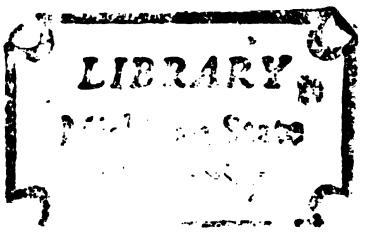
# FAILURE OF PROTEIN SYNTHESIS AND NET RNA SYNTHESIS IN HEAT-KILLED ESCHERICHIA COLI

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

ROGER GLEN DEAN

1976




This is to certify that the

thesis entitled

**FAILURE OF PROTEIN SYNTHESIS AND NET RNA SYNTHESIS IN HEAT-KILLED ESCHERICHIA COLI**

presented by

**Roger Glen Dean**

has been accepted towards fulfillment
of the requirements for

**DOCTOR OF PHILOSOPHY** degree in **Biophysics**

Major professor

Date 6/14/76

O-7639

ABSTRACT

FAILURE OF PROTEIN SYNTHESIS AND NET RNA SYNTHESIS
IN HEAT-KILLED ESCHERICHIA COLI

by

Roger Glen Dean

High temperature treatment (48°C and above) of Escherichia coli causes cell death as determined by the inability of the killed cells to form colonies upon plating. The kinetics of heat-killing, which is pseudo-first-order, lead to the conclusion that one or a small number of events or molecules are the cause of death. In addition to the first-order kinetics of killing, a number of molecular sites of thermal damage have been found in heated cells of various bacteria. These sites of damage include proteins, the membrane, rRNA, and DNA.

There is evidence that much of the thermal damage produced by heating cells is not lethal. This non-lethal damage is expressed by the increased lag time necessary, after heating, for survivors to reinitiate growth. In order to separate more clearly thermal damage which causes death from thermal damage that is non-lethal, it is necessary to compare heat-killed cells and survivors which have been subjected to the same heating conditions.

To accomplish this comparison, cells were heated until a mixed

population of heat-killed cells and survivors was achieved. Treating this mixed population with penicillin causes lysis of the surviving cells. The heat-killed cells, or a class of heat-killed cells, is unaffected by this process and remain largely intact. Labeling the population of surviving and heat-killed cells immediately after heating with [$^{3}$H]uracil or [$^{14}$C]leucine provides for a comparison of protein or RNA synthesis after heat-killed and surviving cells are separated using the penicillin treatment. This technique has shown that protein synthesis and net RNA synthesis fails in heat-killed cells. Furthermore, this failure occurs in the earliest stages of recovery. These results indicate that the lethal damage occurring in heat-killed cells is directly coupled to protein and RNA synthesis and causes the immediate failure of these two synthetic processes.

Several possible explanations of these results are considered. These explanations are based on the types of molecular damage known to occur in heated populations of bacteria. Most explanations are found to be inadequate because they are either inconsistent with the results of this thesis, they are inconsistent with the first-order kinetics of killing, or the types of damage have not been shown to be lethal. One alternative explanation does appear to have some merit and is proposed. This proposal suggests that damage to the nucleoid RNA which holds the DNA together, is the rate-limiting step in heat-killing. This proposal agrees with the data of this thesis and with the first-order survival kinetics.

# FAILURE OF PROTEIN SYNTHESIS AND NET RNA SYNTHESIS IN HEAT-KILLED *ESCHERICHIA COLI*

by

Roger Glen Dean

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biophysics

1976

To my parents, wife, and children

ACKNOWLEDGEMENTS

The assistance of my advisor Dr. Estelle McGroarty with this research is very deeply appreciated. She had the difficult task of advising me in this work, which was outside her immediate area of research. Despite such an obstacle, her insight, patience, and considerable effort were instrumental in the development of this work. In addition I am most grateful for her assistance in the preparation of the text of this dissertation.

I would also like to express my gratitude to the members of my dissertation committee: Dr. Barnett Rosenberg, Dr. Gabor Kemeny, and Dr. Harold Sadoff. Their continued support and assistance with this research was most helpful and is greatly appreciated.

I must also thank my wife Barbara who typed all the rough drafts and the final draft of this dissertation.

I was also supported in this work by the Michigan State University College of Osteopathic Medicine and NIH Training Grant No GM 01422.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

# INTRODUCTION

The study of killing micro-organisms by means of heat has had a very long history. Initial efforts were directed toward developing methods of sterilizing and protecting food products. The early contributions of investigators were of tremendous practical importance to the development of microbiology, but very little was done toward understanding the mechanism of heat-killing. Many studies were carried out in which various organisms, primarily those organisms found in food, were heated in milk, fruit juice, and many other media related to food products. Studies of this kind were not directed towards elucidating the mechanism of heat-killing. When studies were directed towards finding the mechanism of heat-killing, no consistent method of growth, heating, or recovery was used, making it difficult to compare results between different laboratories. In addition, a number of inadequate methods of measuring the thermal resistance of micro-organisms were used in early experiments. Some of these difficulties persist in present experiments, particularly the variations in methods employed.

## Kinetics

An important advance toward understanding the molecular mechanism of heat-killing was made when Chick (16) measured the number of surviving cells in bacterial cultures during heating as a function

of time. The number of surviving cells at various times of heating were determined by plating samples of the heated cells and counting the colonies which grew. These kinetic measurements resulted in the finding that the kinetics of heat-killing was exponential, typical of first-order chemical kinetics. The functional relationship is found to be

$$N/N_o = \exp[-kt] \qquad (1)$$

where N is the number of survivors, $N_o$ is the number of cells in the initial population, and k is the rate constant. This relationship has been a central theme in most work concerned with heat-killing of micro-organisms. It has been interpreted, misinterpreted, and questioned as to its general applicability, but it has always been considered informative as to the molecular mechanism of heat-killing. This relationship is particularly important for the experiments dealt with in this thesis, since the survivor curve has been found to be exponential for Escherichia coli (16).

Most investigators feel intuitively that the first-order rate of heat-killing is explained by events at the molecular level. Alone, first-order kinetics for a process does not imply that the process is the result of molecular events. For example, a population of ants on a well-traveled sidewalk will give an exponential survival curve, yet this killing can hardly be described as due to molecular events. A more important relation which implies the molecular nature of a process is the temperature dependence of the rate constant k. The temperature dependence of the rate constant for the survival of micro-organisms often follows quite closely an Arrhenius equation (28), which means there is an activation energy in the

process, and the events described by the rate constant are molecular. Thus, when applied to the case of the ants, this explanation is consistent with the conclusion that the death in ants is not a molecular event, since it does not show such temperature dependence.

The molecular events which give rise to the logarithmic survival curve are better understood by deriving the relationship from probability theory. The derivation of the exponential survivor curve demands three conditions. First, the process being described is a two-state process, going from state A to state B. In the case of heat-killing, the organism goes from living to dead. Secondly, the process is not reversible. Thirdly, the probability of a cell being killed in a unit of time dt is kdt, where k is the rate constant of the logarithmic survival curve. When these three conditions are met, the derived equation is identical to Equation 1 (see Appendix A). The meaning of the exponential survival curve is contained in the conditions set down which allowed its derivation.

The most straight-forward, and perhaps the only interpretation of these conditions is that a single molecule in each cell makes the transition from state A to state B, which results in the death of the cell. This deceptively simple interpretation makes very good mathematical sense, but when applied to the complexities of the bacterial cell, it is difficult to imagine how this could be true. A number of authors have argued against such interpretation (14), and some have made attempts at other interpretations (19). Biologically, it is easier to think in terms of many molecules denaturing or for many steps to be included in the process. Such possibilities, when examined carefully, are found to be inadmissible in the framework

of exponential killing. First, any process which is made up of several steps, each having a rate constant equal (or close to equal) to the others, requires a shoulder (an initial time when no death is observed) in the exponential survivor curve when plotted as the logarithm of survivors vs. time. This shoulder would occur if the steps are sequential or if there are a large number of molecules, each of which has to be destroyed before death can occur. The shoulder would appear, because the time taken to accomplish the steps prior to the last step is a period when the organisms are still alive. Therefore, a lag time would be necessary for the first steps to be accomplished before death would be due to the last event. This thinking is incorporated formally in the derivation of Equation 1 when we assume that the process of going from state A to state B has a constant probability, regardless of the organism's previous history of heating. If the death process in the organism had proceeded through several steps, then at any time t, some of these steps would have been accomplished, and the organism would have an increased probability of death in the next instant of time. This would not fit the theory. This line of reasoning does not imply that we have ruled out the possibility of many events. There can be any number of events, as long as the rate constants of these events are much greater than the rate constant for one of the events. Thus, the slowest event (the event with the smallest rate constant) becomes the rate-limiting step. Since all the steps before and after this step are rapid, the organism can be considered to live or die based on the rate-limiting step.

Another possible molecular mechanism which would give an

exponential survivor curve is that there are several molecular sites, each of which can be lethal if it is destroyed. If there are n potentially lethal molecular sites in a cell any one of which can cause death when it is destroyed by heating, the relation for the fraction of surviving cells at any time during heating becomes

$$N/N_o = \exp[-nkt] \qquad (2)$$

where N is the number of survivors, $N_o$ is the initial number of cells, and k is the rate constant. This equation gives an exponential survivor curve, but the slope of the curve is nk instead of k (29).

The very significant finding that the order of death for many organisms is exponential has not been without exceptions. Numerous investigators have found, in addition to exponential rates of death, rates which deviate somewhat from a strict exponential decay. Figure 1 shows some of the various kinds of curves obtained from studies of heat-killing. Extensive discussions of the curves have appeared in the literature (26,28,35,38,44).

Curve B of Figure 1 is the typical exponential decline in population which we have discussed. Curve C of Figure 1 shows a population of cells which apparently increases their heat resistance with time. This type of curve is usually explained by either assuming that there is a heterogeneous population of cells (10,57), or that products leaked from the cells during heating have a protective effect on the cells remaining in the later stages of heating.

Curve A shows a population which shows a shoulder before the exponential decline in survivors. In terms of the probability of death with time, this increasing sensitivity to heat can be explained in two ways. First, any sequential reaction which includes the

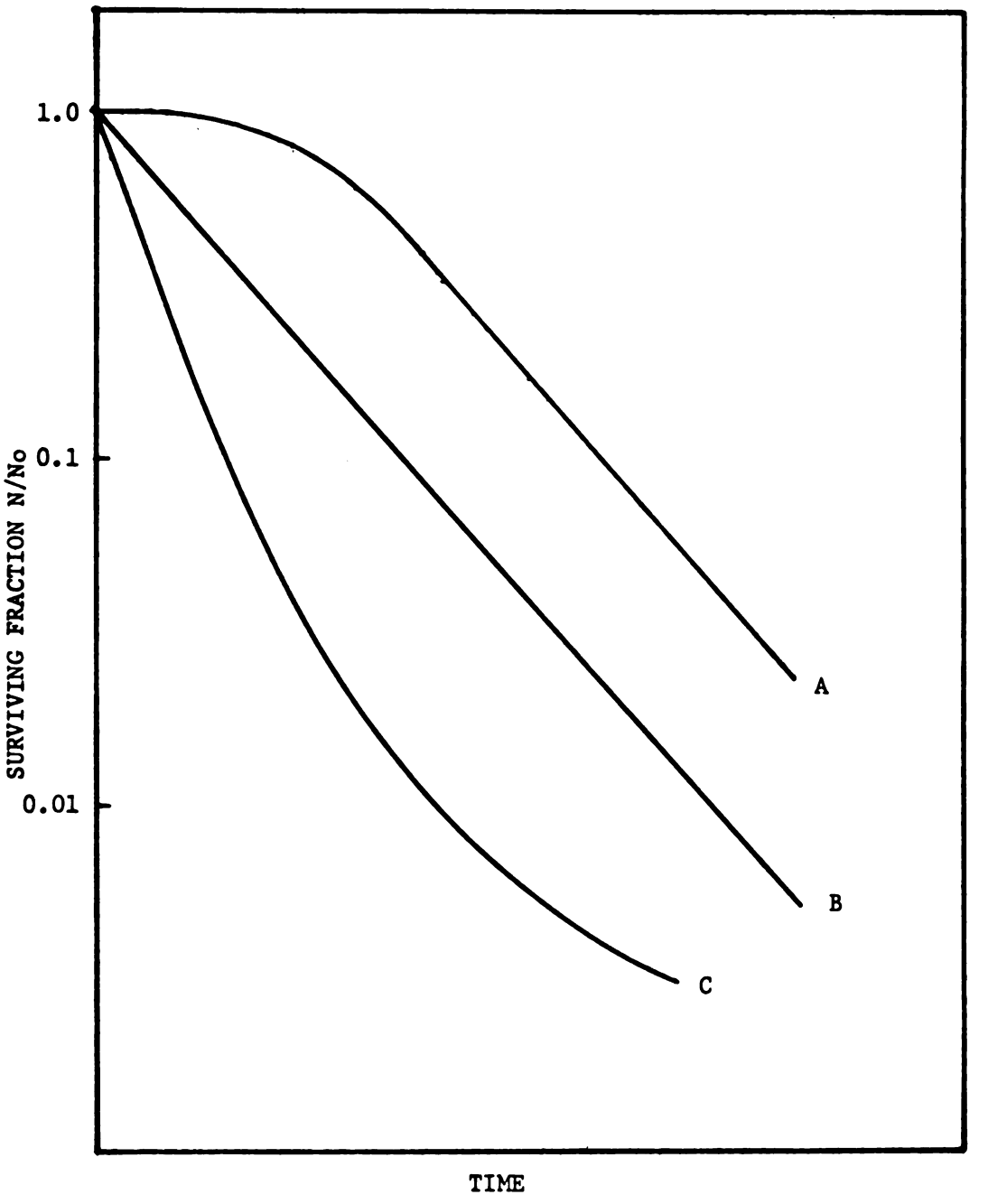



Figure 1. Types of survivor curves observed during heating. N is the number of cells surviving at time t and $N_o$ is the initial number of cells.

lethal damage can exhibit a shoulder if two or more steps have rate constants smaller than the other rate constants yet very close to each other in magnitude. Alternatively, there are two or more identical steps that must be accomplished (not necessarily in sequence) in order for the organism to be killed. The familiar equation from target theory for this process is

$$N/N_o = 1 - [1 - \exp(-kt)]^n \qquad (3)$$

where $N_o$ is the number of cells in the original population, N is the number of survivors at time t, k is the rate constant for the event, and n is the number of events which must be accomplished.

The variations in kinetics as illustrated in Figure 1 has given rise to a great deal of debate about the validity of the exponential order of death. This debate has not been very profitable for several reasons. First, the experimental methods employed may give spurious results as to the shape of the curve (44). Secondly, curves such as curves A and C are usually not very exaggerated. Curve A usually does not possess much of a shoulder, and when calculations are made to find the number of events predicted by this curve, it is found that the number is quite small. Debate over whether the true state of affairs is 1 or 5 events has not been helpful in alerting the researcher as to what the lethal molecular damage might be. The only real question we might have is whether the organisms are somehow mimicking single-event kinetics with events of 1000 or more. The tail of curve C is usually found when the number of survivors is low. If it is true that the tail of curve C is due to a heterogeneous population, this is more a question of the experimental design rather than an objection to the exponential order of death.

## Molecular Sites of Damage

The observation that the kinetics of heat-killing in bacteria is exponential or nearly exponential, gives the deceptive prospect that the molecular mechanism for heat-killing is simple, and therefore, should be easy to detect. Indeed, it may be that the molecular mechanism of heat-killing is simple, but it is not at all easy to detect. At present, there is no adequate explanation of the molecular mechanism of heat-killing.

A number of studies have been done on thermal inactivation of proteins and enzymes *in vitro* (30). More important to the problem of heat-killing, but less well studied, is the inactivation of proteins *in vivo*. A number of enzymes involved with glycolysis and the tricarboxylic acid cycle have been shown to be inactivated *in vivo* in *Salmonella typhimurium* and *Staphylococcus aureus* (8,53) by heating at lethal temperatures. An increase in extinction coefficient has been found at 500 nm in cultures of *E. coli* during heating (4,22). It was proposed that the increase in extinction coefficient may be due to changes in cytoplasmic proteins. Patterson and Gillespie (41) have shown that DNA-dependent RNA polymerase is slightly inactivated when *E. coli* are heated to 44°C, a temperature at which they still grow. Presumably, RNA polymerase is inactivated to a greater degree at higher lethal temperatures.

Some investigators have concluded that protein inactivation causes heat-killing in micro-organisms (52). The best evidence for this conclusion comes from thermodynamics. Absolute rate theory (29) describes the rate constant for first order kinetics by

the equation

$$k_D = \kappa \frac{k_B T}{h} \exp[\Delta S^{\ddagger}/R] \; X \; \exp[-\Delta H^{\ddagger}/RT] \qquad (4)$$

where $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ are the activation entropy and activation enthalpy respectively. It has been found that the activation entropy and enthalpy for various proteins follows a simple compensation law (46).

$$\Delta S^{\ddagger} = a \, \Delta H^{\ddagger} + b \qquad (5)$$

where a and b are constants. In addition, similar treatment of the data for various micro-organisms yields a compensation law relation with the same a and b constants as those for protein (46). The correlation of the a and b constants for both thermal death in micro-organisms and protein inactivation is quantitative evidence for protein denaturation as the rate-limiting step in thermal death in micro-organisms. Considering the extreme redundancy of most enzyme systems, it is difficult to justify protein inactivation as the cause of thermal death which gives exponential survivor curves (45).

The contradiction between the demands of the exponential survival curve and the redundancy found in most enzyme systems have lead researchers to interesting and novel explanations for how a single event of protein inactivation can cause the death of the cell. For example, if a repressor protein repressing a potentially lethal gene were denatured and allowed the gene to be expressed, this would kill the cell (Barnett Rosenberg, personal communication). Such models are quite hypothetical and very hard to demonstrate experimentally. It is safe to conclude that enzyme systems are inactivated at killing temperatures; what is not known is to what extent enzyme inactivation

may qualify as the rate-limiting lethal damage.

There is considerable evidence that heating causes damage to the cytoplasmic membrane (14). It has been found that there is significant leakage of substances from the intracellular pool of Staphylococcus aureus. These substances were found to be made up primarily of RNA-like material absorbing at 260 nm and having a positive orcinal test (25). In addition, amino acids have been found to leak from the intracellular pools (3,25). Russell and Harries showed a similar leakage of 260 nm absorbing material from heated suspensions of E. coli (49,50). In both Staph. aureus and E. coli, very little protein was found to be released (2,50). In addition, protein was found not to be degraded upon heating Staph. aureus (3). Tests have not been made for degradation of protein in E. coli.

Total microscopic cell counts of suspensions of E. coli before and after heating have been shown to remain constant (49), indicating that leakage was not due to lysis. Heated suspensions of E. coli spheroplast also showed no evidence of lysis (50). Therefore, it is evident that the damage to the membrane is not due to its rupture. This suggests that the leakage occurs uniformly in all heated cells, not only in cells that are being killed. Furthermore, correlations between the amount of leakage and the number of cells being killed cannot be made, and it may be that the reason for heat-killing is not leakage of essential intermediates (3).

Degradation of ribosomes and rRNA has been found in several different organisms. In suspensions of Staph. aureus which were heated at sub-lethal temperatures, degradation occurred primarily in the 30S ribosomal subunit and the associated 16S RNA (48). The

degradation of the 30S ribosomal subunit under these conditions varies from 85% to 100% (47). Similar degradation of ribosomes and rRNA has been shown to occur in Salmonella typhimurium (54). In Bacillus subtilis, both the 16S RNA and 23S RNA have been found to be degraded (37). The mechanism of this degradation is not known (48); however, evidence indicates that degradation of the 16S rRNA may be the result of a ribonuclease (47). It has also been shown that degraded rRNA can be lost through the cytoplasmic membrane during heating, and these materials must be resynthesized for survival (25).

Bridges, Smith, and Munson (12) have noted in E. coli strains a close correlation between the sensitivity of cells to ionizing radiation and their sensitivity to heating. These authors suggest that thermal damage and damage due to ionizing radiation act similarly in their lethal effect on micro-organisms. Woodcock and Grigg (58) have found that single- and double-strand breaks occur in the DNA of E. coli that have been heated at lethal temperatures. The damage appears to be restricted to single- and double-strand breaks, since no degradation of the DNA takes place (3,43,58). Furthermore, death due to heating was not dependent upon the strand breaks as much as it was on the repair of these breaks (58).

It is apparent that a number of molecular sites suffer damage at temperatures at or near the killing temperatures. Heat affects every site in the cell (14). Therefore, it is not surprising that a number of molecular sites show damage. Furthermore, not all molecular damage is lethal. Cells that survive heating have no lethal damage, yet they do reflect the fact that there is a great deal of

non-lethal damage due to heating. Cells that survive heating show a significant lag time before they reinitiate growth (27,32,37,55). This lag can only be interpreted as the time necessary for the cells to repair extensive non-lethal damage which has occurred due to heating.

All experiments to date can only show that some particular damage has been doen to heated populations of cells. They have not been able to show that the observed damage is the critical or lethal damage. This failure to single out the lethal damage is explicit or implicit in the way the experiments were done. In these experiments, damage due to heating was observed as a deviation from the behavior of unheated cells. This is a poor comparison. Since there is widespread damage in the cell, the investigator, after observing a particular damage, must rely on ad hoc explanations as to the critical nature of the damage in order to justify killing. Such explanations are hazardous and require great caution.

## EXPERIMENTAL THEORY

The biggest single difficulty in detecting the lethal damage which causes heat-killing is separating the lethal damage done to the cell from all the other non-lethal damage which also occurs. Comparisons based on differences in damage done to heated populations and unheated populations cannot conclusively show if the damage observed is lethal. The only valid comparison that can be made which has a chance of singling out the lethal damage is that of comparing heat-killed cells with survivors of heating. If a comparison of heat-killed cells and survivors can be made, the only difference between them should be the lethal damage which occurred in the heat-killed cells and did not occur in the survivors. Furthermore, the exponential survival curve demands that the lethal damage occurs in a single rate-limiting step. The lethal damage does not accumulate, so survivors should have no record of the lethal damage. All non-lethal damage should appear equally in both survivors and heat-killed cells immediately after heating.

A comparison between heat-killed and surviving cells requires a separation of the two populations so that measurements can be done on each set of cells and then compared. In the experiments that follow, this separation has been accomplished. Before separating heat-killed from surviving bacteria for comparative purposes, we must decide what molecular events are to be compared. Of course it

is not possible to directly isolate and compare the lethal damage, since it is not known what it is or where it is to be found. It is possible to compare specific functions in heat-killed cells to those in the survivors. Furthermore, the lethal damage appearing in the heat-killed cells must express itself as a loss of some function. This same function in the survivors should not be lost at all.

Some caution is necessary before differential loss of function between heat-killed and surviving cells can be interpreted as a direct result of lethal damage. It is obvious that the lethal damage in the heat-killed cells must eventually be expressed broadly in the loss of all functions. Nevertheless, those functions whose loss are expressed first are the functions that are most directly coupled to the lethal damage.

Thus, in determining the correlation between the function observed and lethal damage, we have two criteria. First, the function must be lost in the heat-killed cells and not lost in the survivors. Secondly, the loss of function must be expressed immediately after heating as a difference between heat-killed and surviving cells.

Another question which comes to mind in connection with functional loss in heat-killed cells is whether or not any metabolic activities can be expected to be found in heat-killed cells. It could be said that if a cell is killed, then it should not function at all. It must be remembered that the definition of death that is used in all experiments of this kind is the inability of a cell to produce a colony upon plating. This definition in no way implies that all functions are lost immediately after heating, even though the metabolic action will stop eventually. The immediate functional

loss in heat-killed cells should be coupled to the lethal damage. Indeed, there are cases in which a cell will not produce a colony when plated, but continues to function in a number of way after receiving lethal damage (6,33).

In the experiments to be described, the functions of protein synthesis and RNA synthesis will be measured and compared between heat-killed and surviving cells. It is quite possible that the effects of heating on two such broad and important activities as protein and RNA synthesis will provide some information about the lethal damage which occurs in heat-killed cells.

# MATERIALS AND METHODS

## Bacterial Strain

Escherichia coli K-12, CR63 was obtained from Dr. Loren Snyder and was used throughout the following experiments. This organism is prototrophic, contains a sup D60 mutation, and is lambda sensitive (7).

## Growth of Cells and Plate Counting

Cells were kept on slants of nutrient agar (Difco), 23 g per liter of water, supplemented with (per liter of water): yeast extract (Difco), 2 g; glucose, 1 g; and NaCl, 1 g. These slants were stored at 5°C until used. Cultures were started from these slants by inoculating a starter broth composed of (per liter of water): nutrient broth (Difco), 6 g; yeast extract (Difco), 2 g; glucose, 1 g; and NaCl, 1 g. This culture was grown for about 3 hours, with aeration, and then transferred by diluting (about 1/20) into minimal media, and grown for an additional 6 hours. Minimal media used for growth had the following composition (per liter of water): $Na_2HPO_4$, 6 g; $KH_2PO_4$. 3 g; $MgSO_4 \cdot 7H_2$), 0.15 g; NaCl, 1 g; $CaCl_2$, 0.01 g; $NH_4Cl$, 2 g; and glucose, 3 g. Glass double-distilled water was used in all preparations.

This culture was again transferred to fresh minimal media and grown overnight (10 hours), yielding a very turbid culture. The

final step in growth was the transfer of this overnight culture by dilution (about 1/100) into fresh minimal media. The cells were then incubated at 37°C until they reached an optical density of 0.15 at 560 nm. The time necessary to reach this optical density represented about four doubling times, so that all cells were in logarithmic phase before experiments were carried out. The concentration of cells at the optical density of 0.15 was about $1.5 \times 10^8$ cells per ml. Estimates of growth were made in each experiment by measuring the time required for the culture to double in optical density.

Dilutions for plate counts were done in buffer which was of the same composition as the buffer used in heating. Plate counts were done by the pour plate method, using tubes of liquid agar medium at 41°C. The composition of these materials is given below.

## Assay Buffers and Media

Heating buffer and dilution buffer was made up of (per liter of water): $Na_2HPO_4$, 6 g; $KH_2PO_4$, 3 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; and NaCl, 1 g. Recovery media was made by adding a concentrated 5X solution of amino acids, glucose, $NH_4Cl$, and $CaCl_2$ (recovery concentrate) to the heating buffer. The final composition of the recovery media was (per liter of water): $Na_2HPO_4$, 6 g; $KH_2PO_4$, 3 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; NaCl, 1 g; $CaCl_2$, 0.01 g; $NH_4Cl$, 2 g; and glucose, 3 g. In addition, the recovery media had the following concentrations of amino acids: alanine, glycine, lysine, serine, valine, and glutamic acid--20 ug per ml; threonine, proline, isoleucine, araginine, glutamine, methionine, asparagine, and phenylalanine--10 ug per ml; tyrosine and leucine--2 ug per ml; aspartic acid--16 ug per ml; histadine--5 ug per

ml; tryptophan--1 ug per ml, and cysteine--0.4 ug per ml.

Lysis media was made by adding a concentrated solution (lysis concentrate) of beef extract, yeast extract, peptone (Difco), $MgSO_4 \cdot 7H_2O$, NaCl, and sucrose to the recovery media containing the cells. The final concentration of the added materials (per liter of water) was: beef extract, 0.5 g; yeast extract, 0.5 g; peptone, 1.5 g; NaCl, 2 g; and $MgSO_4 \cdot 7H_2O$, 10 mM. Sucrose was present at either 8% or 5%, depending on the experiment. The composition of the recovery media, except for NaCl, was diluted by 2/3 when the lysis media was made. Potassium penicillin G (E. R. Squibb and Sons) was added to the lysis media at a final concentration of 1111 units per ml or 0.66 mg per ml when lysis was required.

Plating agar was made with minimal media supplemented with the 20 amino acids at the same concentrations found in the recovery media, and 6.5 g per liter of water of Bacto agar (Difco). Except for the agar, the plating agar was identical in every respect to the recovery media. Resuspension buffer, which was used to resuspend the first pellet after lysis, was 50 mM $KH_2PO_4$ and made to pH 6.6 using KOH.

## Heating

Heating was carried out in two steps. The first step was to preheat the cells at 44.7°C for 5 minutes. This was a temperature at which no killing took place, and it served to bring the cell suspension up to a temperature which would prevent heat shock. Heat shock may have taken place if cells at room temperature were suddenly subjected to killing temperatures. This step also reduced the time

necessary for the suspension to come up to the killing temperature when the cells were added to the pre-warmed buffer at killing temperature. The time for the cell suspension to reach the set killing temperature was approximately 45 seconds. Cells were heated at killing temperatures of 49.9°C to 51°C in water baths with bath control model 33 (Fisher) with an accuracy of 0.05°C and a variation of ± 0.01°C. Cell suspensions were aerated during heating.

## Centrifugation

All centrifugation steps were carried out in a model HN-S centrifuge with swing buckets (International Equipment Company) at a range of temperatures from room temperature to approximately 35°C. The increase in temperature was due to moderate heating of the centrifuge at high speeds and long times. Room temperature was used to prevent killing due to low temperature shock (25). Graduated conical centrifuge tubes (15 ml) were used in all centrifugation steps. These tubes had the top lips cut off so that they could be fitted with Bacti-capall covers. These covers prevented dust from entering the tubes.

## Radioactive Labeling and Counting Techniques

Cells were labeled with [$^{14}$C]leucine, uniformly labeled with a specific activity of 325 mCi per m mol in the protein synthesis experiments. In the RNA synthesis experiments, [6-$^{3}$H]uracil at a specific activity of 26.2 Ci per m mol was used. Both radiochemicals were obtained from New England Nuclear. In most experiments, except where otherwise noted, carrier levels for non-radioactive

leucine of 2 ug per ml, or for non-radioactive uracil of 0.1 or 0.3 ug per ml were used.

Two variations of liquid scintillation counting techniques were employed. Most counts were taken from samples collected on membrane filters. Liquid scintillation fluid for counting these filters was 2,5-diphenyloxazole, 6 g; and 1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]-benzene, 0.25 g in 1 liter of toluene. In experiments where only samples collected on filters were counted, the results are given in terms of cpm. Some experiments required counting of liquid samples containing water in addition to counting samples collected on filters. In these cases, the sample was mixed with PCS$^{tm}$ cocktail (Amersham/Searle Corporation) so that the mixture had a water content of 12% or lower. In the experiments where samples containing water were compared with samples collected on filters, results are given in dpm, so that comparisons can be made between the samples. Disintegrations per minute were calculated on selected samples by the internal standardization method using [$^{14}$C]toluene which had an activity of $3.000 \times 10^5$ dpm. This carbon-14 standard was obtained from New England Nuclear. It was necessary to standardize only in the case of experiments utilizing [$^{14}$C]leucine.

All samples were counted in a Packard Tri-carb Liquid Scintillation spectrometer Model 3320. For carbon-14 samples, the gain was set at 8%, and the discriminators were set at 50 and 1000 respectively. For the tritium samples, the gain was set at 70%, and the discriminators were set at 50 and 1000. All samples were counted in glass vials with foil-lined tops.

## Separation Assay

The experimental procedure used throughout these experiments was designed to separate heat-killed from surviving organisms. In addition, radioactive labels were added immediately after heating to measure and compare the biosynthetic processes of heat-killed and surviving cells. The procedure, as outlined below, was modified in various experiments to provide control experiments or to obtain specific information pertinent to the investigation. The methodology for the penicillin and lysis steps is a modification of the Kaback (31) method for isolating membranes. Figure 2 shows a flow chart of the "Separation Assay."

Step 1. During the final growth before heating, an estimate of the growth rate was made by measuring the optical density of the culture using a Beckman model B spectrophotometer. When an optical density of 0.15 was reached, 6.3 ml of the culture was removed from the bubbler tube and centrifuged at 3400 rpm (1400 x g-1900 x g) for 15 minutes. After centrifugation, the supernatant fluid was removed, leaving only the pelleted cells. These cells were resuspended in 4.3 ml of heating buffer, transferred to a heating tube, and preheated as described previously.

Step 2. Four ml of the preheated culture was poured into 14 ml of heating buffer which had been preheated to the killing temperature. The cells were mixed, and a 1 ml sample was withdrawn for plate counting immediately. Heating was continued with aeration for 15 minutes, at which time the culture was quickly cooled and a 1 ml sample was again taken for plate counting so that survivors could

be counted.

Step 3. Immediately after cooling, a 1 ml sample was taken for plate counting, 4 ml of recovery charge was added to the culture, and [$^{14}$C]leucine was added so that the final concentration was 0.417 uCi per ml. For RNA synthesis studies, the concentration of [$^{3}$H]uracil was 1.25 uCi per ml. Exceptions to these amounts are noted.

Step 4. As soon as possible, 2 ml aliquots of the cell suspension were dispensed into each of nine test tubes (25 mm diameter) and incubated at 37$^{o}$C. At 10 minute intervals for 90 minutes, cold leucine was added to one of the 9 tubes so that the final concentration of cold leucine was 714 ug per ml. This addition of leucine severely reduced the incorporation of the labeled leucine by the cells. All the tubes were incubated at 37$^{o}$C for a total of 2.5 hours.

Step 5. At the end of the recovery period, 1 ml of the lysis concentrate and 1111 units of penicillin G were added to each sample. Incubation was continued for 1.5 hours. In some experiments, Triton X-100 was added at 0.008% to 0.01% 1 hour after the addition of the lysis concentrate. After the addition of Triton X-100, incubation was continued for 30 minutes. This step lyses or produces spheroplast in the surviving population.

Step 6. Cells from the incubation tubes were poured into centrifuge tubes and centrifuged at 3200 rpm (1200 x g-1600 x g) for 30 minutes. After centrifugation, the supernatant fluid was carefully removed, leaving a 0.3 ml pellet volume. The supernatant fluid was put in numbered tubes on ice.

Step 7. The pellet was partially resuspended in the 0.3 ml

pellet volume. Then 3 ml of resuspension buffer was added and mixed vigorously to resuspend the cells. The resuspended cells were incubated for 15 minutes at 37°C. One-tenth ml of 100 mM ethylenediaminetetraacetic acid (EDTA) was added to give a 3 mM final concentration to each sample. The samples were incubated an additional 15 minutes; then magnesium sulfate was added to give a final concentration of $Mg^{++}$ of about 8 mM.

Step 8. The cell suspension was centrifuged at 2600 rpm (800 x g-1100 x g) for 30 minutes. After centrifugation, the supernatant fluid was removed, leaving a 0.2 ml pellet volume. This supernatant fraction was put on ice with the first supernatant fraction. The pellet was resuspended in 3 ml of resuspension buffer and put on ice.

Step 9. To the combined supernatant fractions, 0.7 ml of cold 50% trichloroacetic acid (TCA) was added, and to the pellet fraction, 0.35 ml. of 50% TCA was added. This addition of TCA gives a final concentration of about 5%. Both the pellet and the supernatant fractions were placed on ice, and the protein or RNA precipitate was allowed to form for 30 minutes.

Step 10. The precipitates or cells were collected on Metricel™ membrane filters, type GA-8 with pore size of 0.2 um (Gelman Instrument Company). The pellet fraction collected was washed with 12 ml of cold 5% TCA, and the supernatant fraction was washed with 24 ml of cold 5% TCA. The filters were dried and counted.

The above "Separation Assay" describes the method of separating dead from living cells. This separation is achieved because cells that recovered and lived were susceptible to penicillin lysis, while

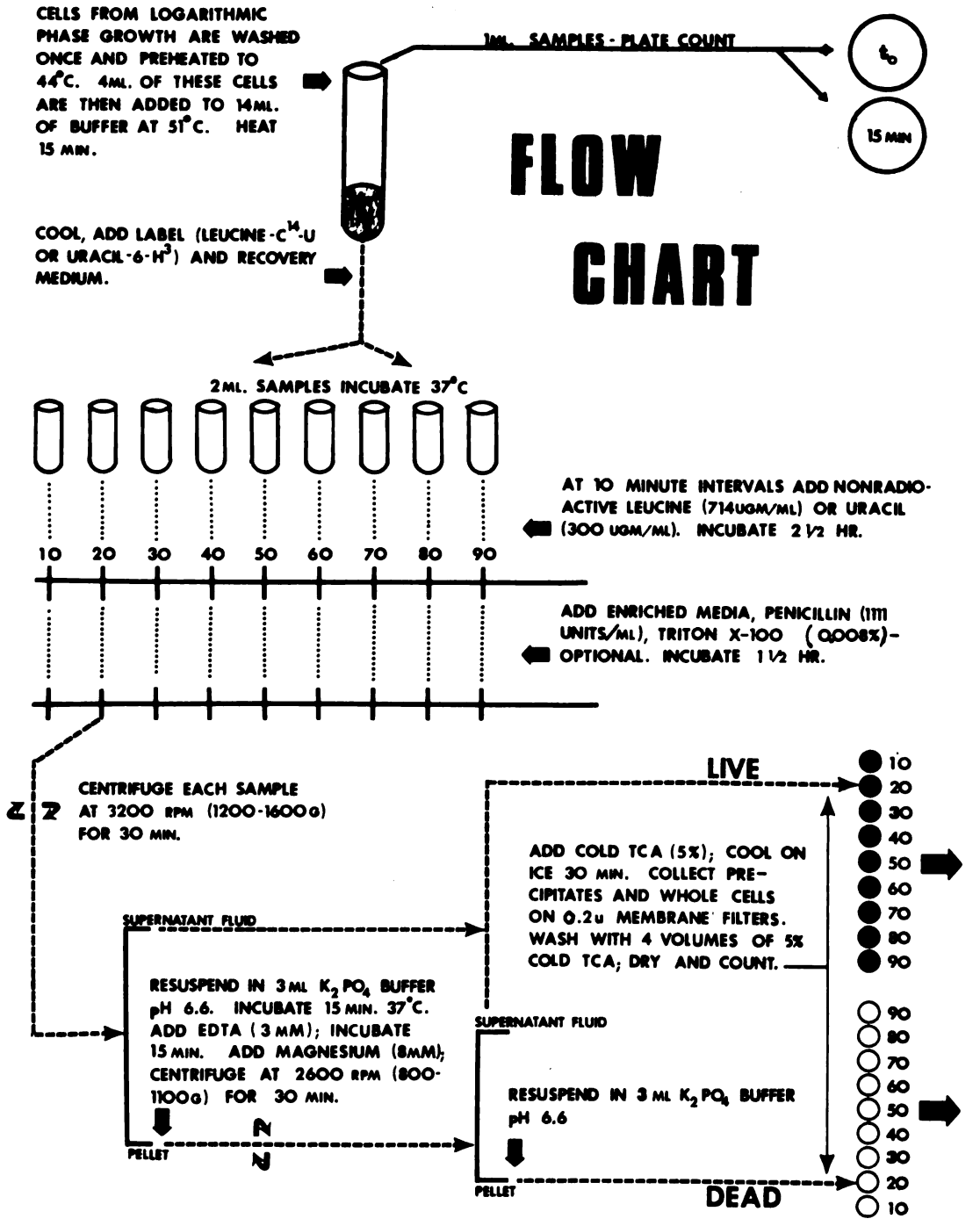



Figure 2. Flow chart of the "Separation Assay."

heat-killed cells could not be lysed by penicillin. During the differential centrifugation steps of this experiment, the debris of surviving cells which lyse should be found in the supernatant, while the heat-killed cells which do not lyse but remain as whole cells would be found in the pellet. Thus, the separation of surviving from heat-killed cells can be viewed as a separation of supernatant fraction from pellet fraction. The terms will be used interchangably throughout this text.

# RESULTS

## Kinetics of Heat-killing and Recovery

Before the method of separating heat-killed from living cells can be employed, it is necessary to inquire about some basic characteristics of the cells regarding their kinetics of death and recovery.

In separating surviving from heat-killed bacteria, it is necessary to be able to generate a mixed population of living and heat-killed cells. The only requirement is that significant numbers appear in both populations so that measurements of biosynthesis can be done. Measurements of the kinetics of death also can give information concerning the number of molecular events involved. The kinetics of heat-killing are shown in Figure 3. Death was approximately exponential, with possibly a slight shoulder at both temperatures used. The 15-minute heating time shows that a significant number of heat-killed cells have been produced, so this time was chosen as the time to be used in further experiments.

Figure 3 does not give an accurate picture of the reproducibility of the death kinetics. At any particular temperature, separate experiments showed rather wide variation in the percent of cells surviving after 15 minutes of heating. The variation ranged from about 35% to 70% survivors. I was unable to eliminate this

Figure 3. Kinetics of heat-killing at 50.0°C (•), and 50.8°C (o). Cells surviving heating (N) as a percentage of the original number ($N_o$) at the end of 15 min of heating were 66% at 50.0°C and 56% at 50.8°C. The bars represent the two standard deviation interval for each measurement.

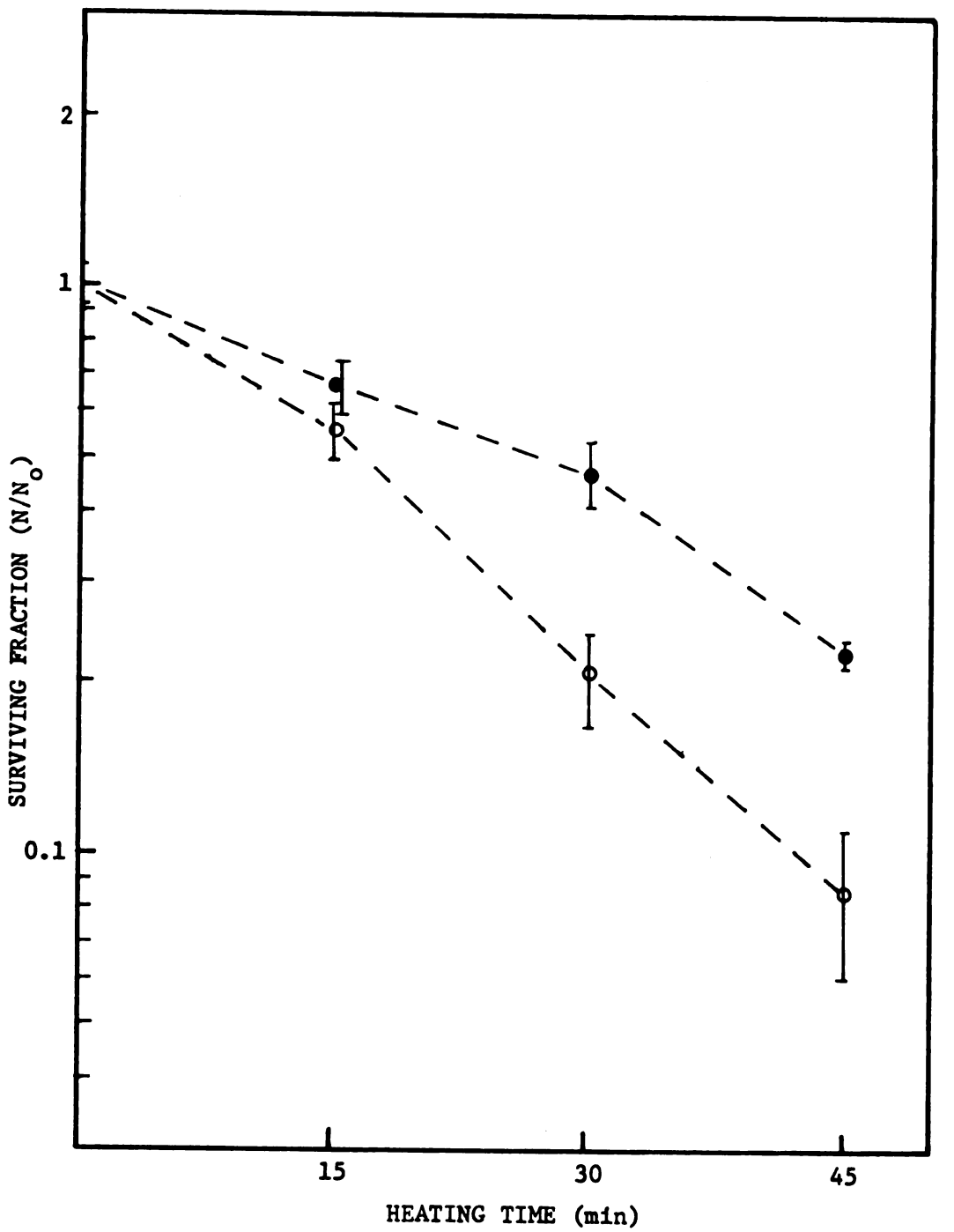



Figure 3.

variation, but in most cases, it was acceptable. This variation would interfere with experiments only if there were too few survivors or too few heat-killed cells to measure their incorporation of radiolabel. Since the percent of cells surviving heating is a better measure of the severity of heating than either temperature or time of heating, the percent of cells surviving heating (percent survivors) will be given in all data.

The kinetics of recovery are shown in Figure 4. Cells were heated, recovery concentrate was added, and the cells were then incubated at 37°C with aeration. The difference in the percent of cells surviving in these two cases illustrates the problem of reproducibility mentioned above. The important quantity that was determined in recovery experiments was the time taken for the cells to recover and grow at the rate at which they would have grown in recovery media had they not been heated. The doubling time of normal cells in the recovery media is about 40 minutes as determined by optical density measurements. In Figure 4, the cells with 83% survival reach this growth rate in approximately 1.5 hours. The cells with 35% survival reach this growth rate at approximately 2.5 hours. The difference in time taken to reach maximum growth rate is apparently related to the percent of cells surviving, which is a measure of the severity of heat-killing. This relation between the percent of cells surviving and recovery time was found to be a common feature in heat-killed cells.

It was found that when a culture of E. coli B is recovering from heating, it is not lysed by ampicillin (a drug similar to

Figure 4. The kinetics of recovery after 15 min of heating was measured by viable cell counts. The temperature of killing in both cases was 49.9°C. This heating resulted in 35% survivors (●), and 83% survivors (o) immediately after heating when recovery was initiated.

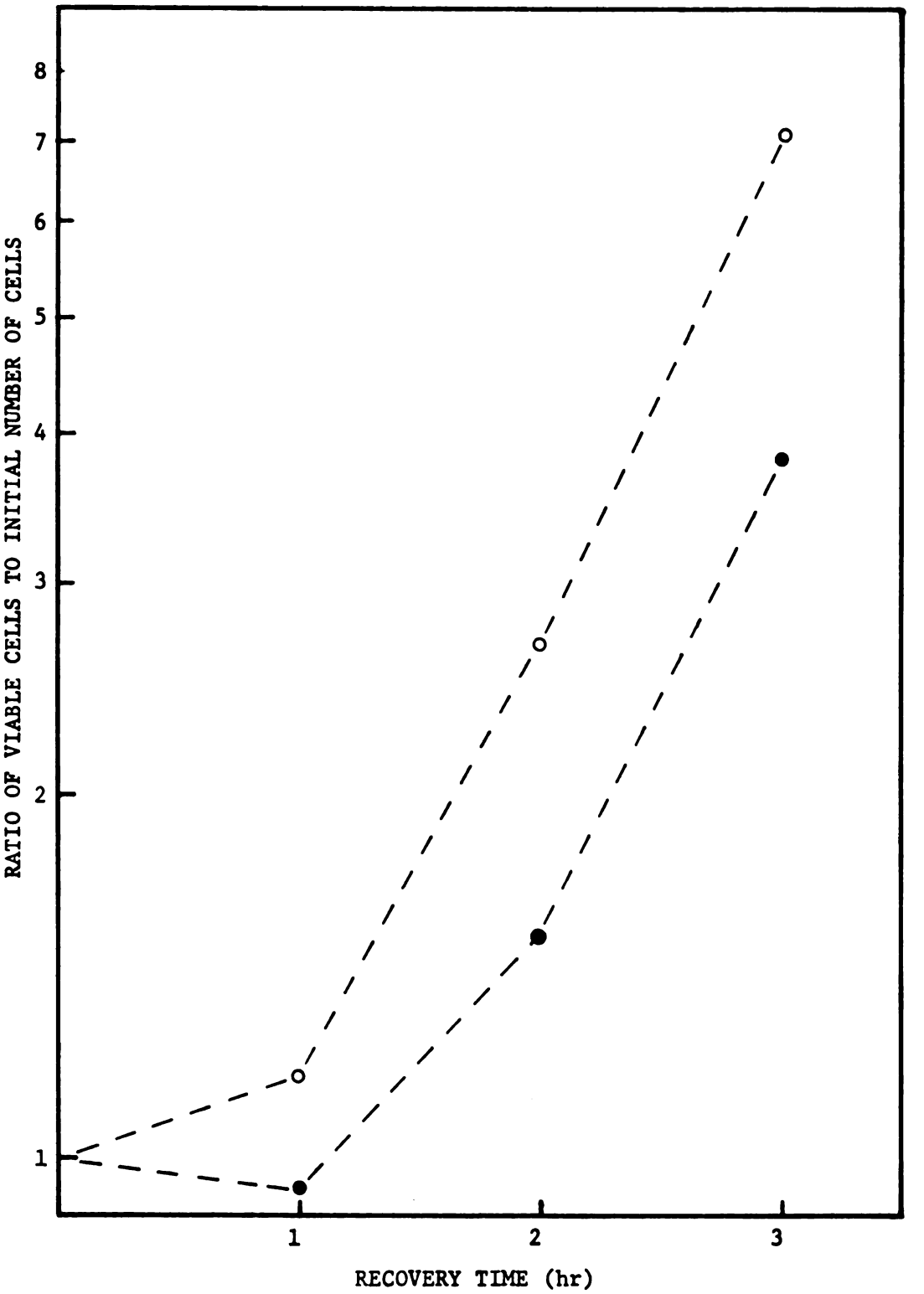

RATIO OF VIABLE CELLS TO INITIAL NUMBER OF CELLS
8
7
6
5
4
3
2
1
1
2
3
RECOVERY TIME (hr)

Figure 4.

penicillin), if the ampicillin is administered soon after heating. This finding of inhibition of penicillin lysis has been found under other conditions (51). Since penicillin and ampicillin have the same mechanism of action on cells, as a precaution against lysis inhibition, cells were allowed to recover until good growth was observed before penicillin was added. The kinetics of recovery suggest that 2.5 hours is a safe time to allow cells to recover so that lysis inhibition may be prevented. This is the time allowed in all experiments involving recovery.

## [$^{14}$C]leucine Contamination of Pellet Fraction

In order to measure biosynthetic processes that might occur in heat-killed cells, it was necessary to show that the separation process produced a population of heat-killed cells which was as free as possible from contamination from surviving cells or their debris. Experimentally, this means that the pellet fraction, containing the heat-killed cells, must be free of contamination. Particularly important is contamination that carries [$^{14}$C]leucine labeled protein into the pellet fraction. To test the "Separation Assay" for contamination of the pellet fraction, the "Separation Assay" described previously was followed with the heat-killing step eliminated. The pellet fraction, in this case, would contain no heat-killed cells, and radioactive label found in the pellet fraction would represent contamination from living cells or the lysate.

Contamination of the pellet fraction with living cells or spheroplasts was probably much less than 1% of the total living population. It was found that only about 1% of the original population of cells

Figure 5. [$^{14}$C]leucine contamination of the pellet fraction. Cells were grown to an OD of 0.075, centrifuged and resuspended in 12 ml of heating buffer with 4 ml of "recovery concentrate" added. [$^{14}$C]leucine was added to a final concentration of 0.417 uCi/ml. Cells were not heated and the "Separation Assay" (Steps 4 through 10) was used to give a supernatant fraction (o), and pellet fraction (•).

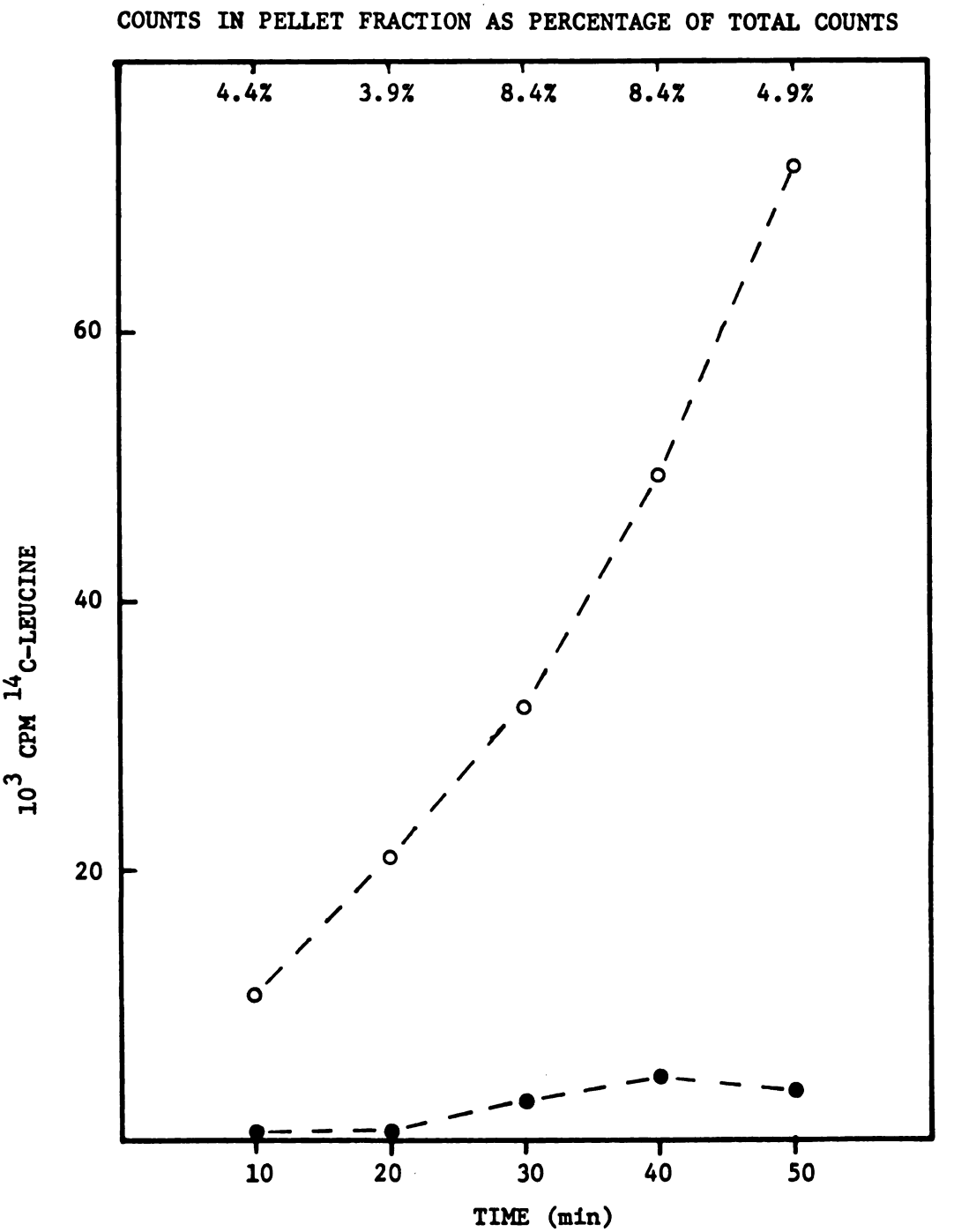

COUNTS IN PELLET FRACTION AS PERCENTAGE OF TOTAL COUNTS
4.4%
3.9%
8.4%
8.4%
4.9%
60
40
20
$10^3$ CPM $^{14}$C-LEUCINE
10
20
30
40
50
TIME (min)

Figure 5.

could form colonies when penicillin was removed, after Step 4 of the "Separation Assay." After resuspension of the first pellet, the number of unlysed cells probably would decrease markedly.

Figure 5 shows the results of this experiment. The lysate from living cells which appeared in the supernatant fluid showed high levels of label that increased as the label was taken up. The pellet fraction, which would normally contain heat-killed cells, but in this case contained only contamination from live cells, contains little label. The contamination of [$^{14}$C]leucine in the pellet fraction as a percentage of the label found in the supernatant fluid with time is given in Figure 4. The average contamination is 6.0% with a standard deviation of 2.0% and a two standard deviation interval of 2.0% to 10.0%. Thus, in an experiment involving heat-killed cells, if the radioactivity found in the pellet fraction was above 10.0%, it would probably be due to incorporation of the label by the heat-killed cells. It was also important that the magnitude of the contamination be low, since high contamination might have obliterated or masked small amounts of synthesis in heat-killed cells.

## Protein Synthesis in Heat-killed and Surviving Cells

Figure 6 shows the incorporation of [$^{14}$C]leucine in surviving and heat-killed cells. The surviving cells showed recovery and a high incorporation of [$^{14}$C]leucine into TCA-precipitable protein. The pellet fraction, or heat-killed cells, showed a very low level of [$^{14}$C]leucine incorporation. The label found in the pellet fraction as a percentage of the label found in the supernatant fraction is, on the average, 6.2% with a standard deviation of 1.3% and a

Figure 6. The measurement of [$^{14}$C]leucine (0.417 uCi/ml) incorporated in surviving cells (o), and heat-killed cells (•) during recovery. The total number of cells before heating was 5.16 x $10^7$. After heating the number of survivors was 2.00 x $10^7$, giving 39% survivors. The procedure followed was that of the "Separation Assay."

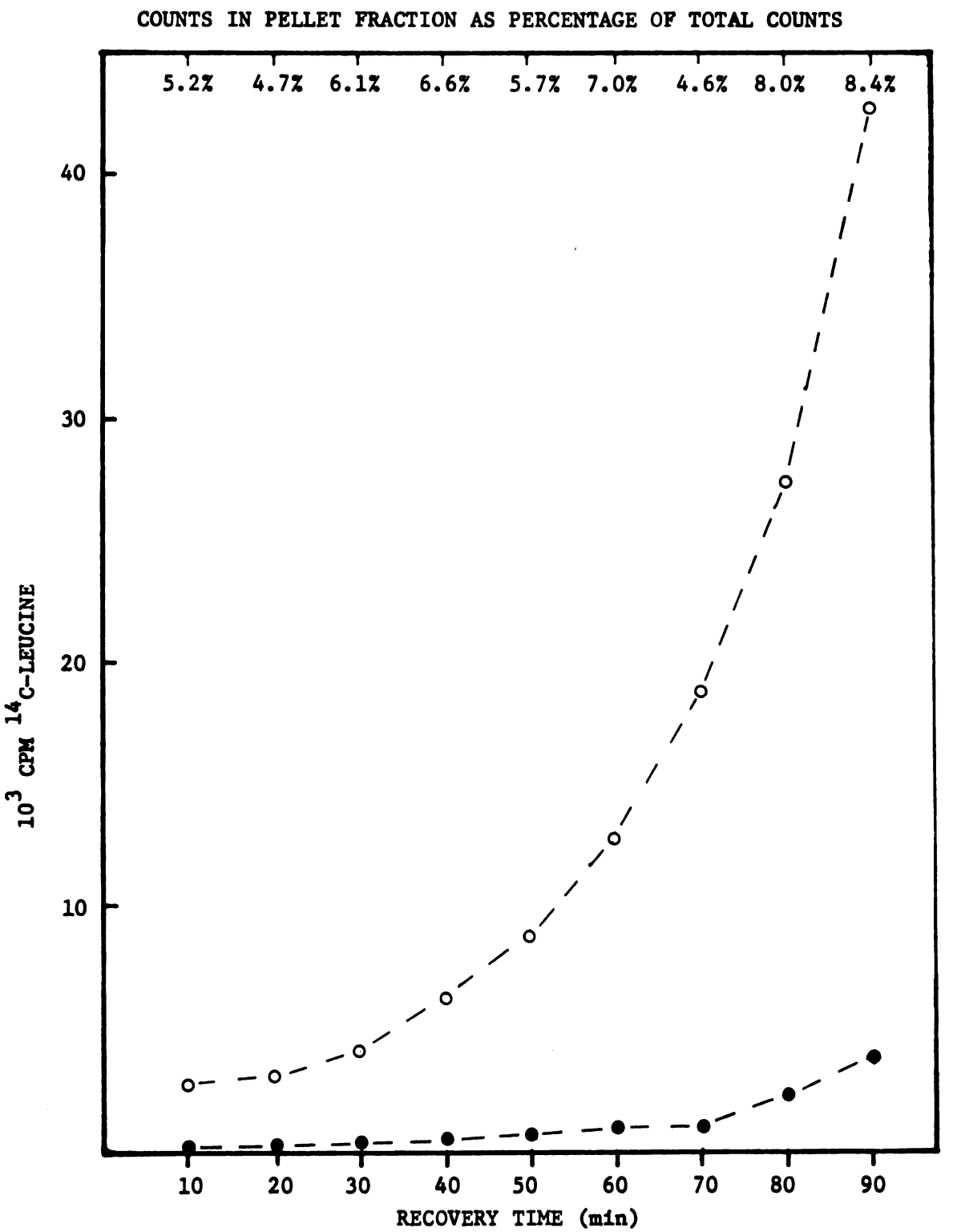

COUNTS IN PELLET FRACTION AS PERCENTAGE OF TOTAL COUNTS
5.2%
4.7%
6.1%
6.6%
5.7%
7.0%
4.6%
8.0%
8.4%
40
30
20
10
$10^3$ CPM $^{14}$C-LEUCINE
10
20
30
40
50
60
70
80
90
RECOVERY TIME (min)

Figure 6.

Figure 7. The rate of [$^{14}$C]leucine incorporation calculated from the 10 min intervals of Figure 6. The rates were calculated by taking the difference in counts for each 10 min interval and plotting these values on the ordinate at the midpoint of the time interval on the abscissa. The dotted line is the extrapolation of the curve.

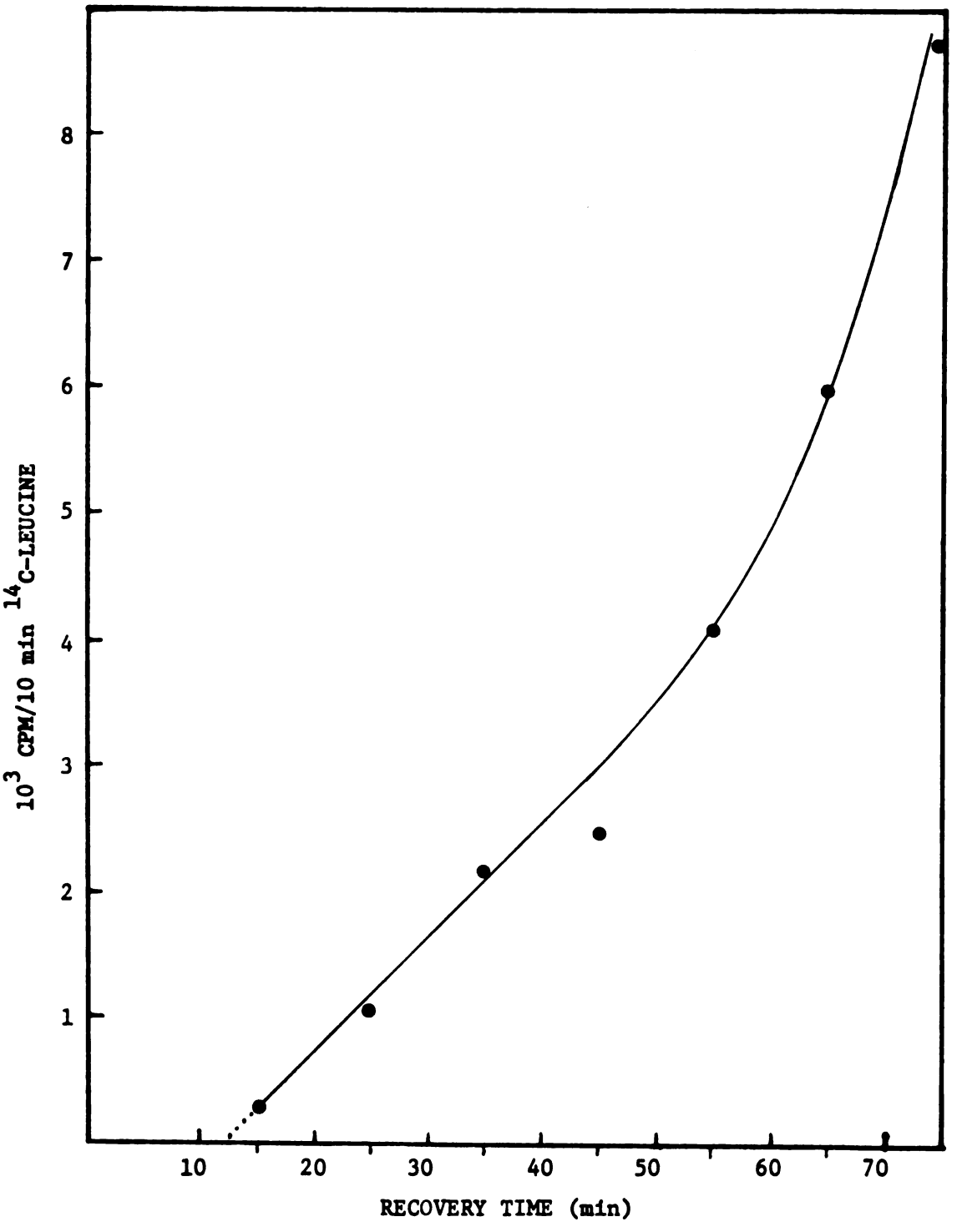

8
7
6
5
4
3
2
1
$10^3$ CPM/10 min $^{14}$C-LEUCINE
10
20
30
40
50
60
70
RECOVERY TIME (min)

Figure 7.

two standard deviation interval of 3.6% to 8.8%. This interval, and all the individual percentages of label found in the pellet fraction (Figure 6), fall within the two standard deviation interval of the experiment described in Figure 5. Thus, this level is thought to be due only to contamination of the pellet fraction. A tentative conclusion is that since the pellet fraction label in this experiment is not above contamination levels, there is no incorporation of the [$^{14}$C]leucine in heat-killed cells.

This experiment has been repeated three times at slightly different temperatures, with widely different percentages of cells surviving, and with slightly smaller pellet volumes. The average percentage of [$^{14}$C]leucine-labeled protein found in the pellet fraction in two of the experiments was 3.8% and 4.2%, lower than in the above experiment. In the third experiment, Triton X-100 was added in Step 5 of the "Separation Assay," and the percentage of [$^{14}$C]leucine-labeled protein found in the pellet fraction was a very low 1.8%.

Figure 7 shows the rates of [$^{14}$C]leucine incorporation as calculated from the 10-minute intervals of Figure 5. The rates of incorporation show a linear plot for the first 50 minutes of incorporation. This period of linear increase of the incorporation rate coincides with the period during which there is no increase in cell numbers. Figure 4 shows that when cells were heated, resulting in 35% survivors, the cell numbers during recovery did not increase for about 1 hour. Thus, in Figures 6 and 7 the first hour of recovery is a time of little or no growth. The increase in cell numbers that

begins at about 1 hour of recovery coincides with the non-linear increase in rate of incorporation shown in Figure 6 beginning at about 1 hour of recovery. Extrapolation of the linear portion of the curve in Figure 7 shows that there was a delay in the incorporation of [$^{14}$C]leucine of about 12 minutes.

## Contents of the Pellet Fraction

The results described above would be conclusive if it could be shown that heat-killed cells appeared in the pellet fraction. Such low levels of label in the pellet fraction could reflect the fact that nothing, not even the heat-killed cells, was in the pellet fraction. Perhaps the heat-killed cells themselves lysed shortly after heating, independent of penicillin treatment.

One fact that prompted the line of research taken in this thesis was the apparent physical stability of heat-killed cells. Microscopic observation of many different strains of *E. coli* after heat-killing revealed that the cells retained their integrity for many hours after heating. This observation was tested on the strain of *E. coli* used in these experiments. Cells were grown, washed, and heated at 49.9°C for 15 minutes. After heating, the cells were cooled, but no recovery concentrate was added to the cells so that survivors were not able to grow. Optical density (OD) measurements were taken immediately after heating, and the cells were incubated at 37°C. Optical density measurements were then taken every hour for 3 hours with no change in OD. There was still no change in OD as long as 8 hours after heating. Phase contrast microscopic counts of the cells using a Petroff-Hauser counting chamber showed no change

in cell numbers for 3 hours after heating. However, cells often appeared to be plasmolysed with some loss of contrast in certain sections of the cell. The loss of contrast in the cells seemed to increase with time, suggesting that the cells might be leaking some cellular constituents. Nevertheless, cells appeared well enough intact so that they could be collected by centrifugation.

In an attempt to further clarify the issue, [$^{14}$C]leucine was incorporated into growing cells before heating, and the amount of [$^{14}$C]leucine recovered in the pellet fraction was measured. Two variations of the "Separation Assay were employed. In the first experiment (Experiment 1 of Table 1), the "Separation Assay" was followed except that no additional [$^{14}$C]leucine was added in Step 3. The second experiment was essentially the same as the first, but Triton X-100 was added to the lysis media as described in the "Separation Assay."

If heat-killed cells remained intact and were separated from surviving cells, the pellet fraction should contain [$^{14}$C]leucine-incorporated label proportional to the number of heat-killed cells in the original cell suspension. The supernatant fraction should contain radioactive label proportional to the number of survivors. This simple relationship is complicated by cross contamination so that the final calculation takes the rather complex form

$$\frac{X}{N_k} = \frac{C_s(N_k + Y) - C_k(N_s - Y)}{(C_k + C_s)N_k} = \text{Percent of total heat-killed cells lost as whole cells or [}^{14}\text{C]leucine} \qquad (6)$$

where X is the heat-killed cells or leucine label lost to the supernatant fluid, Y is the surviving cells or their debris (contamination)

Table 1. Loss of [$^{14}$C]leucine from the heat-killed or pellet fraction during the "Separation Assay."[a]

| expt | supernatant fraction cpm | pellet fraction cpm | plate count survivors | heat-killed[c] | percent[d] [$^{14}$C]leucine lost ±SD (n)[e] |
|---|---|---|---|---|---|
| 1 | 84,812 | 24,600 | $3.16 \times 10^7$ | $2.22 \times 10^7$ | 54.8% ± 2.9% (3) |
| 2[b] | 46,652 | 41,830 | $1.52 \times 10^7$ | $3.67 \times 10^7$ | 34.1% ± 2.7% (4) |

a. Cells were grown and labeled with approximately 0.26 uCi/ml of [$^{14}$C]leucine for 1 hr. Cells were then centrifuged and resuspended in buffer and heated at 51°C for 15 min. The "Separation Assay" (except no additional label was added in Step 3) was employed to separate surviving and heat-killed cells.

b. Triton X-100 was added to a final concentration of 0.01% as described in the "Separation Assay" (Step 5).

c. Heat-killed cell counts were determined by subtracting the number of survivors from the number of cells counted by plating at the beginning of heating.

d. The percent of [$^{14}$C]leucine lost from the pellet fraction was calculated using Equation 6.

e. (n) is the number of data used to compute the standard deviation.

found in the pellet, $N_k$ is the number of cells which are heat-killed (by plate count), $N_s$ is the number of cells surviving (by plate count), $C_s$ is the number of counts found in the surviving fraction or supernatant fluid, and $C_k$ is the number of counts found in the heat-killed fraction or the pellet.

This equation was used to analyze the data from both experiments, and the results are given in Table 1. For the experiment done without Triton X-100 (Experiment 1 of Table 1), it was found that roughly 45% of the incorporated [$^{14}$C]leucine that should have been recovered in the pellet fraction was actually recovered. This means that either 45% of the heat-killed cells were recovered in the pellet, or, in the cells recovered, 45% of the incorporated [$^{14}$C]leucine was retained. This amount of recovery in the pellet fraction should be sufficient to measure the incorporation of [$^{14}$C]leucine in heat-killed cells. The 54.8% loss of incorporated [$^{14}$C]leucine is acceptable only if it does not represent loss due to lysis of heat-killed cells by penicillin. Penicillin lysis of heat-killed cells would mean that some of the heat-killed cells were synthesizing cell wall components and might exhibit other biosynthetic processes. If this were the case, the conclusion that protein synthesis does not occur in heat-killed cells would have to be modified and applied only to those heat-killed cells not lysed by the penicillin treatment.

Experiment 2 of Table 1 shows the results with the addition of Triton X-100 to the lysis media. Surprisingly, the amount of lost [$^{14}$C]leucine label was reduced to an average of about 34% loss. The addition of Triton X-100 either allows more of the heat-killed cells

to be retained in the pellet fraction or prevents heat-killed cells from losing incorporated [$^{14}$C]leucine. The latter proposal is considered unlikely, since Triton X-100 is expected to have a damaging effect on heat-killed cells. In fact, at concentrations of 0.1%, Triton X-100 was found to lyse them. The possibility that Triton X-100 addition enhanced the number of heat-killed cells recovered in the pellet fraction is considered more likely, since the pellets were visibly different.

In experiments involving [$^{14}$C]leucine incorporation, the "Separation Assay" was used without the addition of Triton X-100. In experiments which determined the incorporation of [$^{3}$H]uracil, the "Separation Assay," with the addition of Triton X-100, was employed.

## Pellet Fraction Losses of Incorporated [$^{14}$C]leucine

Although the amount of incorporated [$^{14}$C]leucine recovered in the pellet fraction was sufficient to measure incorporation in the heat-killed cells if it occurred, I did try to determine how the incorporated [$^{14}$C]leucine was lost. The [$^{14}$C] label could have been lost from the pellet fraction in many ways. First, labeled protein in the heat-killed cells might have been excessively degraded and then lost through the membrane of the cell, especially when TCA was added to the cells in Step 10 of the "Separation Assay." Secondly, incorporated [$^{14}$C]leucine might have been lost from the heat-killed cells as whole protein. This loss could have been the result of the heating procedure during which the membrane became sufficiently disturbed so that whole proteins leaked out. The membrane also could have been disturbed after heating by treatments of the cells with

EDTA or sucrose. Thirdly, the possibility of the loss of whole cells from the pellet fraction must be considered. This probably would have occurred in the centrifugation steps or during removal of the supernatant fractions after centrifugation. Lastly, penicillin lysis of heat-killed cells could account for the losses described in Table 1. Direct measurement of this possibility is very difficult. Loss of incorporated [$^{14}$C]leucine by lysis of a subset of the heat-killed cells was assessed by determining the extent to which the aforementioned possible ways of losing incorporated [$^{14}$C]leucine occur and will be described below.

## Degradation of protein

Figure 8 shows the results of an experiment designed to show the level of protein degradation in heat-killed cells. Samples of cells were taken every hour after heating. There was no significant decrease in the counts found in these samples with time, indicating little degradation or leakage of protein. The radioactivity of the cells after heating was on the average 37,500 dpm with a standard deviation of 1,920 dpm. The two standard deviation interval is 33,600 dpm to 41,200 dpm. A 54.8% loss of the incorporated [$^{14}$C]leucine from the heat-killed cells, shown in Table 1. would have resulted in a count of only about 21,500 dpm. This value lies significantly outside the two standard deviation interval described in Figure 8, and should have been easily detected if indeed protein degradation accounted for the losses of incorporated [$^{14}$C]leucine observed in Table 1. Furthermore, if it is assumed that there was protein degradation but that it was masked by the random error of the experiment,

Figure 8. Cells were labeled during growth with about 0.26 uCi/ml [$^{14}$C]leucine for 55 min, then washed and heated for 30 min at 51°C which produced 22% survivors. Cells were cooled and "recovery concentrate" suplemented with cold leucine (714 ug/ml) was added. TCA was added to 2 ml samples at 1 hr intervals and cells were collected on membrane filters (●). The sample taken at 5 hr (o) was collected on a membrane filter but was not treated with TCA. The filtrate of the 5 hr sample was also retained and counted (o).

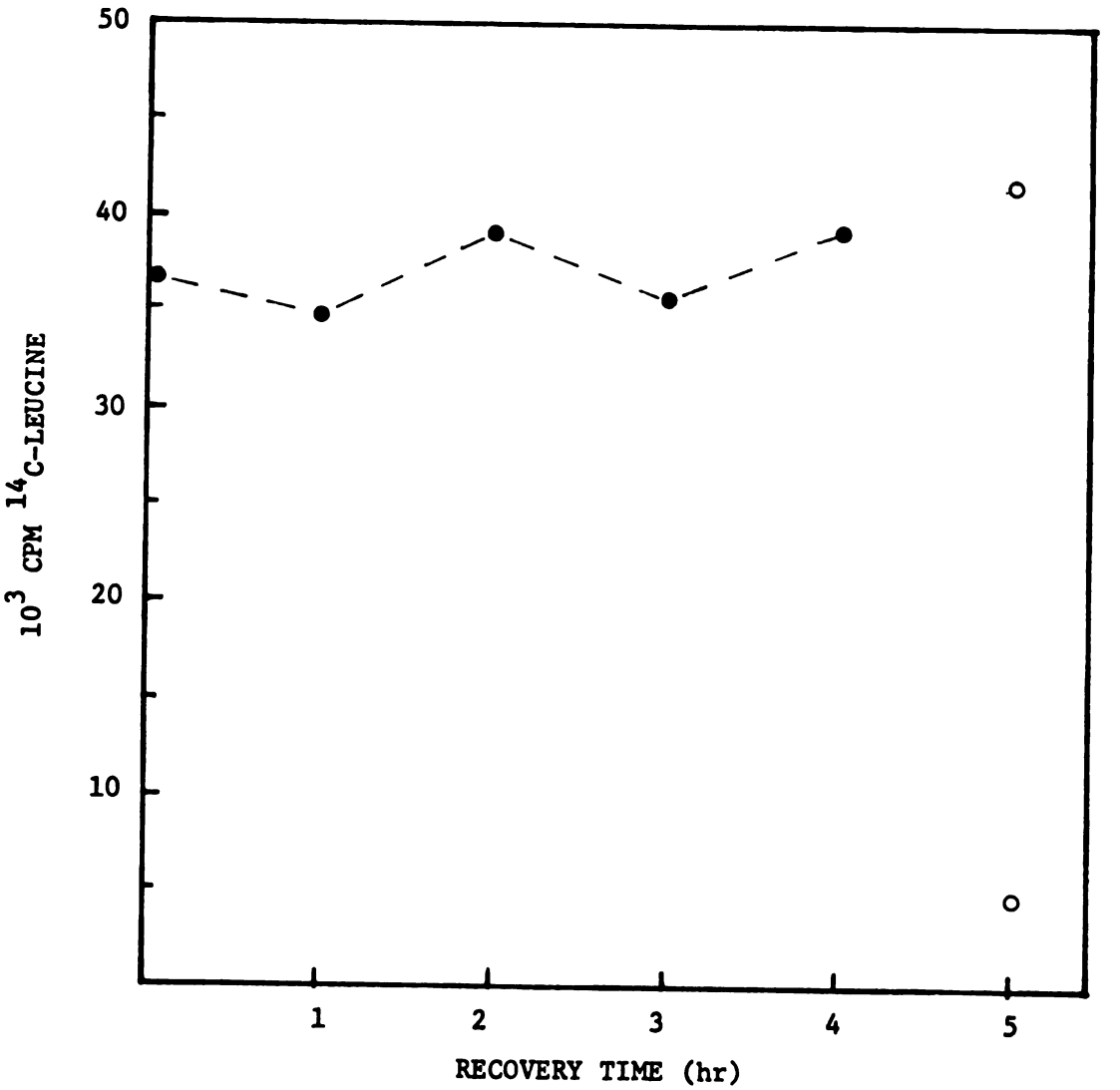



Figure 8.

then two standard deviations is 3840 counts, which is 10.2% of the total. Thus, if degradation was concealed within the two standard deviations of the experiment, it would have been limited to 10.2%.

It is possible that protein was degraded and lost during the time of heating, and therefore, samples at times 0 through 4 hours did not reflect this degradation. Degradation and leakage of protein during heating was unlikely, since it has been found that the rate of protein turnover in E. coli decreases as the temperature of incubation increases to 50°C (42). Furthermore, 30 minutes is a very short time for extensive degradation to occur. Nevertheless, if protein were degraded and leaked into the heating buffer, it should have appeared in the filtrate of samples collected after heating.

A sample was collected after 5 hours of recovery and filtered without treating the sample with TCA (Figure 8). Since cold leucine (714 ug per ml) had been added to the recovery media, it is unlikely that leaked [$^{14}$C]leucine was reincorporated during the 5-hour incubation period. The filtrate from this 5-hour sample contained about 9.5% of the total count found in the sample. The [$^{14}$C]leucine found in the filtrate could have been whole protein, degraded protein, or [$^{14}$C]leucine which had not been effectively removed from the culture before heating. Further experiments were done to clear up these possibilities.

Losses during heating--whole protein

Next it was determined whether the [$^{14}$C]leucine found in the filtrate (samples at hour 5) in the experiment described in Figure 8 was in fact a loss of label from the heated cells, and, if so, whether

or not it was due to loss of whole protein. Great care was taken to insure that [$^{14}$C]leucine was not left in intra-cellular pools prior to the heating step.

Table 2 indicates the level of [$^{14}$C]leucine incorporation in cells labeled prior to heating. It should be noted that the filtrate of the washed cells before they were heated is extremely low in radioactivity, indicating that extracellular [$^{14}$C]leucine was negligible before heating. In addition, samples of the total cell suspension after heating corresponded quite well to the amount of label in the cells before heating.

After heating, multiple samples were filtered on membrane filters to collect the cells, and the filters were washed with phosphate buffer. Both the first filtrate and the wash were counted. As a percentage of the total amount of label in the cells before heating (Table 3), the label in the cells after heating showed 84% retention of the incorporated [$^{14}$C]leucine label. The filtrate and wash, after heating, were found to contain a total of about 11.8% of the [$^{14}$C]leufound in the cells before heating (Table 3). This extracellular [$^{14}$C]leucine appearing in the filtrate must have come from the cells, and it is only slightly higher than the amount of [$^{14}$C]leucine found in the filtrate of the hour 5 sample from the experiment described in Figure 8.

Table 4 shows the results of TCA precipitation of the filtrate collected from cells after heating. Bovine serum albumin was added to the filtrates to a final concentration of 10 ug per ml to insure precipitation of any protein. The TCA precipitates contained very

Table 2. Amount of [$^{14}$C]leucine found in cells before heating and in the total suspension after heating.[a]

| sample | dpm |
|---|---|
| cells before heating[b] | 62,200 |
| filtrate[c] | 586 |
| total suspension[d] | 61,000 |

a. Cells in logarithmic phase were labeled with 0.13 uCi/ml [$^{14}$C]leucine for 2 hr, chased with cold leucine (187 ug/ml) for 30 min and washed once. The cells were then heated at 51°C for 15 min resulting in 64.6% survivors. Cells were then cooled and "recovery concentrate" was added.
b. Cells were collected on membrane filters and washed with cold phosphate buffer.
c. This is the filtrate from the cells before heating.
d. Total suspension included cells and the heating buffer after heating.

Table 3. Amount of [$^{14}$C]leucine found in cells and extracellularly after heating.[a]

| sample | dpm ± SD | (n)[c] | percent[d] |
|---|---|---|---|
| cells after heating[b] | 51,200 ± 480 | (3) | 84.0% |
| filtrate | 5,520 ± 167 | (3) | 9.1% |
| wash | 1,660 ± 130 | (3) | 2.7% |

a. The same conditions of heating, recovery, and labeling were used as in Table 2.
b. Cells were collected on membrane filters and washed with cold phosphate buffer.
c. (n) is the number of data used to compute the standard deviation.
d. Percent is calculated as percentage of total cell suspension after heating from Table 2.

Table 4. TCA precipitable [$^{14}$C]leucine in filtrate from cells after heating.[a]

| sample | dpm ± SD | (n)[d] | percent[e] |
|---|---|---|---|
| cells after heating[b] | 52,800 ± 1130 | (3) | 86.5% |
| filtrate from cells[c] | 311 ± 9 | (3) | 0.5% |

a. The same conditions of heating, recovery, and labeling were used as in Table 2.
b. Cells were collected on membrane filters and washed with cold phosphate buffer.
c. Filtrate was precipitated with 5% cold TCA and collected on membrane filters.
d. (n) is the number of data used to compute the standard deviation.
e. Percent is calculated as percentage of total cell suspension after heating from Table 2.

low counts, indicating that the extracellular [$^{14}$C]leucine which appeared after heating did not occur as TCA-precipitable protein.

Presumably, the leaked [$^{14}$C]leucine shown in Table 3 is low molecular weight peptides or unincorporated [$^{14}$C]leucine which occur in the intracellular pool. Unincorporated [$^{14}$C]leucine is very difficult to account for, since the intracellular pools of leucine in E. coli should be very small (13), especially in this case since the label was chased with cold leucine. Since the label lost from the heated cells was not TCA-precipitable, it would not have appeared in either the supernatant or pellet fractions during the "Separation Assay." Thus, the loss of [$^{14}$C]leucine from the cells after heating would have appeared as the differential loss illustrated in Table 1 only if the heat-killed cells exclusively lost [$^{14}$C]leucine.

Whole cell losses during centrifugation

Table 5 summarizes the results of experiments designed to determine to what extent whole cells are left in the supernatant fractions after centrifugation in Steps 6 and 8 of the "Separation Assay." The [$^{14}$C]leucine activity left on membrane filters was measured after filtering these supernatant fractions. Both surviving and heat-killed cells would have been collected if they were in the supernatant fluid, since these survivors are not lysed. The label found on these filters indicated that about 5% of the total cell population remained in the supernatant fluid after centrifugation at 3200 rpm. Following centrifugation at 2600 rpm, 9.3% of the total cell population before heating remained in the supernatant fluid.

Table 5. Cells remaining in the supernatant fraction after heating and centrifugation steps described in the "Separation Assay."[a]

| sample | dpm[d] ± SD | (n)[e] | percent[f] |
|---|---|---|---|
| 1[b] | 2,590 ± 356 | (3) | 5.0% |
| 2[c] | 4,810 ± 549 | (3) | 9.3% |

a. The conditions of heating, recovery, and labeling were the same as those of Table 2 except that "lysis concentrate" was added to the cells before centrifugation. Cells were collected on membrane filters.
b. Cells were collected from the supernatant fraction after centrifugation at 3200 rpm described in Step 6 of the "Separation Assay."
c. Cells were collected from the supernatant fraction after centrifugation at 2600 rpm described in Step 8 of the "Separation Assay."
d. The dpm are normalized to the concentration found in the recovery media.
e. (n) is the number of data used to compute the standard deviation.
f. Percent is the dpm as a percentage of the count for cells after heating. The count for cells after heating is the average of the count for cells from Tables 3 and 4. This average is 52,000 dpm.

Since we are interested in determining the percentage of heat-killed cells left in the supernatant fluids, this result may be misleading. In order to ascertain the number of heat-killed cells in the supernatant fluids in Table 5, the number of surviving cells remaining in the supernatant fluid was measured and subtracted from the total number of surviving and heat-killed cells in the supernatant fluids.

To make these measurements, living cells were centrifuged under conditions identical to those used for the experiment described in Table 5. The number of living cells in the supernatant fluids was determined by viable plate count. Table 6 shows that the number of living cells remaining in the supernatant fluids after centrifugation was a very small percentage of the original cell population centrifuged. The average percentage of living cells found in the supernatant fluid after centrifugation at 3200 rpm was 0.68% with a range of 0.48% to 0.92% based on one standard deviation of the poisson distribution. The poisson distribution was used since the number of cells in each plating of the supernatant fluid was a small number. The percentage of living cells in the supernatant fluid after centrifugation at 2600 rpm was on the average 0.15% of the total with a poisson standard deviation range of 0.079% to 0.23%.

The data of Table 6 was then compared with that for the radioactivity found in the supernatant fluids described in Table 5. Using 0.646 as the fraction of surviving (living) cells and 1% as a convenient maximum for the percent of living cells remaining in the supernatant fluid after centrifugation at 3200 rpm, the amount of

Table 6. Living cells left in the supernatant fractions after centrifugation.[a]

| sample | number of cells before centrifugation ± SD | cells in supernatant fraction after centrifugation ± SD |
|---|---|---|
| 1[b] | $2.32 \times 10^7 \pm 0.15 \times 10^7$ | $1.6 \times 10^5 \pm 0.4 \times 10^5$ |
| 2[c] | $3.30 \times 10^7 \pm 0.18 \times 10^7$ | $5.0 \times 10^4 \pm 2.2 \times 10^4$ |

a. Cells were grown, centrifuged, and resuspended in heating buffer. "Recovery concentrate" and "lysis concentrate" were added, and the cells were then centrifuged.

b. Cells were centrifuged at 3200 rpm as described in Step 6 of the "Separation Assay."

c. Pellets of cells from the 3200 rpm centrifugation were resuspended in lysis buffer, counted and treated as in Step 7 of the "Separation Assay." The cells were then centrifuged at 2600 rpm as described in Step 8 of the "Separation Assay."

radioactivity due to living cells is then calculated to be

$$0.01\ (52{,}000\ \text{dpm} \cdot 0.646) = 340\ \text{dpm}$$

where 52,000 dpm is the count for cells after heating, which is an average of Tables 3 and 4. When these counts are subtracted from the counts found in the supernatant fluid following centrifugation at 3200 rpm (Table 5), we find that the number of counts which can be attributed to heat-killed cells only is 2250 dpm. Since the maximum percentage for the number of living cells in the supernatant fluid was used, the 2250 dpm figure represents a lower limit estimate of the radioactivity due to heat-killed cells in the supernatant fluid following centrifugation at 3200 rpm as described in Table 5.

The same calculation was carried out for the level of cells appearing in the supernatant fluid following centrifugation at 2600 rpm. Again, a maximum estimate of the number of living cells in this supernatant (0.25%) was used. The radioactivity due to living cells in this supernatant fluid from the data in Table 5 is then calculated to be

$$0.0025\ (52{,}000\ \text{dpm} \cdot 0.646) = 85\ \text{dpm}$$

Therefore, the number of counts in the supernatant fluid following centrifugation at 2600 rpm which is due only to heat-killed cells is 4730 dpm. The total loss in both centrifugation steps is 6980 dpm. This loss is due to heat-killed cells only. The percentage of heat-killed cells in the total population of cells before centrifugation of the experiment described in Table 5 is 35.4%. The amount of radioactivity represented by the heat-killed population is $0.354 \cdot 52{,}000$ dpm or 18,400 dpm. After centrifugation, 6980 dpm appeared in the

combined supernatant fluids and were due exclusively to the presence of heat-killed cells. The percent of heat-killed cells lost to the supernatant was 6980/18,400 or about 38%. This is a low estimate.

This estimate agrees rather favorably with results obtained from microscopic counts of dead cells in the final pellet. The number of dead cells determined from plate counts after heating was $9.12 \times 10^6$. The final pellet showed $6.5 \times 10^6$ cells by microscopic counts. Thus, the loss of dead cells from the pellet fraction was 29%. The only difference between this experiment and the experiment above was that 8% sucrose was used in the lysis media instead of 5% sucrose as in later experiments. It should also be kept in mind that the loss in this experiment represents the total loss, not just centrifugation losses.

The appearance (after centrifugation) of 38% of heat-killed cells in the supernatant fluids would directly reduce recovery of [$^{14}$C]leucine in the pellet fraction. The data of Table 1 imply a loss of 55% of the [$^{14}$C]leucine from the pellet fraction. Since centrifugation losses account for 38%, the loss not accounted for is about 17%. This is a reasonably small percentage and could easily be explained if the data of Table 1 represented abnormally high losses or if the data of Table 5 represented abnormally low losses.

Possible variations in the results of Table 1 or Table 5 would most likely be due to variations in centrifugation. The procedures of centrifugation and removal of the supernatant fraction in the experiments are most sensitive to error, both random and systematic. Removing the supernatant fraction, leaving 0.2 or 0.3 ml pellet

volumes, is difficult to do without disturbing the pellet. Systematic errors can be introduced by simply jostling the test tube rack of samples immediately after centrifugation in one experiment and not in another. In fact, in an experiment very similar to that described in Table 1, the percent of [$^{14}$C]leucine lost from the pellet fraction in three samples was 29%, 56%, and 17%, which is a rather large variation.

## Net RNA Synthesis in Surviving and Heat-killed Cells

Protein synthesis requires some degree of RNA synthesis. Although mRNA synthesis may be particularly important to protein synthesis, it was not possible to measure mRNA synthesis in heat-killed and surviving cells using the "Separation Assay." Net RNA synthesis could be measured and compared with the prospect that such a measurement may provide some information about the failure of protein synthesis in heat-killed cells. In addition, a comparison of net RNA synthesis in heat-killed and surviving cells could give additional information as to what the site of lethal damage might be.

Net RNA synthesis was determined by measuring the incorporation of [$^{3}$H]uracil in heat-killed and surviving cells. The "Separation Assay" was employed to separate heat-killed and surviving fractions. This procedure was identical to that used in the preceeding experiments that measured protein synthesis, with the exception that Triton X-100 was added in Step 5 of the "Separation Assay." Experiment 2 of Table 1 shows that the percentage of heat-killed cells recovered in the pellet fraction was 65.9%, which should be sufficient to measure [$^{3}$H]uracil incorporation by heat-killed cells, if it

Figure 9. The measurement of [$^{3}$H]uracil (1.25 uCi/ml) incorporated in surviving cells (o), and heat-killed cells (•) during recovery. The total number of cells before heating was $4.90 \times 10^{7}$. After heating, the number of survivors was $1.84 \times 10^{7}$, giving 38% survivors. The procedure followed was that of the "Separation Assay."

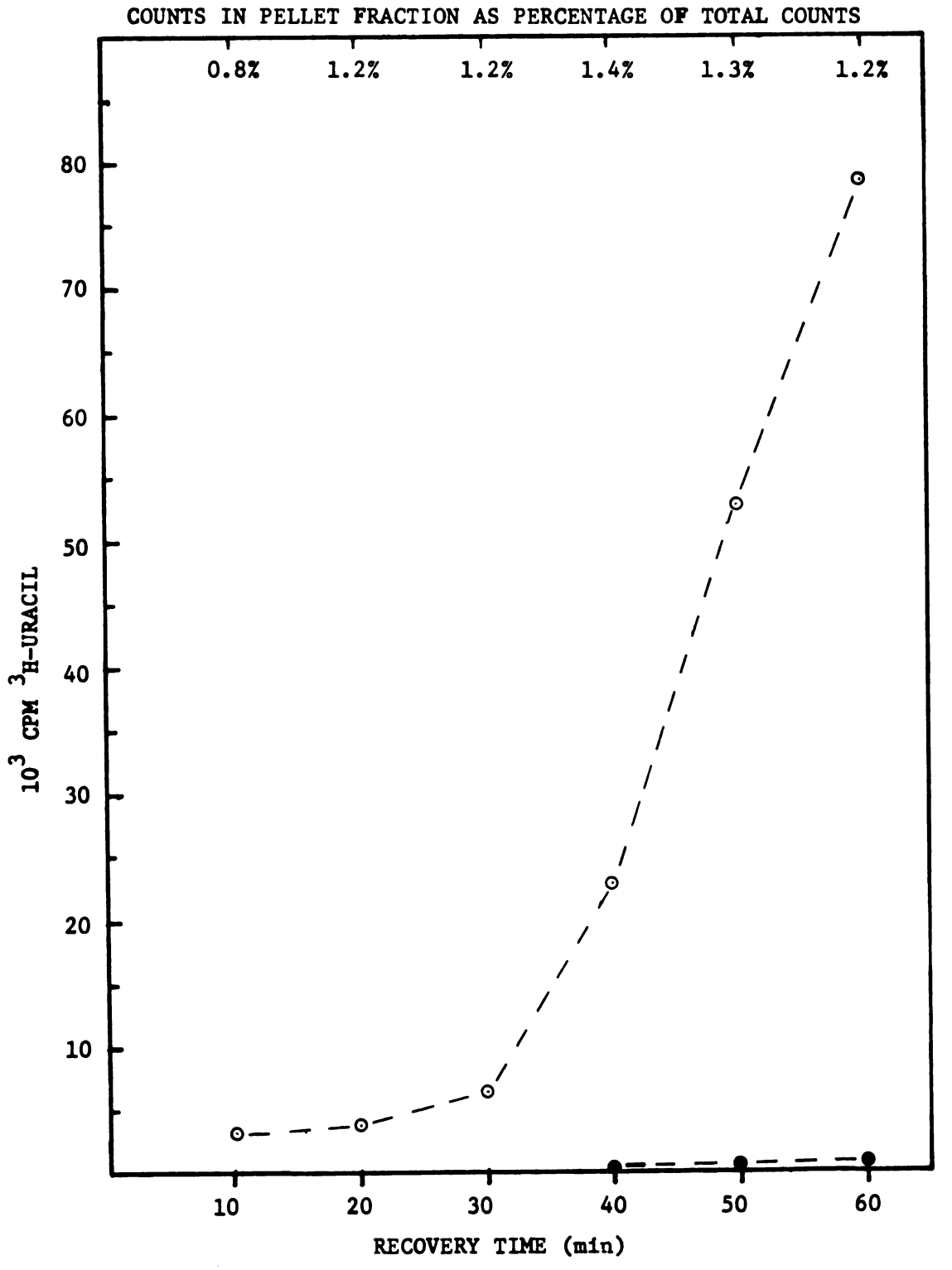

COUNTS IN PELLET FRACTION AS PERCENTAGE OF TOTAL COUNTS
0.8%
1.2%
1.2%
1.4%
1.3%
1.2%
80
70
60
50
40
30
20
10
$10^3$ CPM $^3$H-URACIL
10
20
30
40
50
60
RECOVERY TIME (min)

Figure 9.

Figure 10. The rate of [$^{3}$H]uracil incorporation calculated from the 10 min intervals of Figure 9. The rates were calculated by taking the difference in counts for each 10 min interval and plotting these values on the ordinate at the midpoint of the time interval on the abscissa. The dashed line is the extrapolation of the curve.

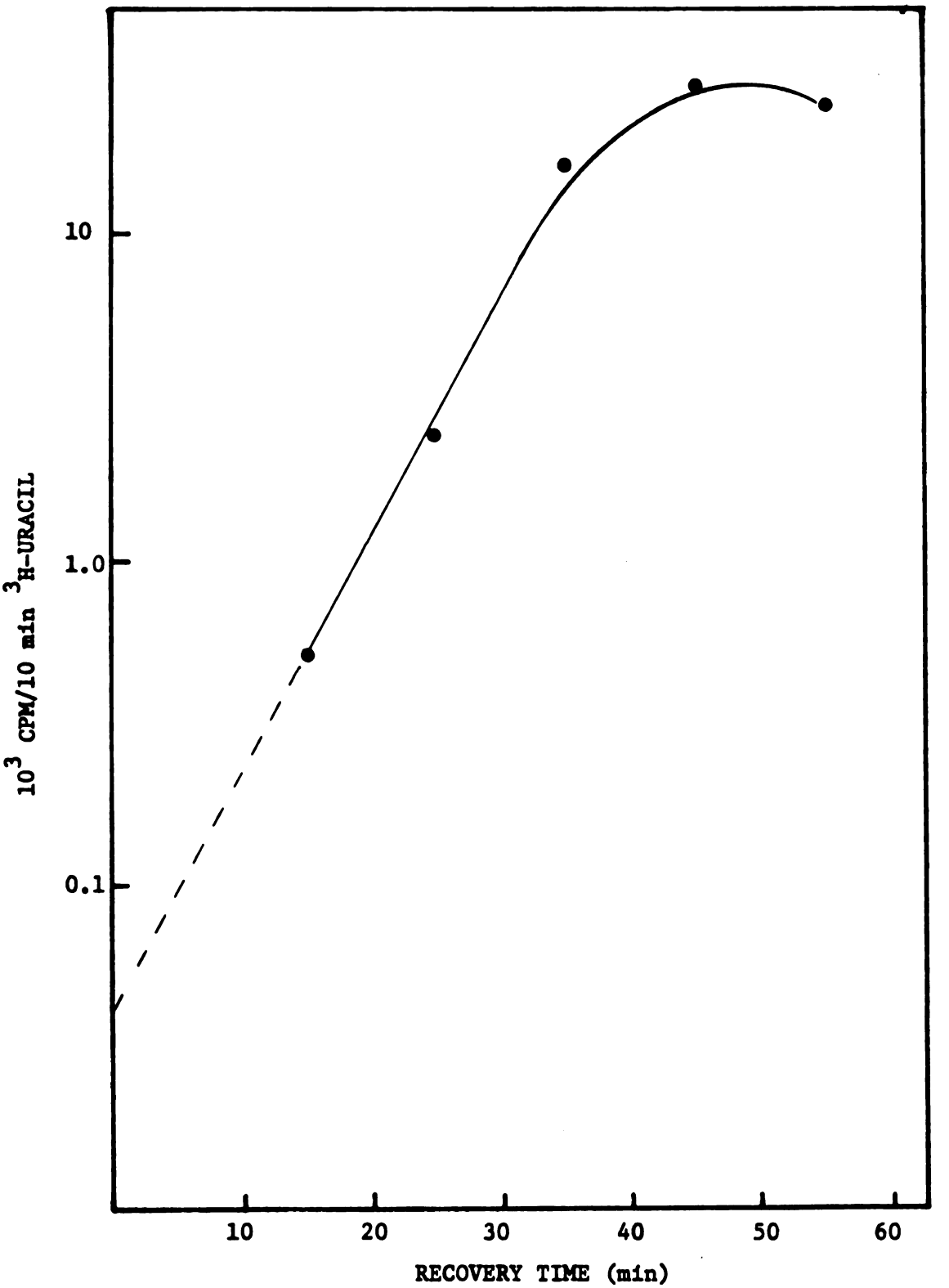

10
1.0
0.1
$10^3$ CPM/10 min $^3$H-URACIL
10
20
30
40
50
60
RECOVERY TIME (min)

Figure 10.

occurred.

Figure 9 shows the incorporation of [$^3$H]uracil by surviving and heat-killed cells. The surviving cells showed recovery and rapid incorporation of [$^3$H]uracil. The heat-killed cells in the pellet fractions showed negligible incorporation of the label. The [$^3$H]uracil found in the pellet fraction as a percentage of the label found in the supernatant fraction was on the average 1.16% with a standard deviation of 0.17%.

Figure 10 shows the rates of [$^3$H]uracil incorporation as calculated from the 10-minute intervals of Figure 9. The rates of incorporation show a logarithmic increase in the rate of incorporation for the 30-minute period immediately after heating. The data in Figure 10 have been extrapolated toward the zero time of the graph. This extrapolation shows that there was no delay in the initiation of RNA synthesis.

## [$^3$H]uracil Contamination of the Pellet Fraction

Although the label found in the pellet fraction of Figure 9 was negligible, it was necessary to ascertain the amount of label in the pellet fraction which could be attributed to contamination from the supernatant fraction or surviving cells. To do this, the same methods were used as in the experiment described in Figure 5. Cells were grown, centrifuged, and resuspended in recovery media with [$^3$H]uracil. The "Separation Assay" was used to separate the pellet and supernatant fractions. Since the cells were not heated, the pellet fraction should contain only the label that represents contamination.

The supernatant fraction (Figure 11) shows the expected rapid

Figure 11. [$^{3}$H]uracil contamination of the pellet fraction. Cells were grown to an OD of 0.07, centrifuged and resuspended in 12 ml of heating buffer with 4 ml of "recovery concentrate" added. [$^{3}$H]uracil was added to a final concentration of 2.5 uCi/ml. Cells were not heated and the "Separation Assay" (Steps 4 through 10) was used with the addition of Triton X-100 (0.008% final concentration) in Step 5. The supernatant fraction (o) and pellet fraction (●) is shown.

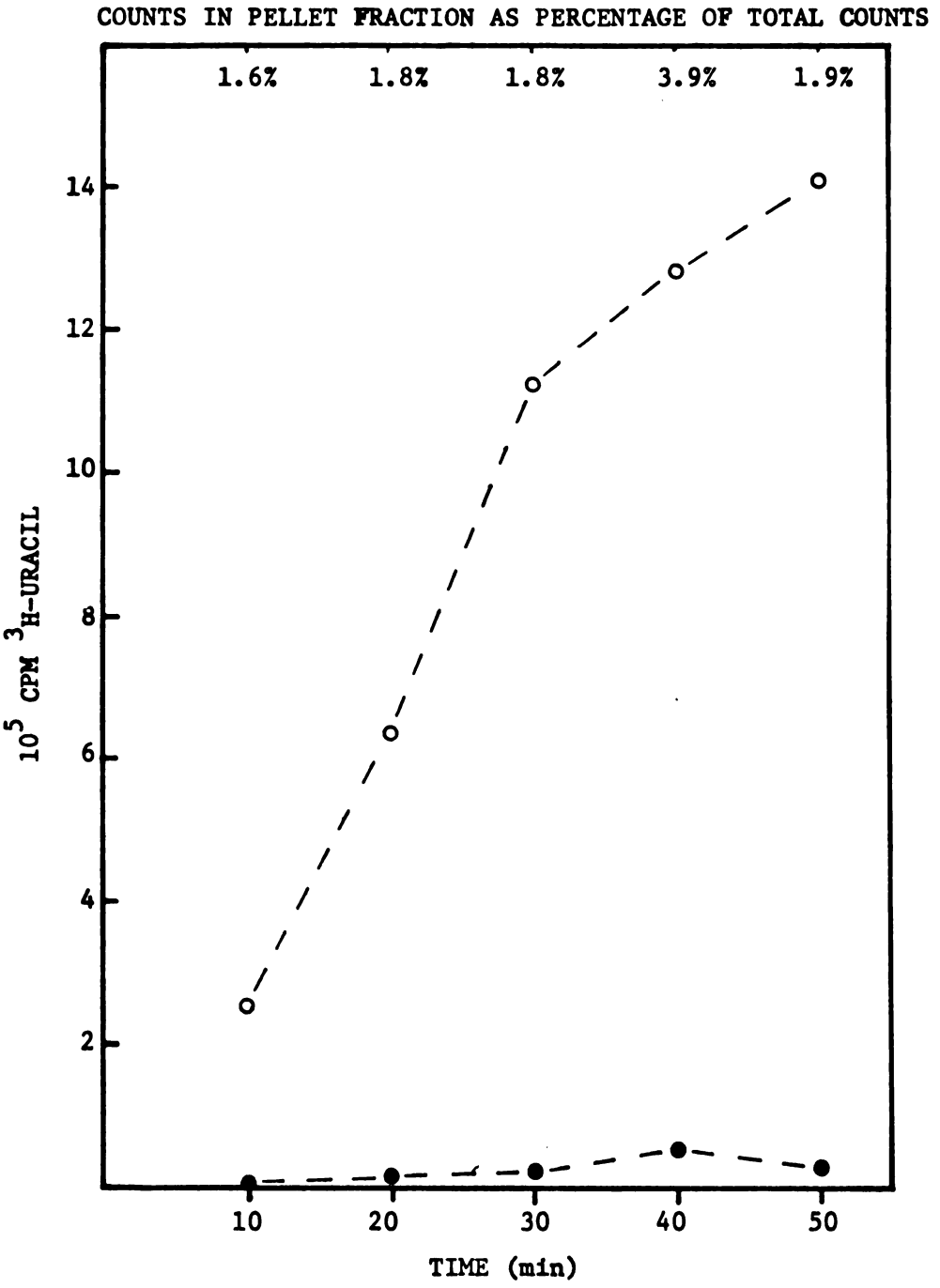

COUNTS IN PELLET FRACTION AS PERCENTAGE OF TOTAL COUNTS
1.6%
1.8%
1.8%
3.9%
1.9%
14
12
10
8
6
4
2
$10^5$ CPM $^3$H-URACIL
10
20
30
40
50
TIME (min)

Figure 11.

incorporation of the label. The pellet fraction, which in this case shows only contamination from the lysate, is very low in radioactivity. The average contaminating radioactivity found in the pellet is 2.20% with a standard deviation of 0.85%. This, the [$^{3}$H]uracil found in the pellet fraction shown in Figure 9 is not above the contamination levels of label found in the control experiments of Figure 11. This result indicates that there was a failure of net RNA synthesis in the heat-killed cells in the pellet fraction of Figure 9.

# CONCLUSIONS

One of the central concerns in using the "Separation Assay" was the possibility that some heat-killed cells may be lysed by the penicillin treatment. If this had happened, then at least some of the heat-killed cells may have recovered biosynthetic functions in the same way as surviving cells. Thus, the conclusion that protein and RNA synthesis do not occur in heat-killed cells would be questionable.

The data in Table 1 (Experiment 1) showed that the heat-killed fraction of cells contained only 46% of the [$^{14}$C]leucine expected. In subsequent experiments it was found that the loss of [$^{14}$C]leucine was accounted for largely by losses of heat-killed cells during the centrifugation steps of the "Separation Assay." These results showed that only 17% of the heat-killed cells remained unaccounted for, after centrifugation losses were considered. This remaining 17% could be due to lysis of the heat-killed cells by penicillin. However, considering the variation in the results of experiments which determined the losses, the 17% could be a spurious result. If this 17% does represent a subpopulation of heat-killed cells which is lysed by penicillin, it can be said that there are a significant number (83%) of heat-killed cells which are not lysed by penicillin. In this case, the conclusions would apply to the 83% of the cells which do not lyse.

From Figure 6 it can be concluded that there is essentially no protein synthesis in heat-killed cells beyond the background contamination levels found in the control data shown in Figure 5. This conclusion does not rule out the possibility that a very small amount of protein is being synthesized in each heat-killed cell. The "Separation Assay" is only designed for comparative purposes. This comparison, when taken to the limit, asks the following questions: What is the initial rate of synthesis of protein in the surviving cells? If this same rate or a lesser rate of synthesis occurs in heat-killed cells, could this synthesis go undetected in the results shown in Figure 6? Figure 7 shows that protein synthesis was not initiated in survivors immediately after heating. Therefore, in comparing the initial synthesis in survivors to possible initial synthesis in heat-killed cells, the comparison is made when synthesis is initiated or at about 12 minutes after heating.

Figure 7 shows that the surviving cells had an initial rate of [$^{14}$C]leucine incorporation equal to 270 counts per 10 minutes in the 10 to 20 minute interval of recovery. If heat-killed cells had this same initial rate, they should have incorporated 195 counts in the same 10-minute interval. This would have made the 20-minute count in Figure 6 for the heat-killed cells 385 counts instead of 148 counts. Appendix B details how these calculations were made. This calculation includes the counts that would be due to an average contamination of 6.0%. This level of incorporation, as a percentage of the total counts in the surviving fraction at 20 minutes, would have been 12.1%. This is a greater amount of label in the pellet fraction

than permitted by the upper limit of the two standard deviation interval for contamination of the pellet fraction. The two standard deviation upper value for contamination is 10.0%. Thus, if the heat-killed cells synthesized protein at the same initial rate as survivors, the "Separation Assay" should have detected it as being more than contamination of the pellet fraction. However, the anticipated initial synthesis of protein in heat-killed cells is quite close to the upper limit of the two standard deviation of contamination. Therefore, any rate of initial synthesis by heat-killed cells lower than that of surviving cells could not have been detected by the "Separation Assay." Despite the insensitivity of the "Separation Assay" in this case, this analysis does show that protein synthesis in heat-killed cells, if it occurs, fails to occur at the same initial level as in survivors. This comparative failure is shown in the earliest stages of reinitiated protein synthesis.

This insensitivity of the "Separation Assay" assumes that protein synthesis in heat-killed cells stops after 10 minutes of synthesis or at 20 minutes of recovery. If the initial rate of synthesis by heat-killed cells was lower than that of survivors, but continued for more than 10 minutes, the cumulative effects could have been detected by the "Separation Assay." For example, if the initial rate of synthesis in heat-killed cells was only half that of survivors but continued for 20 minutes, or until 30 minutes of recovery, the count at 30 minutes would have been 455 counts instead of 267 counts found in Figure 6 at 30 minutes. This calculated count as a percentage of the total count found in the surviving fraction would

be 10.4%. This percentage is very close to the upper value of the two standard deviation interval of contamination and would lead to the suspicion that protein synthesis may have been occurring in heat-killed cells. From the data in Figure 6, it also appears that if a low rate of protein synthesis occurred in heat-killed cells, the rate did not increase as shown by survivors in Figure 7. Increasing rates of protein synthesis in heat-killed cells would be expected to give counts in the pellet fraction at progressively higher levels which could have been detected by the "Separation Assay." It is therefore apparent that if heat-killed cells do synthesize protein, synthesis does not occur for more than a short time (10 minutes), and the rate of synthesis fails to increase with time.

Figure 7 shows that there was a lag time of about 12 minutes before protein synthesis was reinitiated in surviving cells. This lag may be interpreted to mean that certain cellular functions must have resumed and preceeded protein synthesis. One cellular function which might necessarily preceed protein synthesis is RNA synthesis. Figure 10 shows that RNA synthesis has no lag time before it is reinitiated. Other investigators have also shown that RNA synthesis preceeds protein synthesis (55). Thus, RNA synthesis may be particularly important to the survival or death of heated cells.

Figure 9 shows the results comparing net RNA synthesis in heat-killed and surviving cells. It is apparent that there is no net synthesis of RNA in heat-killed cells beyond the contamination levels shown in Figure 11. Again, when the comparison of heat-killed cells and survivors is assessed for the earliest stage of recovery, we find

that a survivor-level of RNA synthesis in heat-killed cells would not have escaped detection of the "Separation Assay."

In order to obtain the earliest rate of [$^{3}$H]uracil incorporation for surviving cells, the curve in Figure 10 was extrapolated as shown by the dotted line. The initial rate of synthesis was 100 counts over the first 10 minute interval. If the heat-killed cells had this same rate of synthesis, then the 10-minute count in Figure 9 would have been 192 counts instead of the 26 counts found. This calculation is made using the result that 65.9% of the heat-killed cells were recovered in the pellet fraction (Experiment 2, Table 1). The calculated 192 counts includes the 2.2% average contamination found in Figure 11. For the method of calculation, see Appendix B. The calculated incorporation of 192 counts at 10 minutes, as a percentage of the total counts found in the surviving fraction at 10 minutes would be 5.4%. The contamination of the pellet fraction in the control experiment (Figure 11) was only 2.2% with a two standard deviation interval of 0.5% to 3.9%. Since 5.5% is well above the upper value of the two standard deviation interval of contamination, it should have been possible to detect this amount of synthesis in heat-killed cells. Furthermore, if the rate of initial RNA synthesis in heat-killed cells was only 2/3 the initial rate of synthesis in surviving cells, the 10-minute count in Figure 9 would have been 152 counts. This count as a percentage of the total counts found in the surviving fraction at 10 minutes would have been 4.4%. This percentage is also above the upper value (3.9%) of the two standard deviation interval of contamination.

The two standard deviation interval of contamination was determined from the percents of contamination found in Figure 11. The percentage of contamination found in Figure 11 at 40 minutes was 3.9% which deviates significantly from the rather uniform percent contamination found at the other times. If the average contamination and two standard deviation interval is recalculated without including the 40-minute value of 3.9% contamination, the average becomes 1.7% contamination with a two standard deviation interval of 1.4% to 2.0%. These values of contamination compare much more favorably to the percentage of count in the pellet fractions found in Figure 9. Based on this reevaluation, the sensitivity of the "Separation Assay" can be recalculated with better results. If heat-killed cells synthesized RNA at the same initial rate as survivors and the average contamination of the heat-killed fraction is taken to be 1.7%, it is found that the 10-minute count in Figure 9 would have been 178 counts. This count would be 5.1% of the total count found in the surviving fraction in Figure 9 at 10 minutes. The reevaluated upper value of the two standard deviation of contamination is only 2.0%. Thus, if heat-killed cells synthesized RNA at the same initial level as survivors, the expected counts found in the pellet fraction at the end of 10 minutes would be more than double the upper value of the two standard deviation interval of contamination. If heat-killed cells synthesized RNA at a lower level than the initial synthesis in survivors, the "Separation Assay" could have detected it. For example, synthesis by heat-killed cells at only 1/3 the initial rate of synthesis in survivors would have given 102 counts at the 10-minute

recovery time of Figure 9. This count as a percentage of the total count in the surviving fraction at 10 minutes is 2.9%. Using the upper value of the two standard deviation interval of 2.0%, the expected count in the pellet fraction would give a percentage (2.9%) above the two standard deviation interval of contamination.

In the above analysis of the sensitivity of the "Separation Assay," it was assumed that RNA synthesis in heat-killed cells, regardless of the initial level, stops after 10 minutes of recovery. If RNA synthesis persisted for longer than 10 minutes in heat-killed cells, the additional counts accumulated would make the "Separation Assay" more sensitive. It also appears that RNA synthesis, if it occurred in heat-killed cells, did not increase in rate as shown for survivors in Figure 10. If heat-killed cells showed increasing rates of RNA synthesis, the resultant net synthesis of RNA would most probably be detected. The above analysis shows that there is a comparative failure of net RNA synthesis in heat-killed cells and that this failure occurs in the earliest stages of recovery.

The obvious question is whether failure of protein and RNA synthesis is the functional expression of the lethal damage done by heating. The answer appears to be yes, since this functional failure fits the necessary criteria, discussed on page 14, in order to qualify as a direct functional expression of the lethal damage. First, the failure of protein and RNA synthesis appear as a difference between heat-killed and surviving cells. Secondly, the difference appears in the earliest stages of recovery or reinitiation of the function.

# DISCUSSION

The conclusion that the lethal damage in heat-killed cells causes an immediate failure of protein and RNA synthesis provides some insight as to what the lethal molecular damage may be. In discussing some of the possible sites of lethal molecular damage, we must attempt to correlate the findings of this thesis with the kinds of molecular damage previously reported to occur in heated cells. In addition, we must assess the implications of the exponential order of death upon the various possible sites of damage.

The findings that single- and double-strand breaks occur in heated populations of E. coli, and that death is enhanced by blocking the repair of these strand breaks, are strong evidence that the lethal damage in heated cells is involved with DNA or its repair (58). It is certain that unrepaired double-strand breaks due to heating would be lethal. However, DNA single- or double-strand breaks alone should not appreciably affect RNA and protein synthesis, and this is in conflict with the findings of this thesis.

Ionizing radiation has been found to be lethal to cells. There is evidence that death produced by ionizing radiation is due to damage to the DNA (11,23). This damage is most commonly breaks in the DNA strands (36,43). It has been found that cells of E. coli which have received lethal doses of ionizing radiation and have lost

almost completely their ability to produce colonies, form filaments and may even divide once or twice (33). Thus, cells that have been killed with ionizing radiation, which produces many DNA strand breaks, continue to synthesize RNA and protein. In the case of heat-killing, if death is due to DNA strand breaks, then we would expect RNA and protein synthesis to continue. The findings of this thesis indicate that continued synthesis of RNA and protein does not occur.

It may be argued that heating causes damage not only to the DNA but to many sites, and that the non-lethal damage to sites other than DNA causes failure of RNA and protein synthesis. If this premise is accepted, then protein and RNA synthesis should also fail in survivors of heating. In other words, when protein or RNA synthesis is resumed, there should be no immediate difference in synthesis between heat-killed cells and survivors. However, an immediate difference was observed. Therefore, DNA strand breakage in itself is not the lethal damage causing cellular death from heating.

Woodcock and Grigg (58) concluded that DNA breakage alone may not be the cause of death in heated cells and that the actual lethal damage may be in the repair system enzymes. This conclusion does not agree well with the damands of the exponential order of death. Most enzymatic systems have a great deal of redundancy, including the enzymes responsible for DNA repair. For example, DNA ligase is estimated to be present in E. coli in over 200 copies (34). If the destruction of these repair enzymes were the rate-limiting step in the death of the cell, then a large shoulder should be observed in the kinetic curve showing death (45). Furthermore, it is not

immediately apparent how destruction of DNA repair enzymes could be responsible for the failure of RNA and protein synthesis in heat-killed cells. The findings of this thesis maintain that when protein or RNA synthesis is resumed, there is an immediate difference in synthesis between heat-killed cells and survivors.

Focusing attention on what may cause the loss of RNA synthesis, we find many possibilities. The destruction of synthetic enzymes (DNA-sependent RNA polymerase) could cause an immediate failure of RNA synthesis. A loss of nucleotide pools through leakage could cause a failure of RNA synthesis. Immediate degradation of nascent RNA would effectively be identical to failure of the RNA-synthesizing capacity of the cell. Finally, inhibition of RNA synthesis by guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) may occur in heat-killed cells. This inhibition may possibly account for the observation that RNA synthesis does not occur in heat-killed cells.

Heat inactivation of DNA-dependent RNA polymerase in vivo has been studied at elevated ($44^{o}C$) non-lethal temperatures (41). It was found that the enzyme was only slightly inactivated at this temperature. It was also found that continued incubation at this temperature resulted in resumption of the normal activity of the enzyme. Presumably, this enzyme would be inactivated to a much greater degree at lethal temperatures of $51^{o}C$. Nevertheless, if destruction of this enzyme were the rate-limiting step in the death of the cell, a large shoulder should be observed in the kinetics of death. This shoulder would again be due to the redundancy of the polymerase molecule in the cell.

Degradation of rRNA is found to occur in several different

bacteria at elevated temperatures (37,47,48). The heating temperatures in these cases were sub-lethal, and, although very extensive degradation occurred, it did not cause the death of cells. Degradation of rRNA also occurs at lethal temperatures (54). The mechanism of this degradation is at present unknown; however, the evidence indicates the possibility of enzymatic degradation (47).

The possibility that heating enhances RNA degradation must be considered, since this is a possible interpretation of the data in this thesis. In particular, we must ask if RNA formed after heating is degraded following heat treatment. Furthermore, could this degradation occur differentially in heat-killed and surviving cells? This is a very speculative area and not easily accessible to investigation.

The measured RNA synthesis summarized in Figure 9 is net RNA synthesis which is primarily rRNA synthesis. Ribosomal RNA is normally quite stable in vivo (17). Under conditions favorable to rRNA degradation (magnesium starvation), only 45% of the rRNA in a cell is degraded after 20 hours (39). In addition, the findings of Tomlins and Ordal (54) showed that significant amounts of rRNA accumulate in recovering cells of Salmonella typhimurium after heating. It is unlikely that rRNA could accumulate in survivors following heat treatment if it were being degraded. Thus, rRNA formed after heat treatment is probably quite stable in the cell after heating.

Although the experiments described in this thesis can give no information about the degradation of mRNA, the degradation of mRNA after heating is quite likely to be accelerated. Messenger RNA is normally degraded in the cell very rapidly (17). It has also been

found that at sub-lethal elevated temperatures (44°C), there is a loss of ribosomes from the polyribosomal region of a sucrose gradient. This unmasking of the mRNA could result in rapid degradation of the mRNA. In addition, since heating causes the loss of ribosomes through degradation, mRNA should be open to nuclease attack for some time after heating.

It is difficult to see how degradation of mRNA could be the rate-limiting step in cell death or how such degradation could occur only in heat-killed cells and not in survivors. If death is due to degradation of mRNA, then the exponential order of death requires that a single event initiates this degradation in heat-killed cells. Also, this event must occur only in heat-killed cells and not in survivors.

Elevated temperatures have been found to damage membranes (49, 50), and cause the leakage of low molecular weight metabolic substances. Furthermore, this leakage could be mediated by a single molecular event. For example, colicins are thought to have a general effect on the whole membrane of a cell that began as a localized effect or single event (18). It is highly speculative that heat should act in the same way; however, if heat could damage the membrane in this way, the requirements of the exponential survival curve would be met.

Thus, it may be argued that membrane damage is the rate-limiting step in heat-killing and that leakage is a further step that occurs rapidly. This assumes that the membrane damage and leakage occurs only in the heat-killed cells. Therefore, a strong correlation between the number of heat-killed cells and the amount of membrane

leakage would be expected. The findings of Russell and Harries (49) showed that such a correlation does not occur. Furthermore, leakage of metabolites has been shown to occur in cells that were heated sublethally (25). These findings indicate that damage to the membrane and subsequent leakage of metabolites does not cause cell death.

Ribosomal RNA synthesis can be inhibited by ppGpp (15). Furthermore, it has been found that heating E. coli at 44°C causes transient inhibition of RNA synthesis in cells that show a stringent response to amino acid starvation (40). In the experiments described in this thesis, cells were preheated to 44.7°C. Thus, rRNA synthesis was possibly inhibited in the total population. The stringent response by cells is not lethal normally. Although inhibition due to heating is perhaps deleterious, it cannot be concluded to be lethal. At the lethal temperature of 51°C, further inhibition of RNA synthesis by the ppGpp mechanism may occur. Again, it would be questionable if this inhibition could be lethal. Nevertheless, if ppGpp inhibition were the lethal difference between heat-killed and surviving cells, it would have to be caused by a single event and occur only in heat-killed cells.

The production of ppGpp, which may be due to a single event, could be traced to the loss of amino acid pools due to leakage through the cytoplasmic membrane. Thus, the lethality of ppGpp inhibition would depend on the lethality of the membrane damage. It has been pointed out that membrane damage is not necessarily lethal. Thus, it is doubtful that ppGpp inhibition of RNA synthesis would occur lethally or only in heat-killed cells.

Examination of why protein synthesis in heat-killed cells should fail produces many possibilities. Invariably these possibilities concern components of the translational system, each of which has high redundancy. For example, a single cell of E. coli contains approximately 15,000 ribosomes (34). If loss of any such component were the cause of death due to heating, the survival curve for the cells being heated would show a large initial shoulder before the decline in survivors became exponential. Therefore, discussion aimed at trying to determine the site of lethal damage at the level of protein synthesis is not very profitable.

Many of the considerations that were examined with respect to the failure of RNA synthesis also apply to the failure of protein synthesis. For example, degradation of protein formed after heating could account for the lack of net protein synthesis in heat-killed cells. Figure 8 shows that degradation of preformed protein does not occur after heating in lethally heated E. coli. It has also been found that protein is not degraded during heating in Staph. aureus (3), and that protein turnover rates begin to decline in E. coli at 50°C (42). Thus, it appears that protein is quite stable in the cell during and after heating. Proteins which have been heated in a cell and denatured are likely to be degraded since unfolding of a protein could enhance degradation (42). Comparatively, newly formed protein which is not denatured would be even more immune to denaturation. Therefore, it is not likely that protein formed after heating is degraded. Furthermore, it is unlikely that protein degradation is initiated by a single event.

Leakage of amino acids through a damaged cytoplasmic membrane could also cause failure of protein synthesis. Leakage of amino acids has been shown in heated populations of Staph. aureus (4,25). It was also our finding that [$^{14}$C]leucine was leaked from heated populations of E. coli (Table 3). Failure of protein synthesis due to leakage of amino acids in heat-killed cells would be dependent on membrane damage; however, membrane damage has not been correlated with cell death.

The molecular sites of potentially lethal damage considered so far have been eliminated either because they are not lethal, they do not agree with the exponential survival curve, or they cannot be shown to cause the immediate failure of RNA and protein synthesis in heat-killed cells. The exponential survival curve requires that one or a few molecules are involved in the rate-limiting step which determines death of a cell. In considering the molecular structure of a cell, the most likely structures which would meet the requirements of the exponential survival curve are the DNA chromosome and the cytoplasmic membrane.

In the previous discussion, I have pointed out that membrane damage and subsequent leakage is not necessarily lethal. Damage to the DNA in the form of double- and single-strand breaks does not account for the immediate failure of RNA and protein synthesis in heat-killed cells. Perhaps DNA or its structure may be involved in another way.

DNA isolated from E. coli with gentle lysing procedures exhibits a very complex form which is termed folded. The nucleoid or folded

chromosome is held in the folded configuration by RNA which gathers the DNA into about 50 negatively supercoiled loops (59). The nucleoid can be isolated with membrane material bound to it under proper conditions of lysis (60).

RNase treatment of the nucleoid attacks the RNA holding the nucleoid together and unfolds it in an "all or none" fashion (59). The nucleoid is either unfolded totally or not unfolded at all by the action of RNase. The "all or none" character of unfolding due to action by RNase suggests that the RNA which maintains the nucleoid is one or a few molecules in number (59). Although it has not been shown, the unfolding of the chromosome by the action of RNase is probably irreversible, considering the vast change in the chromosome that occurs. If such a vast irreversible change in the structure of the chromosome occurred in the cell, it could easily be lethal.

The "all or none" character of nucleoid unfolding, and the probability that only one or a few RNA molecules are involved in a potentially lethal event, are the precise requirements of the exponential order of survival. In addition, the destruction of the RNA species that holds the nucleoid together may be sensitive to heating. The finding that rRNA is degraded during heating (37,47,48) suggests that the nucleoid RNA could also be susceptible to degradation. The denaturation of R17 RNA and rRNA from E. coli and Bacillus subtilis begins at 45°C to 50°C (20). If the nucleoid RNA has a similar susceptibility to heating, partial denaturation of this RNA may open it to attack by RNase. Heat alone, without the action of RNase, has been found to unfold the nucleoid of E. coli; however, the conditions

are somewhat extreme (67°C for 20 minutes in vitro) (21).

It appears that the nucleoid RNA is an excellent candidate for the molecular site of lethal damage due to heating. Thus, in heat-killed cells, the nucleoid RNA would be functionally destroyed, and in surviving cells, the nucleoid RNA would remain intact. In order to agree with the findings presented in this thesis, destruction of the nucleoid RNA should cause the immediate comparative failure of RNA and protein synthesis in heat-killed cells.

Recent findings indicate that initiation of RNA synthesis by RNA polymerase is two to three times greater on the folded chromosome than on the unfolded chromosome (21). This finding applied to total RNA synthesis, including rRNA synthesis. These studies were done in vitro, and the unfolded DNA was produced by heating the nucleoid. Heating may have caused, in addition to the configurational change of the DNA, inactivation of other factors which may promote initiation of RNA synthesis. Thus, it cannot be concluded that destruction of the nucleoid RNA and resultant unfolding of the chromosome was the only possible cause of a lower RNA polymerase initiation rate. However, the possibility that unfolded DNA could cause reduced RNA synthesis cannot be excluded and deserves consideration as an explanation of the data in this thesis. If the nucleoid was unfolded in heat-killed cells during heating and remained folded in survivors, then, using Giorno's data (21), RNA synthesis could be 2 or 3 times less in heat-killed cells than in survivors. This lower rate of synthesis may not be detected by the "Separation Assay." Synthesis of RNA in heat-killed cells at 1/3

the initial level of surviving cells would give pellet fraction counts of 117 counts at 10 minutes of recovery, 168 counts at 20 minutes of recovery, and 262 counts at 30 minutes of recovery in Figure 9. This calculation assumes no increase in the rate of synthesis by the heat-killed cells during recovery. These calculated counts as percentages of the counts found in the supernatant fraction at the same recovery times are 3.3% at 10 minutes, 4.2% at 20 minutes, and 4.0% at 30 minutes. If we use the contamination data from Figure 10 with an average contamination of 2.2% and the upper value of the two standard deviation interval as 3.9%, it is found that the calculated synthesis in heat-killed cells does not exceed by very much the upper value of the two standard deviation interval of contamination. Thus, a threefold reduction in the initial rate of RNA synthesis in heat-killed cells could produce the comparative failure of RNA synthesis shown in Figure 9.

It has been shown that the sensitivity of the "Separation Assay" in detecting RNA synthesis in heat-killed cells is dependent on the evaluation of contamination appearing in the pellet fraction. If the data in Figure 11 is used without the 40-minute value of contamination, then the average contamination is 1.7% with an upper value of the two standard deviation interval of 2.0%.

Using these values of contamination, synthesis of RNA in heat-killed cells at 1/3 the initial level of synthesis of survivors would produce pellet counts of 102 counts at 10 minutes of recovery, 151 counts at 20 minutes of recovery, and 235 counts at 10 minutes of recovery. These calculated counts as percentages of the counts

found in the supernatant fraction at the same recovery times are 2.9% at 10 minutes, 3.7% at 20 minutes, and 3.6% at 30 minutes. These calculated values for RNA synthesis in heat-killed cells exceed the upper value of the two standard deviation interval (2.0%) of contamination by a great enough margin so that the "Separation Assay" would detect this level of synthesis. In this case, a threefold reduction in RNA synthesis in heat-killed cells would still allow the remaining synthesis to be detected.

In the analysis above, it was assumed that an unfolded nucleoid would give a threefold reduction in RNA synthesis compared to the folded nucleoid. This assumption was derived from the results shown by Giorno (21) from *in vitro* studies. It is not known what the comparative difference in RNA synthesis between the folded and unfolded nucleoid would be *in vivo*. The comparative difference could conceivably be much greater. A number of other assumptions have been used in the above analysis which attempts to show quantitatively if the unfolding of the nucleoid could be responsible for the observed comparative failure of RNA synthesis in heat-killed cells. Although the unfolding of the nucleoid may not explain rigorously the comparative failure of RNA synthesis in heat-killed cells, it is the best explanation of those considered. In qualitative terms, the unfolding of the nucleoid as the rate-limiting step in the death of cells could produce a comparative difference in RNA synthesis between survivors and heat-killed cells. Regardless of the magnitude, this difference in RNA synthesis is the kind of difference required by the data of Figure 9.

Unfolding of the nucleoid may have other effects which inhibit RNA synthesis. In vitro studies with negatively supercoiled phage DNA have shown that, within a certain range, RNA polymerase activity increases with increasing negative superhelicity of the DNA (9,56). Although negative helicity may not be a consideration in vivo under normal conditions (59), heating cells may produce conditions which allow negative superhelicity to exist. Under these conditions, single- or double-strand breaks, as shown by Woodcock and Grigg (58), in one of the loops of the nucleoid would cause the loss of the negative superhelix in that loop. Other loops of the nucleoid would not be affected unless they also had breaks occur (34). The loss of negative superhelix in one loop would be expected to cause some inhibition of RNA polymerase activity in that loop. Since there are approximately 50 loops, the inhibition of RNA synthesis in one loop would not greatly affect RNA synthesis. However, if the nucleoid unfolded when a break in the DNA was present in one loop of the nucleoid, negative superhelicity would be lost in the total DNA chromosome. In this case, total RNA synthesis may be reduced due to the unfolding of the nucleoid. This reduction in RNA synthesis may add to the reduction in RNA synthesis due to unfolding of the nucleoid found by Giorno (21). This further inhibition of RNA synthesis due to the unfolding of the nucleoid would enhance the comparative difference in RNA synthesis found between survivors and heat-killed cells.

The reduction of RNA synthesis due to unfolding of the nucleoid in heat-killed cells can also explain the apparent loss of protein synthesis in heat-killed cells shown in Figure 6. If total RNA

synthesis, including mRNA synthesis, is reduced in heat-killed cells, then it would be expected that protein synthesis would also be reduced. The magnitude of the reduction of protein synthesis in heat-killed cells can only be guessed, but the possibility that a reduction in RNA synthesis could lead directly to a reduction in protein synthesis provides the best explanation of the data in Figure 6. The data of Figure 7, showing a time lag in the reinitiation of protein synthesis, support the above explanation. The time lag shown in Figure 7 indicates that some cellular function must be renewed and preceed the reinitiation of protein synthesis. The most likely function to preceed protein synthesis is RNA synthesis, and the data of Figure 10 show that RNA synthesis does preceed protein synthesis in surviving cells. Thus, if it is necessary that RNA synthesis preceed protein synthesis, a reduction in RNA synthesis in heat-killed cells could lead directly to a reduction of protein synthesis.

In conclusion, nucleoid RNA merits further investigation as the site of lethal molecular damage from heating. The requirements of the exponential survival curve, the agreement with what is known about the susceptibility of RNA to heating, and the apparent agreement with the findings of this thesis point in the direction of nucleoid RNA.

# RECOMMENDATIONS

My recommendations center on the "Separation Assay." Particularly important is the resolution of the two populations of surviving and heat-killed organisms. Although the pellet fraction in the "Separation Assay" was quite free of contamination, especially when Triton X-100 was added to the cells during lysis, there was a significant loss of heat-killed cells to the supernatant fraction. I think initial efforts toward improving these experiments should be directed at achieving a greater recovery of heat-killed cells in the pellet fraction. It is obvious from the data of Table 1 that Triton X-100 helps in the efficient recovery in the pellet fraction of heat-killed cells. Therefore, the use of Triton X-100 is recommended in all future experiments.

In addition, better methods of centrifugation should be used. Higher centrifugation speeds would produce an increased recovery of heat-killed cells in the pellet fraction. A constant temperature being held during centrifugation would increase the reproducibility of recovery of heat-killed cells in the pellet fraction.

Another factor which could improve the method and perhaps increase the efficiency of recovery of heat-killed cells is shortening the length of time of the recovery, lysis, and centrifugation treatment. The time of recovery could be shortened by using a more

enriched, yet defined, media during recovery. This media would include bases, nucleosides, and vitamins as well as the amino acids. This same media, rather than minimal media, should be used during the growth of the cells, and possibly during the heating steps. Thus, consistency in growth, heating, and recovery conditions would be maintained. Enriched recovery media will promote rapid recovery of survivors so that penicillin may be used earlier and more effectively.

The centrifugation steps may be shortened also. Centrifugation times of 15 or 20 minutes, rather than 30 minutes, could be tried. It is also possible that Step 7 of the "Separation Assay" may be eliminated entirely which would shorten the procedure. The purpose in shortening the time necessary to separate surviving and heat-killed fractions is two-fold. First, experiments can be done more quickly. Secondly, during the time necessary for separation, heat-killed cells could be undergoing changes that effect the results. Shortening the time of treatment would reduce the chance of these changes occurring.

Another improvement in technique would be the use of an organism which had much more reproducible death kinetics. With such an organism, the time necessary for recovery could be much more closely controlled. The time at which penicillin treatment should be initiated could be more precisely determined. It would also be worthwhile to attempt separation of surviving and heat-killed cells of types of bacteria other than E. coli.

The "Separation Assay" should be able to determine if certain functions continue in heat-killed cells after heating. Both RNA

synthesis and protein synthesis showed a comparative failure after heating. It would be a vindication of the method if a function were measured in heat-killed cells which continued to be expressed for a short time after heating. This would show that the "Separation Assay" did not select specifically for non-functional cells, and that if functions did persist, they would be detected.

The findings of this thesis are consistent with the idea that nucleoid unfolding is the cause of heat-killing. I would suggest that experiments designed to determine directly if nucleoids were unfolded in heat-killed cells should be done.

# APPENDICES

## APPENDIX A

Suppose a living cell can be described as being in either of two states. State A is equated with life, and state B is equated with death. If the probability that a cell dies in any unit of time dt is constant, then we may write this probability as

$$P_d = kdt \tag{7}$$

where $P_d$ is the probability of death and k is a constant.

The probability of survival ($P_s$) is

$$P_s = 1 - P_d = (1 - kdt) \tag{8}$$

The probability of surviving n time periods, each dt in length, or for a time t = ndt is

$$P_s(t) = (1 - kdt)^n = (1 - kt/n)^n \tag{9}$$

in the limit as $dt \to 0$ and $n \to \infty$, we have

$$P_s(t) = \lim_{n \to \infty} (1 - kt/n)^n \tag{10}$$

The right-hand side of this equation is defined as

$$\lim_{n \to \infty} (1 - kt/n)^n = e^{-kt} \tag{11}$$

The total surviving cells in a population at time t is simply the original number of cells times the probability of survival after time t has elapsed or

$$N = N_o e^{-kt} \tag{12}$$

where $N_o$ is the original number of cells and N is the number of cells surviving. Equation 12 is identical to Equation 1.

## APPENDIX B

Assuming that heat-killed cells synthesize protein or RNA, the number of counts appearing in the pellet fraction of any sample depends on the counts due to contamination in addition to the counts due to incorporation of the label. The average number of counts due to contamination for a particular sample (we will use the 10-minute sample as an example) is found to be

$$\text{Average contaminating counts} = \text{APC}\ (C_s + C_k) \qquad (13)$$

where APC is the average percent of contamination from Figure 5 or Figure 11, $C_s$ is the counts in the supernatant fluid for the 10-minute sample, and $C_k$ is the counts in the pellet fraction for the same sample.

To calculate the counts in the pellet fraction that might appear due to incorporation of the label, an assumption must be made about the incorporation rate of heat-killed cells. I have assumed in the text that this rate is equal to or some fraction of the initial rate of incorporation of the surviving cells. The initial rate of incorporation of label for surviving cells ($IR_s$) is determined from the extrapolations shown in Figures 7 and 10. The counts due to incorporation of the label is found to be

$$\text{Incorporated counts} = IR_s\ (N_k/N_s \times PR_p) \qquad (14)$$

where $N_k$ is the number of cells killed, $N_s$ is the number of cells

surviving, and $PR_p$ is the percent of the dead cells recovered in the pellet fraction (Table 1).

The addition of the incorporated counts to the average contaminating counts gives the number of counts that would appear in the pellet fraction of the 10-minute sample, assuming the heat-killed cells synthesize protein or RNA.

# BIBLIOGRAPHY

BIBLIOGRAPHY


1. Adesnik, M., and C. Levinthal. 1970. The synthesis and degradation of lactose operon messenger RNA in E. coli. Pages 451-460 in Cold Spring Harbor Symposia on Quantitative Biology. Volume 35. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

2. Allwood, M. C., and A. D. Russell. 1967. The leakage of intracellular constituents from heated suspensions of Staphylococcus aureus. Experientia. 23:878-879.

3. Allwood, M. C., and A. D. Russell. 1968. Thermally induced ribonucleic acid degradation and leakage of substances from the metabolic pool in Staphylococcus aureus. J. Bacteriol. 95:345-349.

4. Allwood, M. C., and A. D. Russell. 1969. Thermally induced changes in the physical properties of Staphylococcus aureus. J. App. Bact. 32:68-69.

5. Allwood, M. C., and A. D. Russell. 1969. Growth and metabolic activities of heat-treated Staphylococcus aureus. J. Appl. Bact. 32:79-85.

6. Anderson, T. F. 1948. The growth of T2 virus on ultraviolet-killed host cells. J. Bacteriol. 56:403-410.

7. Bachmann, B. 1972. Pedigrees of some mutant strains of Escherichia coli. K-12. Bacteriological Rev. 36:525-557.

8. Bluhm, L., and Z. J. Ordal. 1969. Effect of sublethal heat on the metabolic activity of Staphylococcus aureus. J. Bacteriol. 97:140-150.

9. Botchan, P., J. C. Wang, and E. Harrison. 1973. Effect of circularity and superhelicity on transcription from bacteriophage DNA. Proc. Nat. Acad. Sci. 70:3077-3081.

10. Brannen, J. P. 1970. On the role of DNA in wet heat sterilization of micro-organisms. J. Theor. Biol. 27:425-432.

11. Bridges, B. A., and R. J. Munson. 1968. Genetic radiation damage and its repair in Escherichia coli. Pages 97-188 in M. Ebert and A. Howard, eds. Current Topics in Radiation Research. Volume 4. North-Holland Publishing Co., Amsterdam.

12. Bridges, B. A., M. J. Ashwood-Smith, and R. J. Munson. 1969. Correlation of bacterial sensitivities to ionizing radiation and mild heating. J. Gen. Microbiol. 58:115-124.

13. Britten, R. J., R. B. Roberts, P. H. Abelson, D. B. Cowie, and E. T. Bolton. 1963. Studies of Biosynthesis in Escherichia coli. Carnegie Institution of Washington Publication 607. Washington, D. C.

14. Brown, R. R. W., and J. Melling. 1971. Inhibition and destruction of microorganisms by heat. Pages 4-37 in W. B. Hugo, ed. Inhibition and Destruction of the Microbial Cell. Academic Press, New York.

15. Cashel, M. 1970. Inhibition of RNA polymerase by ppGpp, a nucleotide accumulated during the stringent response to amino acid starvation in E. coli. Pages 407-413 in Cold Spring Harbor Symposia on Quantitative Biology. Volume 35. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

16. Chick, H. 1910. The process of disinfection by chemical agencies and hot water. J. Hyg. 10:237-286.

17. Davern, C. I., and M. Meselson. 1960. The molecular conservation of ribonucleic acid during bacterial growth. J. Mol. Biol. 2:153-160.

18. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. Barry Wood. 1973. Microbiology, 2nd ed. Harper and Row, New York.

19. Dean, A. C. R., and C. Hinshelwood. 1966. Growth, Function, and Regulation in Bacterial Cells. Oxford University Press, London.

20. Dunlap, B. E., and F. Rottman. 1972. The influence of secondary structure on the translation of ribosomal RNA. Biochim. Biophys. Acta. 281:371-380.

21. Giorno, R., T. Stamato, B. Lydersen, and D. Pettijohn. 1975. Transcription in vitro of DNA in isolated bacterial nucleoids. J. Mol. Biol. 96:217-237.

22. Harries, D., and A. D. Russell. 1967. A note on some changes in the physical properties of Escherichia coli after heat treatment. J. Pharm. Pharmac. 19:740-743.

23. Haynes, R. H. 1964. Molecular localization of radiation damage relevant to bacterial inactivation. Pages 51-69 in L. Augenstein, R. Mason, and B. Rosenberg, eds. Physical Processes in Radiation Biology. Academic Press, New York.

24. Hegarty, C. P., and O. B. Weeks. 1940. Sensitivity of Escherichia coli to cold-shock during the logarithmic growth phase. J. Bacteriol. 39:475-484.

25. Iandolo, J. J., and Z. J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bacteriol. 91:134-142.

26. Ingraham, J. L. 1962. Temperature relationships. Pages 265-296 in I. C. Gunsalus, and R. Y. Stanier, eds. The Bacteria. Volume 4. Academic Press, New York.

27. Jackson, H., and M. Woodbine. 1963. The effect of sublethal heat treatment on the growth of Staphylococcus aureus. J. Appl. Bacteriol. 26:152-158.

28. Johnson, R. H., H. Eyring, and B. J. Stover. 1974. The Theory of Rate Processes in Biology and Medicine. John Wiley and Sons, New York.

29. Johnson, F. H., H. Eyring, and M. J. Polissar. 1954. The Kinetic Basis of Molecular Biology. John Wiley and Sons, New York.

30. Joly, M. 1965. A Physico-Chemical Approach to Denaturation of Proteins. Academic Press, New York.

31. Kaback, H. R. 1969. Bacterial membranes. Pages 99-120 in J. M. Lowenstein, ed. Methods in Enzymology. Volume 14. Academic Press, New York.

32. Kaufmann, O. W., L. G. Harmon, O. C. Pailthorp, and I. J. Pflug. 1959. Effect of heat treatment on the growth of surviving cells. J. Bacteriol. 78:834-838.

33. Labaw, L. W., V. M. Mosley, and R. Wyckoff. 1953. Development of bacteriophage in x-ray inactivated bacteria. J. Bacteriol. 65:330-336.

34. Lehninger, A. L. 1975. Biochemistry, 2nd ed. Worth Publishers, New York.

35. Lemcke, R. M., and H. White. 1959. The heat resistance of Escherichia coli cells from cultures of different ages. J. Appl. Bact. 22:193-201.

36. McGrath, R. A., and R. W. Williams. 1966. Reconstruction in vivo of irradiated Escherichia coli deoxyribonucleic acid; the rejoining of broken pieces. Nature. 212:534-535.

37. Miller, L. L., and Z. J. Ordal. 1973. Thermal injury and recovery of Bacillus subtilis. Applied Microbiol. 24:878-884.

38. Moats, W. A. 1971. Kinetics of thermal death of bacteria. J. Bacteriol. 105:165-171.

39. Nakada, D., and M. J. Marquisee. 1965. Relaxed synthesis of ribosomal RNA by a stringent strain of Escherichia coli. J. Mol. Biol. 13:351-361.

40. Patterson, D., and D. Gillespie. 1971. Stringent response of RNA synthesis in E. coli produced by a temperature shift-up. Biochem. Biophy. Res. Comm. 45:476-482.

41. Patterson, D., and D. Gillespie. 1972. Effect of elevated temperatures on protein synthesis in Escherichia coli. J. Bacteriol. 112:1177-1183.

42. Pine, M. J. 1967. Response of intracellular proteolysis to alteration of bacterial protein and the implications in metabolic regulation. J. Bacteriol. 93:1527-1533.

43. Pollard, E. C. 1970. The effect of ionizing radiation on the molecular biology of Escherichia coli. Pages 53-127 in M. Ebert and A. Howard, eds. Current Topics in Radiation Research. Volume 6. North-Holland Publishing Co., Amsterdam.

44. Rahn, O. 1930. The non-logarithmic order of death in some bacteria. J. Gen. Physiol. 13:395-407.

45. Rahn, O. 1944. Physical methods of sterilization of microorganisms. Bacteriological Rev. 8:1-47.

46. Rosenberg, B., G. Kemeny, R. Switzer, and T. Hamilton. 1971. Quantitative evidence for protein denaturation as the cause of thermal death. Nature. 232:471-473.

47. Rosenthal, L. J., and J. I. Iandolo. 1970. Thermally induced intracellular alteration of ribosomal ribonucleic acid. J. Bacteriol. 103:833-835.

48. Rosenthal, L. J., S. E. Martin, M. W. Pariza, and J. I. Iandolo. 1972. Ribosome synthesis in thermally shocked cells of Staphylococcus aureus. J. Bacteriol. 109:243-249.

49. Russell, A. D., and D. Harries. 1967. Some aspects of thermal injury in Escherichia coli. Appl. Microbiol. 15:407-410.

50. Russell, A. D., and D. Harries. 1968. Damage to Escherichia coli on exposure to moist heat. Appl. Microbiol. 16:1394-1399.

51. Rye, R. M., and D. Wiseman. 1967. Some effects of ampicillin on growing and glucose-starved cells of Escherichia coli. J. Pharm. Pharmac. 19:45-85.

52. Sykes, G. 1958. Disinfection and Sterilization. D. Van Nostrand Company, Inc., New York.

53. Tomlins, R. I., M. D. Pierson, and Z. J. Ordal. 1971. Effect of thermal injury on the TCA cycle enzymes of Staphylococcus aureus MF 31 and Salmonella typhimurium. Can. J. Microbiol. 17:759-765.

54. Tomlins, R. I., and Z. J. Ordal. 1971. Precursor ribosomal ribonucleic acid and ribosome accumulation in vivo during the recovery of Salmonella typhimurium from thermal injury. J. Bacteriol. 107:134-142.

55. Tomlins, R., and Z. J. Ordal. 1971. Requirements of Salmonella typhimurium for recovery from thermal injury. J. Bacteriol. 105:512-518.

56. Wang. J. C. 1974. Interactions between twisted DNAs and enzymes: the effects of superhelical turns. J. Mol. Biol. 87:797-816.

57. Wood, T. H. 1956. Lethal effects of high and low temperatures on unicellular organisms. Advan. Biol. Med. Phys. 4:119-165.

58. Woodcock, E., and G. W. Grigg. 1972. Repair of thermally induced DNA breakage in Escherichia coli. Nature New Biology. 237:76-79.

59. Worcel, A., and E. Burgi. 1972. On the structure of the folded chromosome of Escherichia coli. J. Mol. Biol. 71:127.

60. Worcel, A., E. Burgi, J. Robinton, and C. L. Carlson. 1973. Studies on folded chromosome of Escherichia coli. Pages 43-51 in Cold Spring Harbor Symposia on Quantitative Biology. Volume 38. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.