1·0
0·1
0·01
PERCENT SURVIVAL
110
220
330
DOSE IN ERGS/mm2

Figure 11. Effect of different near UV doses on DNP reactivation of far UV irradiated E. coli B. Cells were exposed to different near UV doses, then irradiated with far UV once and plated on plain nutrient agar and DNP agar (Nutrient agar plus 0.0002 M DNP). Percent survival is plotted on log scale against near UV doses.

Symbols correspond to: O-, plated on plain nutrient agar; ▲-, plated on DNP agar.

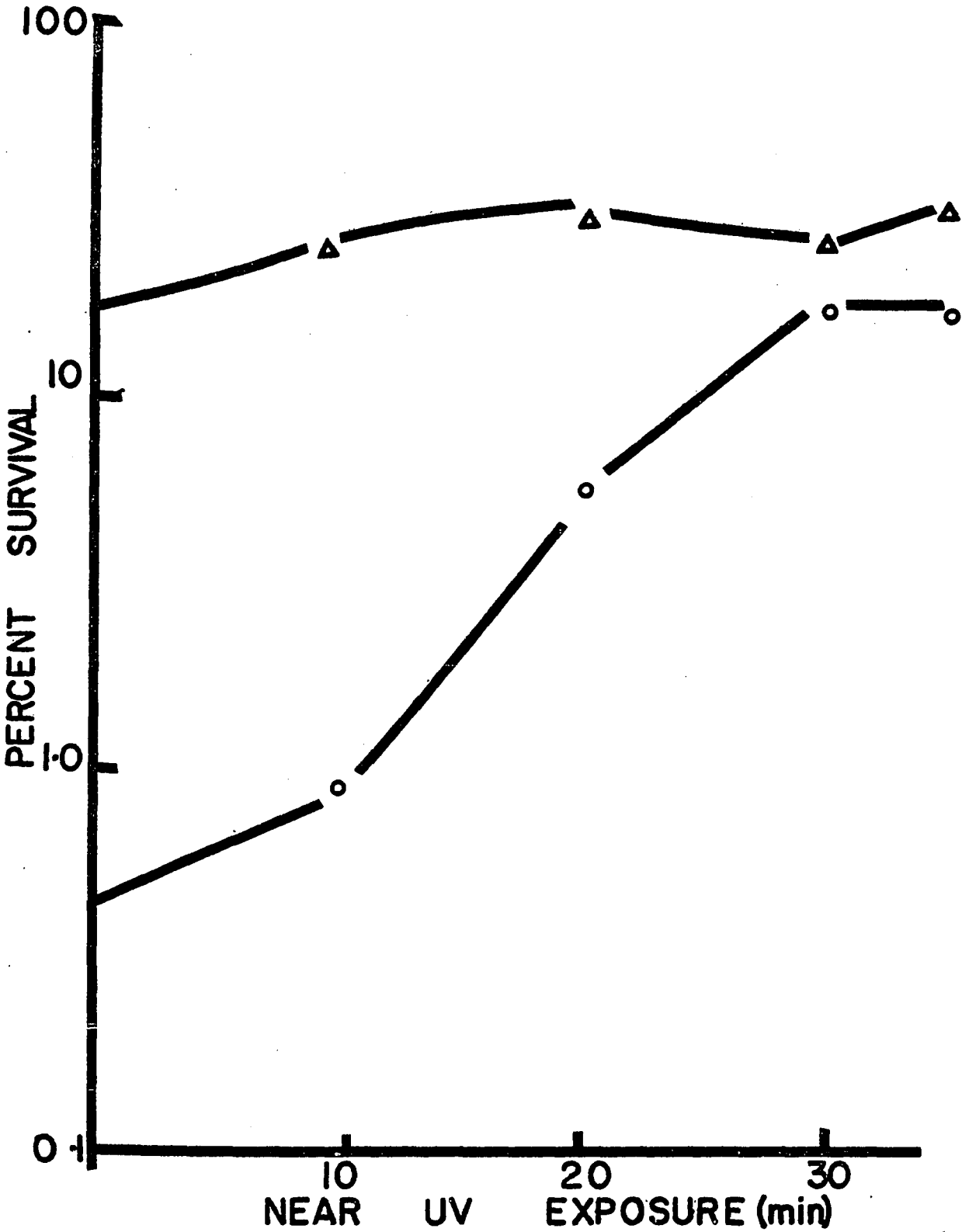
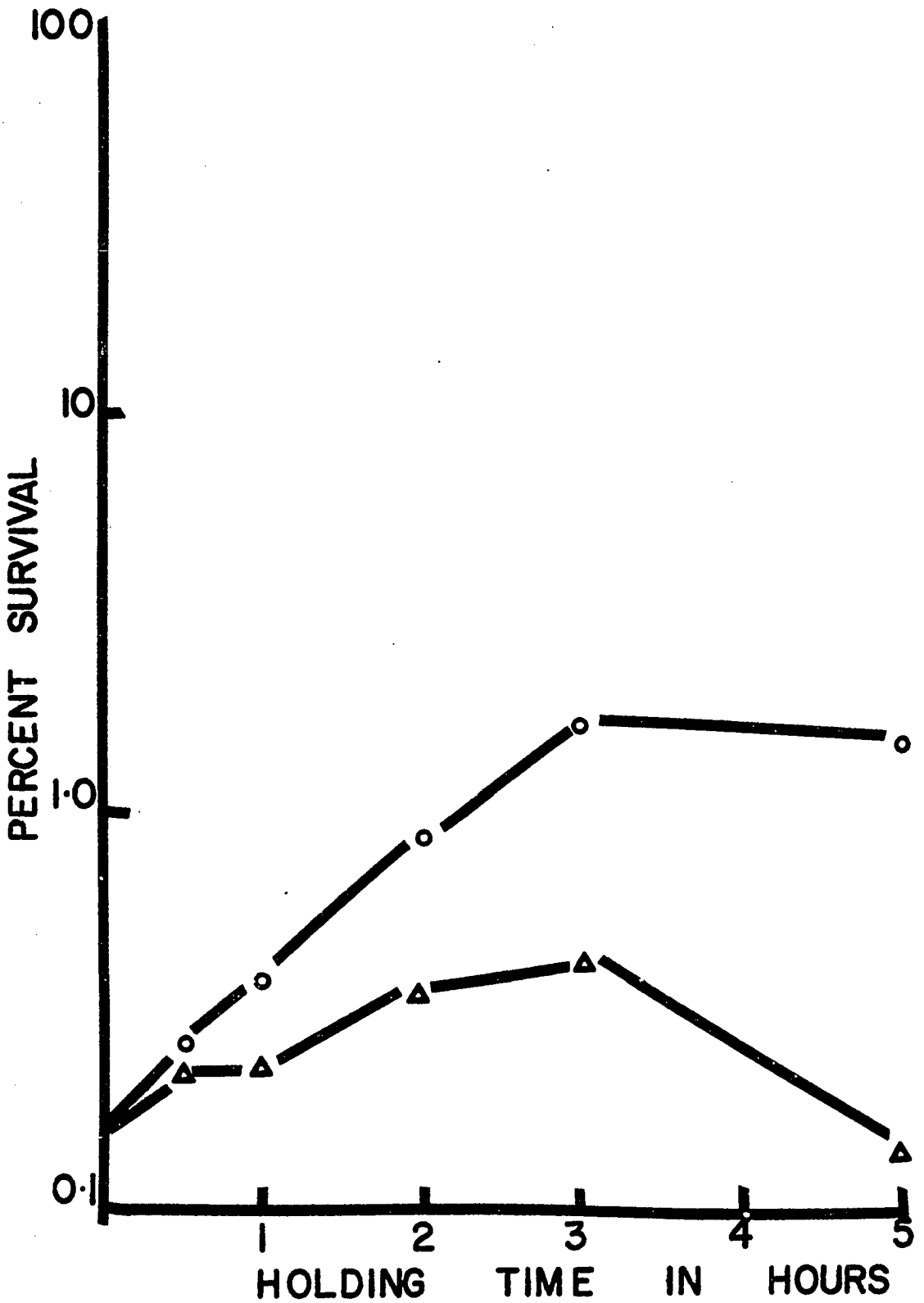

100
10
1·0
0·1
PERCENT SURVIVAL
10
20
30
NEAR UV EXPOSURE (min)

Figure 12. Inhibition of liquid holding recovery by DNP. Percent survival is plotted on log scale against holding time.

Symbols correspond to: ▲-, holding in phosphate buffer with 0.0002 M DNP; O-, holding in phosphate buffer without DNP.


100
10
1·0
0·1
PERCENT SURVIVAL
1
2
3
4
5
HOLDING TIME IN HOURS

energy level in the cell caused by DNP. Therefore, it was necessary to test the effect of DNP in the presence of an energy source such as glucose. The inhibitory effect of DNP, however, was reversed by the addition of glucose to the holding fluid. These results are shown in Figure 13.

The recovery effect of DNP is not limited to far UV damage but is significantly effective against X-ray and HN2 inactivated cells. These results are shown in Figure 14 and 15. This strain of E. coli B is not photoprotectable against X-ray inactivation but shows PP against HN2. PP treatment appears to be slightly more effective than DNP in recovery of HN2 inactivated cells. Figure 16 suggests that the combination of these two treatments against HN2 is not much more effective than PP alone.

The effect of 2,4,6-trinitrophenol is shown in Figure 17. This compound is closely related to DNP in that both of these chemicals combine with proteins, but this chemical, unlike DNP, does not uncouple oxidative phosphorylation from respiration. Both 2,4,6-trinitrophenol and DNP cause an increase in UV survival, but the concentration of 2,4,6-trinitrophenol must be ten times greater than that of DNP to obtain the same level of survival.

Figure 13. Effect of glucose on the inhibition of liquid holding recovery by DNP. Percent survival is plotted on log scale against holding time.

Symbols correspond to: ▲-, cells held in buffer with 0.0002 M DNP; O-, cells held in buffer only; ●-, cells held in buffer plus 1% glucose; ▲-, cells held in buffer with 1% glucose and 0.0002 M DNP.

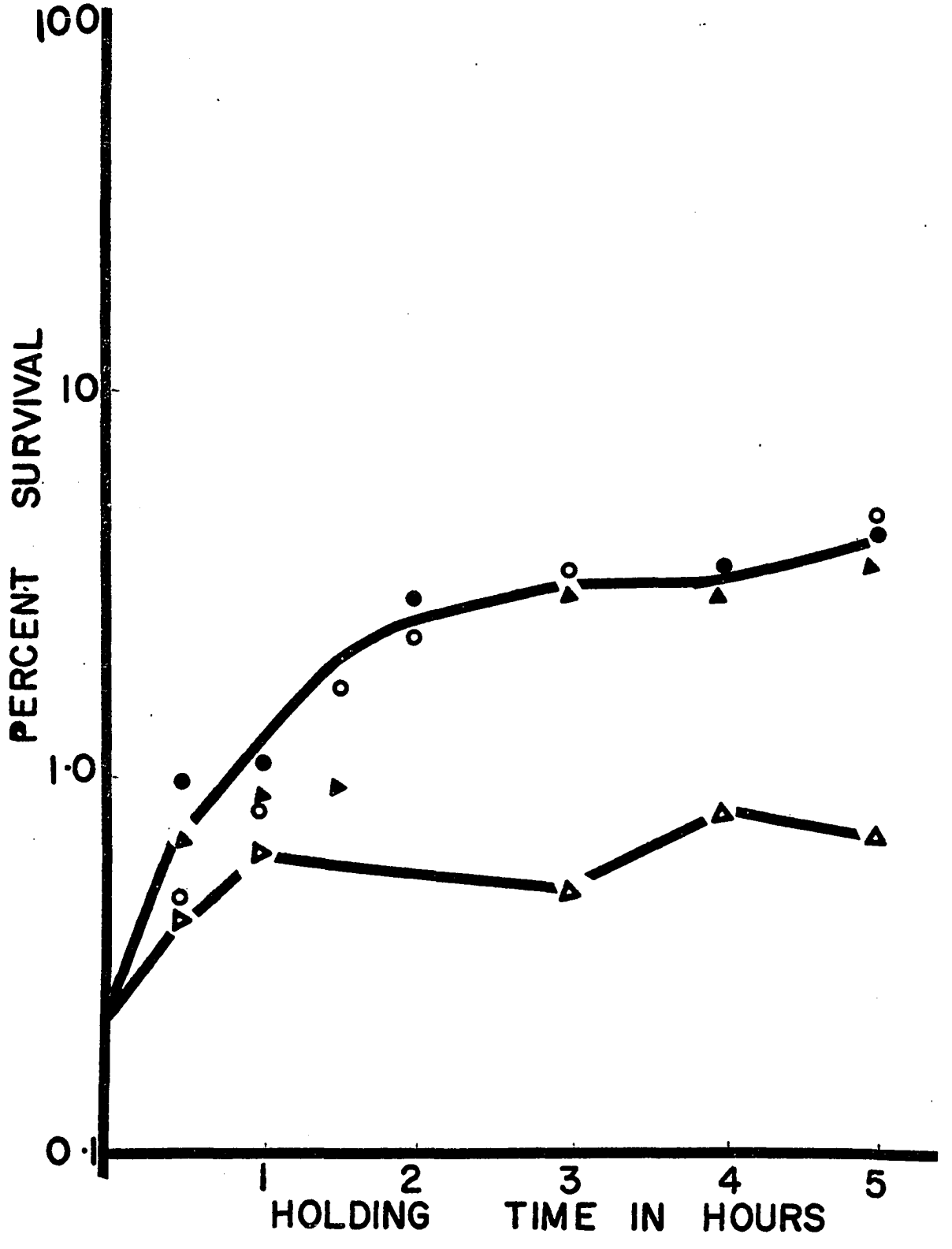

100
10
1·0
0·1
PERCENT SURVIVAL
1
2
3
4
5
HOLDING TIME IN HOURS

Figure 14. Effect of DNP on the survival of X-ray irradiated E. coli B. Percent survival is plotted on log scale against X-ray dose.

Symbols correspond to: ●-, cells plated on plain nutrient agar; ▲-, cells plated in DNP agar (nutrient agar plus 0.0002 M DNP).

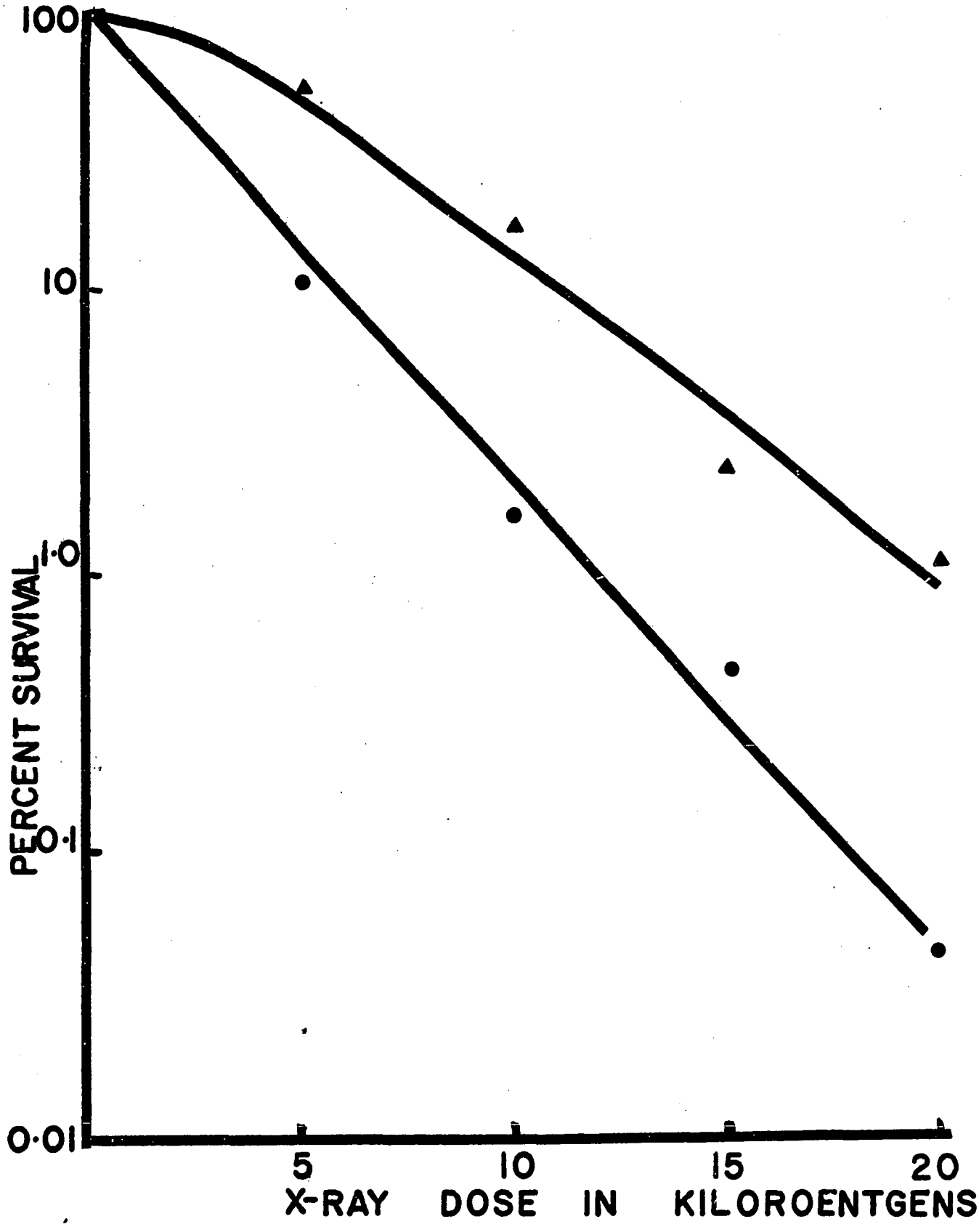

100
10
1·0
0·1
0·01
PERCENT SURVIVAL
5
10
15
20
X-RAY DOSE IN KILOROENTGENS

Figure 15. Effect of DNP on the survival of HN2 inactivated E. coli B. Percent survival is plotted on log scale against time of exposure to 0.3 mg/ml HN2 in phosphate buffer at 37 C.

Symbols correspond to: ●-, cells plated on plain nutrient agar; ▲-, cells plated on DNP agar (nutrient agar plus 0.0002 M DNP).

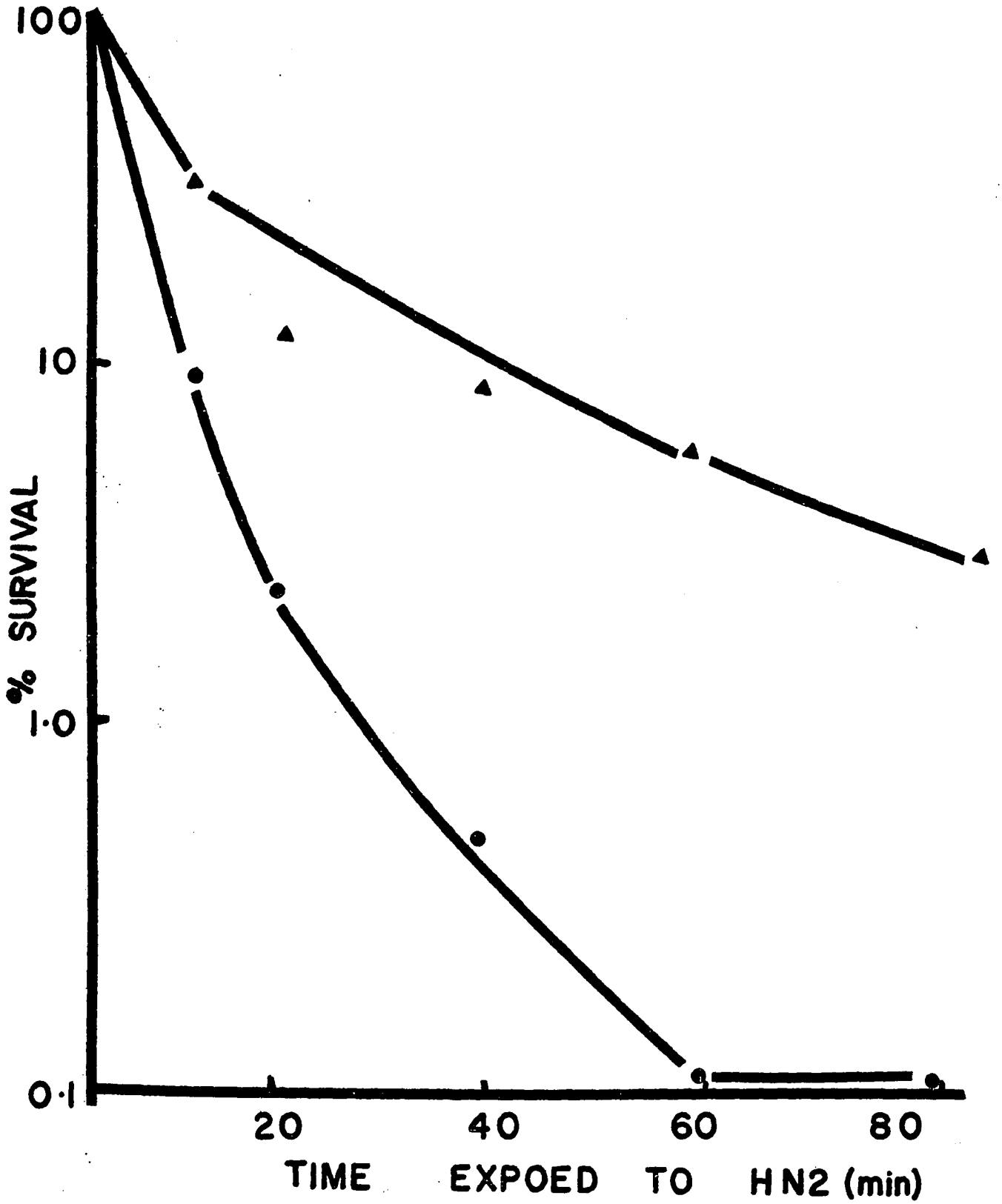

100
10
1·0
0·1
% SURVIVAL
20
40
60
80
TIME EXPOED TO HN2 (min)

Figure 16. Photoprotection, DNP reactivation, and photoprotection plus DNP reactivation of HN2 inactivated E. coli B. Percent survival is plotted on log scale against time of exposure to HN2 (0.3 mg/ml) in phosphate buffer at 37 C.

Symbols correspond to: O-, cells plated in plain nutrient agar; ▲-, cells plated in DNP agar (nutrient agar plus 0.0002 M DNP); ▲ , cells exposed to near UV (25 min) prior to HN2 treatment and plated on plain nutrient agar; ● -, cells exposed to near UV (25 min) prior to HN2 treatment and plated on DNP agar.

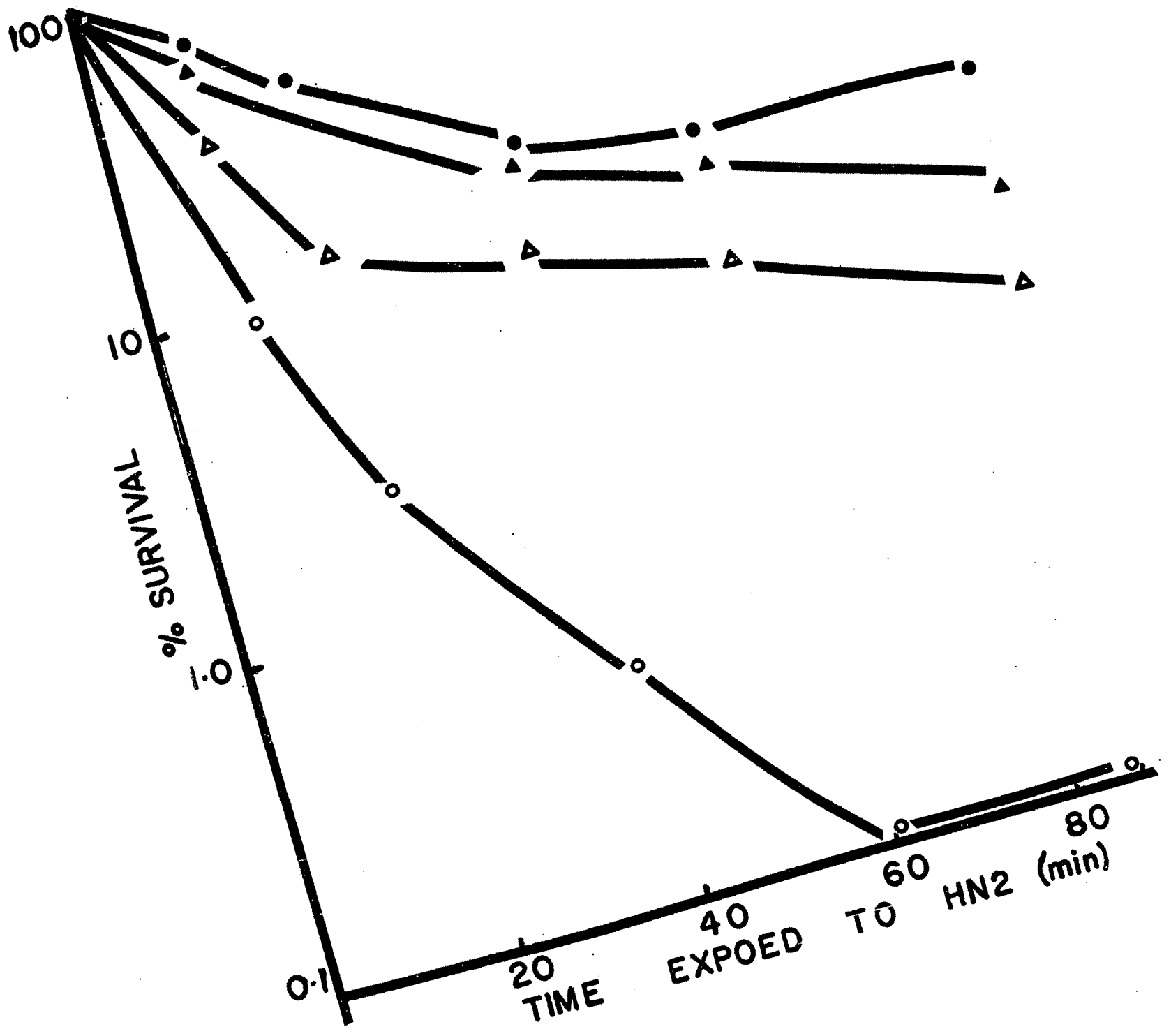
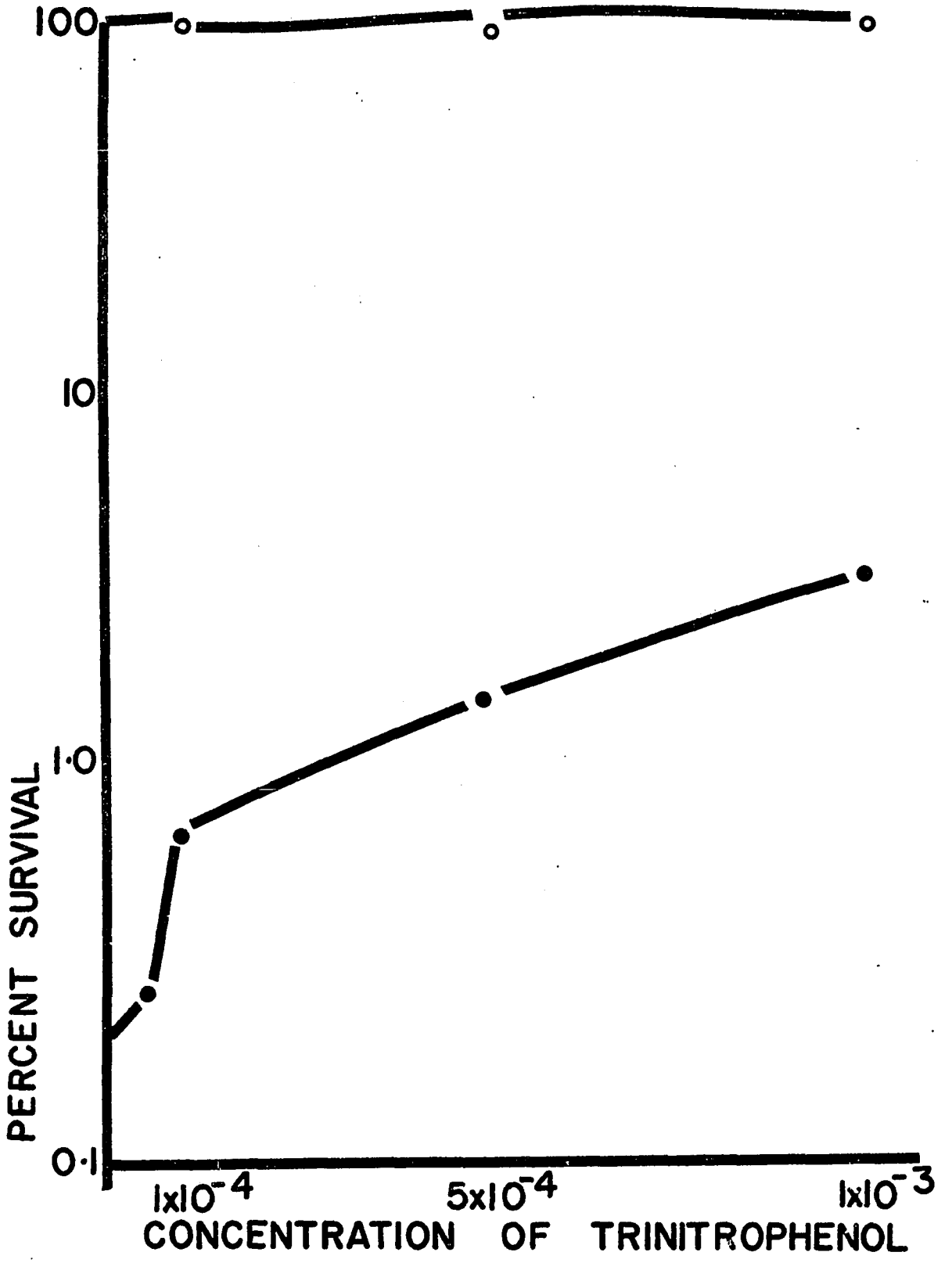

100
10
1.0
0.1
% SURVIVAL
20
40
60
80
TIME EXPOED TO HN2 (min)

Figure 17. Effect of different concentrations of 2,4,6-trinitrophenol on the recovery of UV irradiated *E. coli* B. Percent survival is plotted on log scale against concentrations of 2,4,6-trinitrophenol.


100
10
1·0
0·1
PERCENT SURVIVAL
1x10⁻⁴
5x10⁻⁴
1x10⁻³
CONCENTRATION OF TRINITROPHENOL

CHAPTER IV

DISCUSSION

DNP, cyanide and near UV interfere with the oxidative metabolism of the cell. DNP is a well known uncoupler of oxidative phosphorylation from respiration in mitochondria, but it is reported to have little effect on the oxidative phosphorylation of bacteria (14). Nonetheless, there are many conflicting reports in the literature which suggest that DNP may cause from 30 to 60 percent uncoupling in E. coli, depending upon the concentration of the chemical and pH of the system (21,29,40). Cyanide is a classical inhibitor of the respiratory chain, which is known to inhibit cytochromes $a_1 + a_2$ of E. coli, while near UV radiation is reported to cause destruction of quinone compounds (29,30). These reactions result in the inhibition of respiration and thus probably reduce the rate of oxidative phosphorylation. Thus one common feature to all these agents is their ability to decrease the level of ATP in the cell. This decrease may lead to a growth delay.

According to Jagger's hypothesis, this growth delay may provide more time for the operation of dark

repair enzymes. If this is the only mode of action of these three agents, then there should be a direct correlation between the extent of growth delay and increase in the survival due to each agent. The data presented here indicate that no such simple correlation exists. Cyanide, which is most effective in causing a growth delay, results in the smallest increase in the UV survival, whereas DNP produces the least growth delay, but causes the highest increase in UV survival. Near UV shows intermediate effects.

Inconsistency of cyanide effects with Jagger's hypothesis can be explained. Recently Harm and Haefner (17) have indicated that higher concentrations of KCN (0.002 M) inhibited LHR in E. coli B and B/r. In other words, cyanide at this concentration is capable of inhibiting dark repair. Similar observation was made during the course of this investigation. It appears that the concentration of cyanide is of utmost importance in determining the effect on survival of far UV irradiated cells. Lower concentrations (0.0006 M) in the plating medium cause an increase in survival while higher concentration shows a decrease. This is probably due to a dual effect of cyanide on survival. An increase in survival may result due to an induction of growth delay. A decrease in survival may be due to interference of this chemical with the dark repair process. Higher (but non-lethal to

unirradiated cells) concentrations seem to interfere with the dark repair process to such an extent that a small increase in survival due to growth delay is completely masked. However, lower concentrations, not being extremely favorable to inhibitory processes, result in a net increase in survival. Thus the mode of action of cyanide in the recovery of UV irradiated cells does not appear to be any different than that of near UV, with the exception that cyanide also causes an inhibition of dark repair.

DNP induced effects are also inconsistent with Jagger's hypothesis. The growth delay caused by DNP is so small that it should be inadequate for the increased level of survival obtained. One explanation is as follows. According to Jagger's hypothesis destruction of quinone compounds by a photochemical reaction induced by near UV is responsible for growth delay and consequently for PP. If this is true, then one may expect that the near UV effects (PP and growth delay) would disappear as soon as these quinone compounds are resynthesized. Once this takes place the cell may restore its normal metabolism and thus some of the far UV induced damage may remain unrepaired. On the other hand DNP is present during all post-irradiation growth period and thus would allow ample time for recovery. However, the evidence seems to be against this. A slow decay of PP is shown by Jagger (25). It has been reported by Jagger (25) that a 50% decay

occurred over a period of 28 hours. Therefore, it seems likely that near UV irradiation provides sufficient decrease in the growth rate to allow the time period necessary for repair of all repairable damage by this mechanism. But there may be some damage which is not repaired by this mechanism.

The additive effect of near UV and DNP on UV survival cannot be explained on the basis of growth delay. It appears that either one of the agents is capable of slowing down the metabolism to such an extent that all the repairable damage could be repaired. Therefore, the additional recovery obtained when DNP and near UV are used in combination does not appear to be due to extended growth delay. However, it seems possible that DNP might allow the repair of that damage which is left unaltered after near UV effected dark repair.

Therefore, it appears that mode of action of DNP may be somewhat different than PP. Part of the total increase in survival caused by DNP may be due to growth delay. Since anaerobic conditions enhance the effect of DNP, it may be possible that there is a greater reduction in the ATP level which would result in a more prominent growth delay and thus give a better survival. Mosin (39) has reported a greater reduction in ATP level under anaerobic conditions. The main effect of DNP seems to be due to a mechanism different from growth delay.

Effects of this chemical, other than being an uncoupler of oxidative phosphorylation, have been largely ignored until recently. It has been shown that DNP could combine with many proteins and could inhibit enzymes such as hexokinase, skeletal muscle phosphorylase, purified isocitrate and malate dehydrogenases (56). Picric acid (2,4,6-trinitrophenol) can form a complex with many proteins but cannot uncouple oxidative phosphorylation from respiration (56). Although an approximately ten times higher concentration is required, this chemical is also effective in increasing the UV survival of E. coli.

On the basis of experimental evidence available at present, the uncoupling action of DNP does not appear to be solely responsible for the increase in UV survival. It may very well be possible that part of the total increase in UV survival may arise as a consequence of uncoupling action. Selective binding of DNP to some normal DNA replication enzymes, thus providing a better opportunity for the action of repair enzymes (by eliminating the competition between repair and normal DNA replication), seems to be unlikely for the following reason. This type of binding should give rise to a more prominent growth delay. However, one simple way by which DNP could cause such increase may be due to stimulation of dark repair enzymes. Another alternative to this could be action of DNP on some photochemical damage, which is normally ignored

by dark repair system.

DNP inhibits LHR, which seems inconsistent with what has been previously stated i.e. DNP helps in dark repair and LHR is a type of dark repair which is quite similar to PP. However, it should be borne in mind that these two recoveries take place under entirely different conditions. During the dark repair process that is enhanced by PP, the cells are in a complete medium and can thus initiate normal DNA replication. The energy for normal replication and repair processes is derived from the nutrients obtained from the surrounding medium. On the other hand, during LHR the energy for the repair process is derived from the endogenous metabolism. Most likely during this process there is no normal DNA replication and repair processes. However, any change in the amount of energy available for LHR would certainly influence the recovery. The proof for this comes from the fact that inhibition of LHR can be reversed by addition of glucose. Addition of glucose would provide an additional energy source and thus there would be sufficient energy available for this process. For some unknown reason, DNP does not increase the recovery beyond normal level after adding glucose. Normal level here refers to the level of recovery obtained without DNP in the holding liquid.

DNP and near UV seem to be almost equally effective

against HN2 damage. When both these agents are used in combination on HN2 damage, unlike far UV, there is no additive effect on survival. This may be due to the following reason. Treatment with either agent gives complete repair of all the repairable damage. Recovery effect of DNP against X-ray inactivation indicates that DNP is capable of helping the recovery of these lesions while near UV has no effect on these. Thus DNP appears to be capable of acting on a different kind of damage.

CHAPTER V

SUMMARY

The effect of inhibitors of respiration and oxidative phosphorylation was studied on the recovery of irradiated or nitrogen mustard inactivated E. coli B cells. Near UV and potassium cyanide were used as inhibitors of respiration while 2,4-dinitrophenol was used as an uncoupler of oxidative phosphorylation from respiration. The optimum dose for each treatment was determined by exposing irradiated and unirradiated cells to different concentrations of KCN or DNP (or for different time intervals in case of near UV). These chemicals were incorporated into the plating medium, while near UV was applied to cells prior to far UV irradiation. That concentration (or dose in case of near UV) which gave maximum increase in the survival of irradiated cells without altering the survival of unirradiated cells, was selected for experimental work. These included 0.0006 M KCN, 0.0002 M DNP and 25 min exposure to near UV. Exposure to near UV for 25 min prior to UV radiation or HN2 inactivation gave a significant increase in the survival. Pre-exposure to DNP or KCN was ineffective in changing the survival after

UV irradiation. Near UV did not protect the cells against X-ray inactivation.

Growth delays caused by near UV, KCN, and DNP were compared. It was found that cyanide was most effective in causing the growth delay and least effective in increasing the UV survival. DNP caused maximum increase in UV survival but showed only a very slight growth delay. Near UV had an intermediate effect.

The mode of action of each agent was analyzed. Near UV and KCN may be effective in increasing the UV survival due to growth delay and thus providing more time for the operation of dark repair system. An additional effect of KCN acting as an inhibitor of dark repair system at higher concentrations, originally reported by Harm and Haefner, was confirmed. The dual effect of cyanide: 1. induction of growth delay, thus helping the recovery; and 2. inhibition of dark repair, have been discussed.

The mode of action of DNP in the recovery of irradiated cells appears to be somewhat different than that of near UV. It has been postulated that DNP either stimulates dark recovery and/or helps the recovery of some photochemical damage that is normally ignored by dark repair system even after PP treatment. It was also shown that a closely related compound 2,4,6-trinitrophenol at a concentration ten times higher than DNP also caused an increase in UV survival which was comparable to that caused

by DNP. It should be noted that this chemical cannot uncouple oxidative phosphorylation from respiration.

Effect of DNP on LHR was determined. Inhibition of LHR by DNP was reversed by addition of glucose to the holding fluid. Possible reasons for this have also been discussed. When near UV and DNP were used in combination, the net survival was approximately the sum of the survivors obtained by using each agent separately. This may be due to the following reason. Near UV treatment does not cause a complete repair of all the repairable damage and thus leaves some of the repairable damage unrepaired. This damage is repaired by DNP. In addition DNP may be able to cause some recovery of the photochemical damage that is normally not carried out by this system.

Finally, an attempt was made to show that all these treatments effective against far UV damage also show a very high increase in survival after HN2 inactivation and a somewhat less increase in survival after X-ray inactivation.

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