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KINETIC AND THERMODYNAMIC MEASUREMENTS
IN AN AGING MODEL
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KINETIC AND THERMODYNAMIC MEASUREMENTS IN AN AGING MODEL BASED ON THE MAMMALIAN ERYTHROCYTE

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ABSTRACT

KINETIC AND THERMODYNAMIC MEASUREMENTS
IN AN AGING MODEL
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The kinetics and thermodynamics for thermal lysis in red blood cells was studied. This cell is used as a model system for unicellular aging without repair mechanisms. RBC's were separated according to age on a Ficoll density gradient. Younger and older populations of cells were taken off the gradient and their kinetics of thermally induced lysis examined over the temperature range 42-56°C. The kinetics differ between the populations; they are not exponential in form but are best described by Gompertz and power law kinetics. Use of Arrhenius plots give both activation enthalpies and entropies for the young and old populations. These values indicate that protein denaturation is the cause of the lysis event. Use of the Weibull distribution indicates that this event consists of three steps each with the same value for the activation enthalpy. The correlation of the results and implications to other aging systems is discussed.

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INTRODUCTION

One of the first phenomena that is noticed in the study of living matter is that a living system does not remain the same with the passage of time. There are, in fact, dramatic changes involved in the spawning of life, rapid changes during the growth of an organism and slow but noticeable changes as the organism increases in age. These changes are the manifestations of the living system's monumentus task of organizing atoms and molecules from its unorganized surroundings into complex systems. Systems capable of localizing and transferring information, and capable of rebuilding and renewing the organism while constantly battling the surrounding forces which fight this localization and ordering of matter and energy.

The growth of an organism is awesome in its complexity, in its ability to fight the increase of entropy within its boundries and in the rate at which it increases in size. It battles these forces so successfully that when it ceases to grow, when less energy is required to be handled and organized by the organism, it would seem that it would be very easy to continue at a steady state of no growth forever. This is not the case, however, when an organism ceases to grow, it begins to age, to lose capacity to function, and eventually to die. This has bothered mankind longer than the enigma of growth and much time has been spent studying this process of aging and death. The purpose of this thesis is to present one more piece of information in this study of aging and death.

There have been many theories presented over the years on many different aspects of the aging phenomena. A brief review of some of

them will be given later. No new theories will be given here, but some insight into the interrelation of the current ones may be forthcoming.

The original impetus for the following experiment was as a pilot study to investigate the possibility of using the Red Blood Cell as a model for an aging system. This is made possible by the apparent aging of the RBC in vivo over a period of about five months in large mammals. Cells are continually produced in the bone marrow to replace old red cells thus providing constant relative populations of young and old cells. Unlike epithelial cells, which are also present in young through old cell populations in layers of the epithelium, the red cells are easy to obtain and separate. As red cells grow old they become more dense and therefore can be successfully separated on a density gradient.

The model was used to determine if the rate of lysis of the red cell followed first order kinetics or kinetics similar to that of dying organisms such as the Weibull distribution or the Gompertz distribution. It was also used to test the differences in activation enthalpies for lysis of the RBC's between young and old populations of cells.

The death rates of bacteria have been believed to be first order processes because they follow reasonable exponential kinetics. The denaturation of protein at high temperatures also exhibits this. The death rates of the multicellular organisms however have not shown first order kinetics but have been described by the Gompertz function which reasonably represents the sigmoid shape of these mortality kinetics.

Another function, the Weibull distribution, also represents the data equally well. This has been shown on death rates in humans and Drosophila[1]. This distribution was originally introduced by W. Weibull in 1938 to describe fatigue failure in solids and was applied to biological systems by Armitale and Doll (1954)[2] and Rosenberg (1971)[3]. Preliminary work on red cell lysis showed that a sigmoid curve described the process and not a simple exponetial. Therefore, both the Gompertz and Weibull distributions were fitted to the data and their parameters examined for useful characteristics to see if one of the distributions had only one temperature dependent parameter so that a possible activation enthalpy could be determined.

It has been shown that there exists a high activation enthalpy for the death event in fibroblast cells and in drosophila[3,1]. Both of these were obtained by studying the rate of population decline or death over a range of temperatures and then using the Arrhenius relation to obtain an activation enthalpy of the rate limiting step that leads to death. In using a range of elevated temperatures, the aging phenomena is artificially accelerated in these systems implying that they went through a "sped up aging" process taking them from their young state to an old state and finally to death at a faster rate than normal. The activation enthalpy measured is that of the rate limiting step in this process, but it is not known when during the course of the lifespan this step occurs. In the above experiments only young organisms were used. If older organisms were also treated, a difference in activation enthalpy might be apparent if the rate limiting step occurred appreciably before death or if it is composed

of several steps, one or more of which occurred before old age and death. If the rate limiting step or steps occur immediately before the death of the organism then the activation energy for the older organism would be identical to the young.

In order to determine if there is more than one step for death in the RBC model and if there is, whether these steps are spread out or are close together at the end, young and old cells were both treated in the same manner at the high temperature range; the kinetics that best fit them determined; and their respective activation enthalpies obtained.

In brief, the results show that whatever process is occurring occurs shortly before death. The kinetics show a sigmoid shape for both young and old cells and are describable by the Gompertz and the Weibull distributions. The Weibull distribution has only one temperature dependent parameter which allows the use of the Arrhenius equation to determine an activation enthalpy for each age. The activation enthalpies obtained appeared to be identical for young and old and were of a very high value. The other parameter of the two parameter Weibull function suggests a 3 step process is occurring and the activation enthalpy of each step for both young and old are the same. If one assumes that each step in this process is a first order reaction then an activation entropy can be calculated. The corresponding activation enthalpy and entropy for each step resembles those that are associated with protein denaturation suggesting that alterations of macromolecules are involved in the process. This could be the breakage of covalent bonds or one of more hydrogen bonds.

In the following discussion, I will give some background into the aging phenomena and some of the theories dealing with aging and death. This will lead into a description of the influence of temperature on aging, the usefulness of the activation enthalpy, and a discussion of Gompertz and Weibull kinetics. Then the RBC model will be presented and the experiment performed on the model followed by detailed conclusions and discussion.

CHAPTER I

AGING

The theories of aging can be broken down into a series of four hierarchical steps: systematic aging, cellular aging, molecular mechanisms of aging, and the fundamental processes dictating these mechanisms.

Systematic aging deals with the gross changes in the organism with time, such as the physiological decline in the organs and tissues, the susceptibility to diseases and their permanent impairing effect on the organism, and the rate of mortality and survival in a population. Cellular aging attempts to explain some of the above by examining the different types of cellular aging and death. The cells of organisms are basically divided into those cells which do not divide at all, those which divide for a number of divisions and then stop and those which appear to divide indefinitely. Degradative processes affect the first two by decreasing the performance capability of the cells and reducing their numbers in the organs which results in a loss of physiological potency characterized by systematic aging. Theories of molecular mechanisms have been postulated to explain these observed cellular changes. These are divided into two areas, the deterioration theories and the pleiotropic theories. The deterioration theories are those that consider somatic mutations, the ever increasing accumulation of error in protein synthesis, and the spontaneous denaturation of macromolecules as the major causes of dysfunction

and death of cells. The pleiotropic theories hypothesize that the genomic expression is advantageous to the organism during development but these same genes produce harmful products in the later stages of life, leading to the death of the organism. This means that the cells are preprogrammed to die and this is how these theories explain the cell death and loss of function. The fundamental processes that underlie these mechanisms seem to fall in two general areas; the intrinsic inability of a living system to function in a steady state situation forever because of the laws of nature and the extrinsic, environmental factors that bombard the organism resulting in damage. Very little work has been done on learning more about these fundamental processes, but the correct perspective of the whole aging phenomena depends on determining the general underlying causes of the mechanisms involved.

Systemic theories in multicellular organisms usually consider generalizations of how combined cellular behavior can result in aging and death. They site such changes as the loss of cells in organs, the decrease in proliferative capacity of germ lines, the transformation of cells, and the susceptibility to disease. These are changes that are characterized by a decrease in physiological function and this is assumed to result in the eventual death of the organism. This loss of vitality follows a linear rate of decline and has been shown to exist for many organs and their function (eg. basal metabolic rate, cardiac index, vital capacity, standard renal plasma flow, maximal breathing capacity)[5]. These different systems show varied changes with time and it is hard to suggest which system is more

important, if any, or how they all fit together to result in the total aging picture. Models have been proposed that deal with this on a theoretical level. They correlate this linear decline in function to the mortality rate which is an easily measurable quantity and a direct consequence of the total aging phenomena.

Strehler[6] gives a good review of the theories of Brody-Failla[7], Simms-Jones[8], Simms-Sacher[9], and Strehler-Mildvan[10]. These theories analytically attempt to derive the Gompertz function of mortality for human beings assuming different aspects of physiological decline and the effects of environment. (More will be discussed about the Gompertz relation in Chapter Two.) Other theories have correlated the onset of diseases to the observed mortality. Multiple factor theories of disease and aging have been formulated by Armitage and Doll[2], Burch[10], Burnet[11], and Curtis[12]. They suggest that degenerative diseases are caused by a sequence of n steps (usually 6) and that these lead to death. This predicts a power relation for the mortality kinetics which is related to the Weibull distribution (also to be discussed in Chapter Two).

These theories do not suggest any mechanisms that might be responsible for the degeneration of cells and tissues but they only try to reproduce observed kinetics of mortality.

The theories of cellular aging are very closely allied to those dealing with the molecular mechanisms. There has literally been an ad nauseum cataloging of every cell and system and macromolecule in living organisms of a wide variety to see if they are affected by aging. What this has shown on the cellular level is that there are

three basic modes that cells can be in, one in which they do not divide at all, one in which they divide for a specific length of time, and one in which they appear to divide indefinitely[13]. The cells that do not divide and those with limited proliferative capacities are believed by almost everyone in the field to be the cause of systemic breakdown. They are the ones that collect damage and if not replaced permanently impair the tissue. Disagreement enters the field when investigators try to explain why there are these three types of cellular behavior and why degradation has the affect it does in two of the cases.

Many theories of specific mechanisms have emerged. The most important ones are the somatic mutation theory, the Orgel error hypothesis, and preprogrammed senescence.

The somatic mutation theory postulates that spontaneous mutations occur in the somatic cells, causing them and their progeny to perform a different function or no function at all. This results in diseases of dysfunction and malfunction in the tissues which then leads to death. Only cells which are dividing at fast enough rates to always allow for cellular selection are unaffected. Cells which do not divide build up damage and cells which are dividing slowly collect sufficient damage in every cell and cellular selection cannot occur. These cells become impaired and die[14].

Orgel first proposed that errors in protein synthesis, once having started, can be an escalating process which leads to cell death[15]. He proposed that random errors in proteins begin to build up and cause the formation of faulty protein synthesizing machinery which then leads

to proteins that contain more faults. Holliday and Tarrant[16] gave evidence to support this. They have found that diploid human fibroblasts accumulate heat labile enzymes as they age. That there is an increasing fraction of these enzymes that show this with age and a decreasing fraction that appear to be normal. Orgel concluded that if cells were dividing fast enough damage to proteins could be small enough and diffuse enough to allow somewhat damaged protein to synthesize protein with less damage. This would explain the ability of some cell lines to undergo divisions indefinitely. If the rate was too slow the cell line would eventually build up lethal amounts of damage. If the cell was not dividing at all, then the rate of protein synthesis would be the determining factor, but even that could not protect the DNA from damage if it wasn't being replicated and so the cell must have a finite lifetime[17].

Programmed cell death was first suggested by Hayflick[18], when he showed the limited number of divisions in fibroblasts. It has been adopted by many others to explain the phenomena of cellular aging. Some cells live for only a matter of hours while others live for years, this follows directly from an idea of programmed lifespan. Franks[13] argued that the short lifespan of certain cells is due to the fact that they are the product of the last division of a differentiating cell line. They exist for a while and then they die (white blood cell for example). Cells such as nerve cells are thought to be caught in an intermediate state in the cell division sequence. They live a long time because they have not been able to divide the necessary number of times.

Work done by Dell'Orco supports this hypothesis[19]. He took several diploid cell strains and showed that they can be arrested by alterations in their medium for as long as three or four times their normal lifespan and then returned to normal growth. When they finally die they attain or surpass the controls in the total number of population doublings. This indicates that in vitro it is not the length of time the cells are alive that determine their lifespan but the number of divisions.

All of these theories have many supporting experiments and this is probably because they all have some truth and value. They don't seem to answer the question of the underlying cause of aging, however. They do not deal with what caused the mutations, the original protein error, or the molecular basis of the programmed death mechanism. Of all the possible intrinsic and extrinsic factors that could be responsible for these it is possible the one stands out and is truly the one that must be dealt with and is the one that ties all these theories together.

Intuition suggest that either there is an intrinsic inability of a living system to function forever due to the laws of nature, or an inability to fight the environmental insults indefinitely, or perhaps both. The answer to this inquiry lies in the physical laws of the universe and this then would give the fundamental processes involved in aging and death.

The empirical laws of thermodynamics describe a living system as an energy-entropy system far from equilibrium with respect to the environment. Living matter has two functions, therefore, one is to

perform the required metabolic processes and the other is to fight the increase in entropy to maintain structure and organization. In order to do this it must manipulate both matter and energy. Matter, even in its quantum mechanical sense, is localized and hence more easily controlled than energy which has many forms and is not easily contained or directed. The statistical distribution of energies, for which the measurement of temperature only represents the mean, continually contains high energy fluctuations in the extremes of the distribution. These are always a nuisance to the living system's organization and functioning tasks. These energies are high enough to break bonds or denature macromolecules. There is no way to control this intrinsic nature of energy, so organisms have relied on replacement of damaged parts and elaborate removal of their deteriorated components. Even this is only a respite and so the process of reproduction has evolved to continue where the individual organism fails.

Extrinsic insults are also thrown at living systems. These come in many forms but the two most dangerous are UV light of the sun and the chemical poisons in the environment. Since UV light has been present through all of evolution, organisms have successfully dealt with its damage at normal levels of intensity. Higher exposures to this radiation, however, are quite lethal. The potential number of chemicals that can poison the organism are almost limitless. The enzymes of the cell, although very specific, can be permanently blocked by substrate analogs or can be induced into action on foreign substrates resulting in products that are even more harmful

or in the excess build-up of extraneous material in the cell. Here again, there seems to be no escaping the continual attack of these poisons because every system of the organism is vulnerable to one degree or another.

The effects of extrinsic insults upon aging are not examined in this study. Rather, the intrinsic factors of the mean temperature and the fluctuations around this temperature are of main concern to determine their influences on the aging process.

Temperature has long been known to be important in the optimal performance of biological systems. In single cell organisms, tissue cultures, and poikilotherms the alteration in temperature is known to affect the aging rate and the total lifespan. In homeotherms the temperature is held constant to within a few degrees. Increases in this optimal temperature can lead rapidly to death of the organism, while decreases can be tolerated much more by the homeotherm. Since the average body temperature is constant except for cases of disease and dysfunction, changes in the mean temperature are not important in the aging phenomena in homeotherms. Localized changes in temperature within the organism, however, may play a role in this phenomena.

Within organisms there exists the possibility that the temperature varies from organ to organ and tissue to tissue depending on the metabolic rates of these tissues. Sacher[21] suggested that the organisms ability to distribute heat evenly throughout its whole system is directly related to the lifespan of the animal. He mentioned the correlation of lifespan of different animals to their

brainsize, this being the important difference that dictates the ability of an organism to control its internal environment to minimize the unequal distributions of temperatures. The cebus monkey and the opossum are the same body weight so their heat dissipation to body ratio is the same and hence they would expect to metabolize at about the same rate, and yet the cebus monkey lives eight times longer than the opossum. The fact that the cubus monkey has a brain which is 70g. as compared to the 6g. brain of the opossum suggests that it has very highly developed methods for dealing with temperature regulation. The quick and efficient equalization of heat within the organism keeps highly active cell groups or organs from operating at unusually high temperatures which would lead to their deterioration at an accelerated rate.

A further question involving a macroscopic thermal phenomena is the heat flux through tissues and individual cells. The ratio of surface area to volume in an organism and the temperature gradient from inside to outside determine the flow of heat through the tissues of the organism. This heat flow in small organisms would be greater than in larger organisms even though the temperatures would be close to the same. Similarly, when the external temperature is low the gradient is high and the heat flux increases because metabolism increases to counteract heat loss from the skin. The effect of changes of heat flux under constant temperatures as related to aging is an area that needs to be studied.

Another intrinsic factor which the organism has no control over is the microscopic distribution of energies around the temperature

mean. This is a distribution in both space and time and hence at any microscopic point in the system fluctuations occur in the energies with time. An experiment of Johnson[20] showed that the fluctuations in the thermal bath can explain the observed rate of death in fibroblast cells at physiological temperatures. This suggests strongly that the most important factor is the living organism's fight against the inherent noise in the thermal energy distribution of its own constituent parts. This distribution of energy is the Boltzmann's distribution representing the frequency of energy states above and below the mean energy level which is defined as temperature. The high energy fluctuations inherent in the upper regions of the distribution are microscopic localizations of high energy states distributed randomly in space and time within a homogeneous system. This suggests that at the right time such a fluctuation could occur at such a location within a macromolecule that a covalent bond is broken. This could lead to the denaturation of the molecule and its inactivation. The denatured macromolecules can lead to mutations, they can lead to protein synthesis error, and they could lead to the expression of masked genes. Due to the rarity of the coincidence of these low probability high energy states occurring at the right place and at the right time the denaturation of macromolecules would be a slow but still significant process as shown by Johnson. It is even possible, as he suggests, that they account for the majority of the age related deterioration that is observed in cells.

To obtain a comprehensive view of aging and death, therefore,

metabolic processes must be examined, the effects of temperature and heat flux must be determined, and theories that deal with temperature fluctuations need to be formulated and tested. Chapter II shows some of the work done already in the area of temperature and high energy fluctuations.

CHAPTER II

KINETICS AND THERMODYNAMICS

TEMPERATURE

Temperature is an extremely important factor in the process of life on this planet. A small change in the temperature can cause drastic changes in organisms and whole eco-systems. It was said once that if the mean daily temperature of the earth was changed by 20°C either up or down, all life would perish. The chemical reactions contained in organisms are very temperature specific and speed up or slow with increases and decreases in temperature. Consequently, in lower organisms, their metabolic rates vary up and down with the temperature. This seems to be an acceptable condition for lower forms of life, but most higher forms of life seem to need a stable temperature for the proper functioning of the many, many interreactions of their parts. They have evolved separate temperature control mechanisms to keep their bodies at a constant temperature level. Not surprisingly, this has allowed these organisms to be more versatile in terms of habitable climates and total activity. This has given mammals a tremendous advantage in the development of the world animal population. It is probably through this homeostasis that hemo sapiens was able to evolve to its present level. An excellent review of temperature, its affects in biology, and temperature optima is given by Johnson, Eyring, and Pollisar[22].

The point of interest in this discussion is how does temperature affect aging and more importantly, how can this be used to learn more

about the aging process. As was discussed in the last chapter, the one factor that seems to link the whole aging process together is the idea of localized "temperatures" being so high as to damage the cells and the organism. It has been known that changing the temperature of poikilotherms changes their lifespan. This has two effects: the first is that as the mean thermal level changes the high energy fluctuations that are always present in a distribution of energies also change in number. These fluctuations are responsible for the denaturation of macromolecules. The second effect is the increase in the metabolic rate which increases the number of hot spots in the organism. These are different from the normal high energy fluctuations that are present in an assumed uniform distribution. It is the combination of these two processes that result in shortened lifespan when the temperature is increased.

In order to give evidence to all this, it needs to be shown that improbable fluctuations can lead to observed mortality rates, that temperature changes are tied to changes in rates, and that macromolecular denaturation can be linked to death or similar catastrophic events associated with aging in the cell or organism.

In order to accomplish this I will present some work that has been done along these lines in the area of energetics and in relating mortality rates to improbable events through the theory of extreme values. In the next chapter I will present the red blood cell model and the experiments which show that the catastrophic process of cell lysis resembles mortality in multicellular organisms and that the energetics suggest protein denaturation.

ENERGETICS

The absolute rate theory establishes a relation between the rate of a chemical reaction and temperature. This is given by:

$$K = \frac{kT}{h} e^{\left[\frac{\Delta S^{\ddagger}}{R}\right]} e^{\left[\frac{-\Delta H^{\ddagger}}{RT}\right]}. \qquad (1)$$

Where K is the rate, k is Boltzman's constant, h is Plank's constant, R is the gas constant, $\Delta S^{\ddagger}$ is the activation entropy, $\Delta H^{\ddagger}$ is the activation enthalpy, and T is temperature. $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ represent constants of the system which determine the rate of the system. These are important to determine because they represent a connection to the mechanism involved in the reaction. In an aging system, these constants can give a clue to what processes are responsible for aging. The problem with the aging system is that it is a very complicated system and absolute rate theory was derived for simple systems.

Crozier and collaborators[22] methodically applied eqn (1) to many biological phenomena and determined that the relation held almost invariably. They determined the constants $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for such processes as heart beat, cricket chirping, luminescence of bacteria, oxygen consumption in the clam, division rate of the sea urchin, the hydrolysis of sugar, the alpha rhythm in the cortex of humans, and a multitude of other complicated processes. The activation enthalpies determined were usually between 10 - 30 Kcal/mole. Equation (1) has also been applied by others to the death rates of fibroblasts, yeasts, bacteria, Drosophila, and other poikilotherms. In all cases, an activation enthalpy was determined, ranging from

25 Kcal/mole to 200 Kcal/mole[23].

These high activation enthalpies for the death rates indicate that a very low probability reaction is occurring. They are rare with respect to the normal processes of an organism, such as those measured by Crozier, which have activation enthalpies much lower, around 10 - 12 Kcal/mole. It also suggests that a denaturation process is occurring, such as the denaturation of protein.

Rosenberg et al. gave further evidence to the idea of protein denaturation as a cause of death[23]. They showed that the activation enthalpies and activation entropies of both the rates of protein denaturation and mortality followed a simple compensation relation, given by:

$$\Delta S^{\ddagger} = \frac{1}{T_c} \Delta H^{\ddagger} + b \quad . \qquad (2)$$

Where b and T_c are constants equal to approximately -65 cal/mole/K°, and 330°k respectively. This is the first quantitative evidence relating the normal death of an organism to a molecular process. An improbable event like death follows well from an improbable event like macromolecular denaturation.

RATE FUNCTIONS

Use of eqn (1) for relating the mortality rates of organisms to temperature is good only if the measured rate is a constant with time, more explicitly, a first order rate constant. Unfortunately, there are many mortality rates that vary with time. In fact, most multicellular organisms and what now appears to be some unicellular organisms show mortality rates that vary with time. In 1825, Gompertz presented a new function to describe human mortality curves, which has been used widely since then to describe mortality rates for a variety of organisms. The Gompertz function[24] relates the mortality rate, $\mu(t)$ to an exponential function of time. It is given by:

$$\mu(t) = \frac{1}{N}\frac{dn}{dt} = R_o e^{\alpha t} \quad , \qquad (3)$$

where R_o and α are constants. The parameter α has the dimensions of a rate constant and hence would be the logical choice for eqn (1). This does not prove satisfactory, because the parameter R_o is also temperature dependent and also has the dimensions of a rate constant.

Another equation has been used (25, 26) which is basically an alteration of simple first order kinetics. The simplest way to derive it is to consider the probability of an operational site being incapacitated, call it q. Therefore, $q=1 - \exp(\alpha t)$, if simple first order kinetics are assumed. If n sites need to be incapacitated before death, then the fraction of survivors, f, is:

$$f = 1- (1-e^{-\alpha t})^n$$

This equation has the benefit of attempting to describe a multistep

reaction and it contains a rate constant, α, directly relatable to a first order rate constant and only it would be temperature dependent. However, this formulation only seems to fit death rates of simple organisms. At large t the function approaches an exponential of slope $-\alpha n$. This is not seen in the mortality curves for multicellular organisms. Figure 1 shows the survival curve for human beings[26]. Here, the log of the fraction of survivors is plotted versus time. It is obvious that no portion of this curve at large times is a reasonable straight line. Figure 2 shows similar plots for Drosophila[27]. Here again, as time increases, the rate constant (slope) continues to increase.

There is a subtle problem with the formulation of eqn (4). It assumes an exponential distribution for a reaction as if it was a separate reaction, a common reaction in the cell. But what is actually occurring is an aberent reaction, a low probability case of a larger group of possible ones. The deactivation of a site is the result of a very high energy fluctuation of an improbable nature causing a "normal" reaction to occur out of place and out of time. The study of this kind of phenomena is called the statistics of extremes or the theory of extreme values. It is used to describe those phenomena which do occur but only very, very seldom. The theory describes the distribution of rare events in an ensemble. A population of organisms is a set of almost exact copies of a single system, it is an ensemble. The distribution of rare events in the population, resulting in death, is thus a distribution of extreme values in an ensemble. It is this theory that must be considered

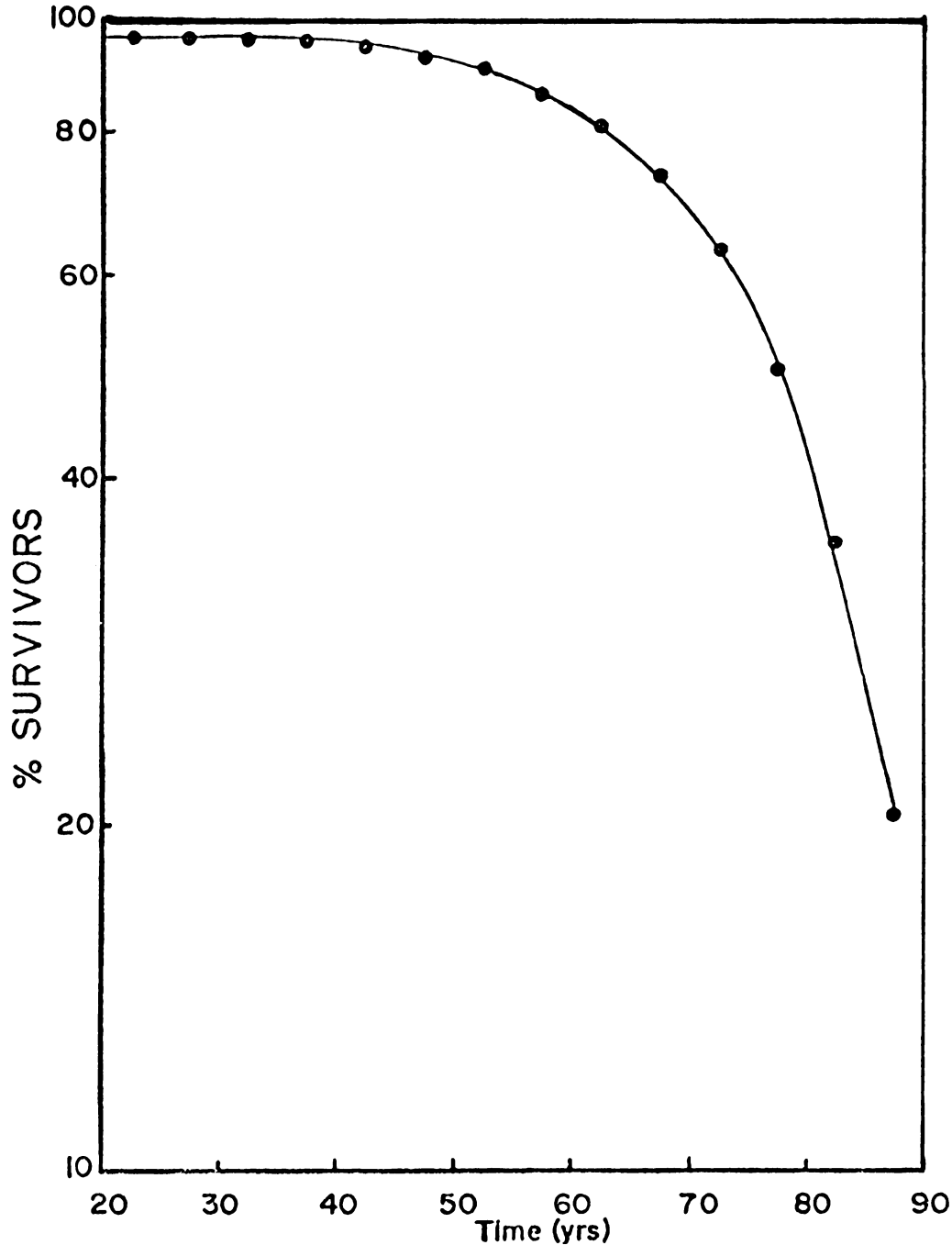

Figure 1. Percent human survivors versus age

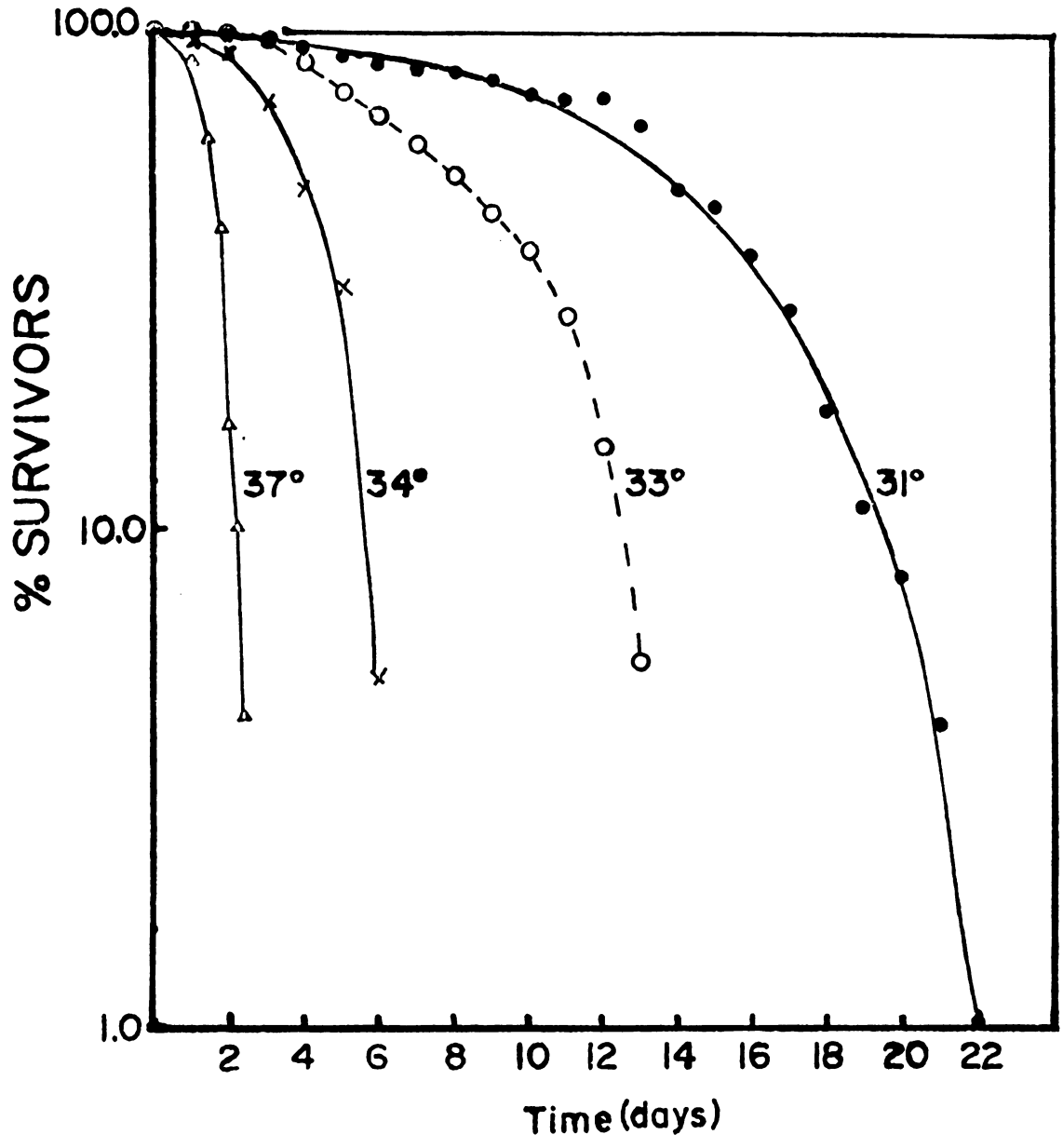

Figure 2. Percent Drosophila survivors versus age for four different temperatures

in describing aging and death.

There are three asymptotic distributions in the theory of extreme values[28]. The derivation, stability criterion, or detailed discussion of each of these is beyond the scope of this paper. Two of these distributions are of interest, however, in describing the phenomena of aging populations:

$$\lambda(t) = 1 - e^{-e^{t}} \tag{5}$$

and

$$\psi(t) = 1 - e^{-t^{\alpha}} \tag{6}$$

Where $\lambda(t)$ and $\psi(t)$ represent probabilities, t is time and α is a constant. The corresponding mortality rates from these are:

$$\mu(t)\big|_{\lambda} \propto e^{\alpha t} \tag{7}$$

$$\mu(t)\big|_{\psi} \propto \alpha t^{\alpha-1} \tag{8}$$

Skurnick[29] showed that a simple model can relate the linear decline in function to the distribution for death shown in equations (7) and (8). The model is represented by N linked systems each able to receive damage randomly. As the damage builds up on each link the probability increases for the disfunction of that link. The combination of this increasing probability and the N independent systems each capable of acting similarly results in the system following either equation (5) or (6) for the probability of the disfunction of a link and thus the death of an organism. The build-up of damage to each link is hypothesized to be a multistep process with the rate constant for each of these steps equal. When n steps have occurred the link is highly prone to break. With less than n

steps, probability is finite but not as great. These combined n steps constitute the rate limiting process for the breakage of a link and the death of the organ or organism. As each link collects damage randomly this represents the linear decline in physiological function because the chain of N links is weaker, i.e. less damage could result in the breakage of a link.

This model is able to imply the observed kinetics (eqn 7 and 8) through the use of the theory of extreme values, but the converse is not necessarily true. It cannot be assumed those hypotheses follow from the fact that the data fits eqn (7) and (8). In particular, the model suggests that the rate limiting process is characterized by n steps. It is worthwhile to investigate the data in such a way as to assume n steps and then see if this assumption leads to information which correlates to other knowledge. This would lend support for the model but it would not imply its truth.

Data of mortality rate fit both equation (7) and (8). Equation (7) is the function used by Gompertz and equation (8) is the Weibull distribution. The Gompertz distribution has been used for many years for the description of mortality but the Weibull distribution has not. It first was used by W. Weibull in 1938 to describe distributions of breaking strength and life expectancy in mechanical systems[30]. It wasn't until many years later that the equation was used for life expectancy distributions in living organisms. Curtis as well as others used a modified form of the Weibull function to describe the effects of radiation on biological systems. He also suggested a model system of aging which is describable by the Weibull

distribution[14]. Rosenberg et al. used this distribution to analyze the mortality rates of humans and Drosophila. They showed that mortality rates could be described by this function equally as well as by the Gompertz equation. They also showed that, if the formulation:

$$\mu(t) = At^{n-1} \tag{9}$$

is used, the A parameter is the only parameter which is temperature dependent. This allowed an attempt to relate A to $\Delta H^{\ddagger}$ though an Arrhenius type equation

$$A = A_o \, e^{\frac{-\Delta H^{\ddagger}}{RT}} , \tag{10}$$

where K_o contains the first two factors of eqn (1). The results showed that A behaves like K giving a $\Delta H^{\ddagger}$ constant equal to 190 Kcal/mole for Drosophila. The A parameter does not have the dimensions of rate, however, and so a $\Delta S^{\ddagger}$ could not be determined.

They also found that the n value which is not temperature dependent is a low valued constant that could be an integer. This, unlike the Gompertz function, suggest a tie to the model of Skurnick. If n is assumed to be representative of the number of steps in the rate limiting process, then a logical further assumption is that each step of this process is represented by a first order rate constant. For the combined process to be the rate limiting process each step must, therefore, be equal or, in other words, each rate constant must be identical. If $1/\tau$ is this rate constant, then equation (6) becomes:

$$\psi(t) = 1 - e^{-(\frac{t}{\tau})^n} \tag{11}$$

and equation (9) becomes:

$$\mu(t) = \frac{1}{\tau} \left(\frac{t}{\tau}\right)^{n-1} \qquad (12)$$

The A parameter in (9) is thus equal to $(1/\tau)^n$. $1/\tau$ is a first order rate constant and can be used to determine a new $\Delta H^{\ddagger}$ from equation (10) and a corresponding $\Delta S^{\ddagger}$ from equation (1). These represent the activation enthalpy and entropy of each step. Whether or not these values have any meaning is a question that must be answered by determining their values for different systems and evaluating their reasonability.

The experiment described in Chapter III determines these values for the lysis process of the red blood cell and shows that the Weibull distribution is a useful method for examining this process and that the formulation of equation (12) represents a physically meaningful function to work with.

The discussion of this chapter has shown that there is already work showing that highly energetic, highly improbable events result in what appears to be molecular denaturation and that this is responsible for mortality. Also an uncommonly used equation, the Weibull function, which has its origins in the distributions of extreme values needs to be examined for possible use in the description of mortality. It appears to have parameters that are more meaningfully representative of physically tangible quantities than the more commonly used Gompertz function.

CHAPTER III

THE EXPERIMENT

THE EXPERIMENTAL MODEL

It was discussed in Chapter I that there are probably many causes of aging. All of those that have been studied and all of those on which models have been based are probably real phenomena in the realm of aging. Some of them appear to be only symptoms and some are truly causal, but all stem from the problem the organism has in dealing with environmental energies that are spontaneous, erratic, and from uncontrollable causes. These include high energy radiations, low energy radiations (heat fluctuations) free radicals, highly reacting foreign molecules, nuclear decays etc. The purpose of this experimental model is to show that thermal energies can cause a population of cells which are not reproducing to accumulate damage and to cease functioning with kinetics that are very similar to death kinetics of other closed, non-reproducing organisms: to show that increasing temperature effects the survivor kinetics of the model organisms in a manner that can be described by the theory of rate processes: moreover, to show that an activation enthalpy, $\Delta H\dagger$, exists for the "death" event in the model implying a molecular event as key to "death" and is of the same high magnitude as that for poikilothermic organisms. (Since the temperature must be varied within the organism to determine the activation enthalpy homeothermic organisms cannot be compared.)

The model system is the mammalian red blood cell. It has been shown to be an aging system, with a life span of about 5 months in mammals. It does not divide nor even replace its own molecules if they

become inactive because it contains no nucleic acid. It is a closed system that simply decays with time and is replaced when considered by the organism to be less valuable than the space it occupies. It makes a good model because it is a continuously decaying system very much like multicellular organisms and like most multicellular organisms, it does not divide and lose its identity. It has the simplicity of a single cell with the general characteristics of an aging multicellular organism.

In order to follow the rate of death in the RBC a useful characteristic is needed that can be easily measured in a population of cells. There are many characteristics of the cell which could be examined for this decline. Since this experiment was designed as a pilot, the most conveniently measureable one, the lysis of the cell membrane, is the one chosen. The proteins, lipids, glycoproteins, etc. in the membrane are undergoing continuous degradation as are the other components in the cell. Therefore, the ease at which the membrane will lyse increases with damage and time. This has been shown by others in measuring the membrane fragility of RBC's.[31,32,33,34]

The plan of the experiment is to follow the rate of lysis in a population of cells over a range of temperatures. The curves that describe the kinetics at each temperature will give some clue as to the type of process occurring, eg., whether it is a first order reaction or a multistep process. Following the change in these curves over a range of temperatures will give an indication of whether or not an activation energy exists for the process of cell

lysis. This will be done by using the Arrenhius equation relating a temperature dependent parameter from the kinetic curves to the inverse of temperature. This will all be done for two populations of RBC's, one young and one old, in order to see if there are any differences in young and old lysis kinetics and if the activation energy changes for this process.

THE EXPERIMENT

The experiment contains two parts: the verification of the separation of red blood cells according to age on a density gradient, and the kinetic studies of following the population decline of the cells at different temperatures.

I. Separation and Verification Methods

A Ficoll density gradient was used to separate sheep red blood cells following the procedure outlined by Rahman et al[35]. To assure the technique was producing the desired results, ^{59}Fe-citrate was used as a measurable parameter for the distribution. This is selectively taken up in newly formed RBC's and remains with a cell until it is removed from the blood stream. A pulse was given allowing for a labeled population of cells that could be followed as they age. If the gradient is successfully separating cells according to age, a band of radioactive cells should move from the less dense regions of the gradient to the more dense region as these pulsed cells age.

(1) ^{59}Fe Incorporation into Erythrocytes

A one year old sheep was obtained from the Michigan State University Sheep Barn and was used as source of blood. The sheep was given a single dose of ^{59}Fe-citrate injected into the jugular vein. Approximately 0.4 mCi was dissolved in isotonic saline to produce total injection volume of 1.0 ml.

(2) Blood Preparation

The sheep was exsanguinated at regular intervals in the course of the life span of the erythrocyte. This was also done through the jugular vein. The blood was collected into a heparinized vacutaner

and stored at 4°C until ready for use. The whole blood was diluted approximately 2:1 with a balanced buffered saline solution (BBS)[36] and this was used for the gradient centrifugations.

(3) Ficoll Gradient Preparation

Ficoll was dissolved in the same balance buffered saline solution as above. Non linear gradients were made using the gradient maker designed by Bock and Ling[37]. The gradient ranged from 50% Ficoll at the bottom of the gradient to 28% at the top. The absolute density ranged from 1.173 - 1.096 g/cm^3. A 1.0 ml sample of the blood preparation was layered on the top of the gradient and the gradient was spun on a Beckman L3-40 ultracentrifuge at 15000 rpm for 90 minutes. Longer centrifugation time did not change the pattern of banding. After centrifugation 3 ml fractions were taken from the bottom of each tube with the use of a narrow aspirator needle lowered to the bottom of each tube.

(4) Measurement of the ^{59}Fe Radioactivity

Each fraction was washed and the blood cells spun down into a pellet. These were then digested with Protosol and a fluorescent cocktail was added. This was counted on a Packard Tri-Carb Scintillation Spectrometer for ten minutes with background subtracted from each sample.

II. Kinetic Studies of Population Decline

(1) Blood Preparation

A four year old ovine ewe was used as the source of red blood cells. It was exsanguinated from the jugular whenever blood was needed. The blood was collected in a 10.0 ml vacuutaner containing 1.4 ml of a

whole blood preservation CPD (.016 M citric acid; .016 M monobasic sodium phosphate; .14 M glucose; .089 M sodium citrate). This was to preserve the freshness of the cells for future use and also acted as an anti-coagulant. A sample of this blood was diluted approximately 2:1 with BBS and layered on gradients ranging in density from 42-24% Ficoll and absolute density 1.145 - 1.082 gm/cm^3. The gradients were fractioned into 12 fractions which were kept at 4°C while they were examined and washed and stored.

(2) Separation of Young and Old Populations

The index of refraction was determined for each fraction of the gradient. The fractions were then washed and resuspended in BBS and the absorption of each determined on a Bausch and Lomb Spectronic 20 at 540 nm. This determined the distribution of the red cells on the gradient and correlated them to specific densities via the index of refraction. The peak occurred at an index of refraction of approximately 1.3800, and the band stretched from about 1.3720 to 1.3880. A fraction was taken with an index of refraction of about 1.3750 for the young fraction and one from about 1.3850 for the old fraction. These were then washed and stored in BBS at 4°C.

(3) Determining Population Lysis Kinetics

A water bath with a Fisher Unitized Bath Control was used to keep the temperature accurate to within $\pm$ .01°C. The red cells were diluted to approximately 1/1000 of normal blood cell concentration or about 4×10^6 cells/ml. This suspension was placed in a 1 gram vial with glass beads which were used for stirring purposes and this was placed in the water bath. The time was monitored and samples were

removed at intervals. Approximately 50 µl of the blood cell suspension was removed at each sampling and was placed on a Bright-Line hemocytometer and counted under a microscope. The experiment continued until less than 5% of the starting number of cells remained. This was repeated for both young and old and for each temperature. The temperature range was from 42°C - 56°C.

(4) Computer Fitting

A multipurpose curve fitting program, KINFIT, from the computer library of Michigan State University was used on a CDC 6500 to determine the best parameters for the Gompertz, Weibull, and Arrhenius functions. All results and curves shown are least squares best fit to the data. The differential forms of the Gompertz and power law were used (eqns. 7 and 8) and were integrated numerically by the computer program. For the Arrhenius equation the best fit of eqn. 10 was given to the data.

RESULTS

I. Age Separation and Gradient Reliability

In order to determine if the gradients were reliable in separating cells according to density irregardless of age, cells from two fractions, one high density and one low, were relayered and respun on separate gradients to see if they banded at the same densities from which they came.

Four gradients were used in order to obtain enough cells. Fractions with indexes of refraction approximately equal to 1.3780 were pooled and washed and another set was taken and pooled with an approximate index of refraction of 1.3860. These represent fractions from the top and bottom of the red cell band respectively. These were then layered on separate gradients and spun as mentioned in methods. The gradients were then fractionated and the results are shown in Figure 3.

The solid and dashed lines show the decrease in index of refraction and density with movement up the gradient. They represent two gradients and show their reproducibility. The densities of the pooled fractions from the original gradient are indicated by where the vertical bars cross these two lines. The two peaks show where those fractions banded on the second gradients. It is clear that the cells have returned to their original density and that there is little overlap between these two populations. Since these two densities are approximately those used later as young and old respectively, it is important that they do not overlap markedly.

In order to determine if the gradient is separating cells according

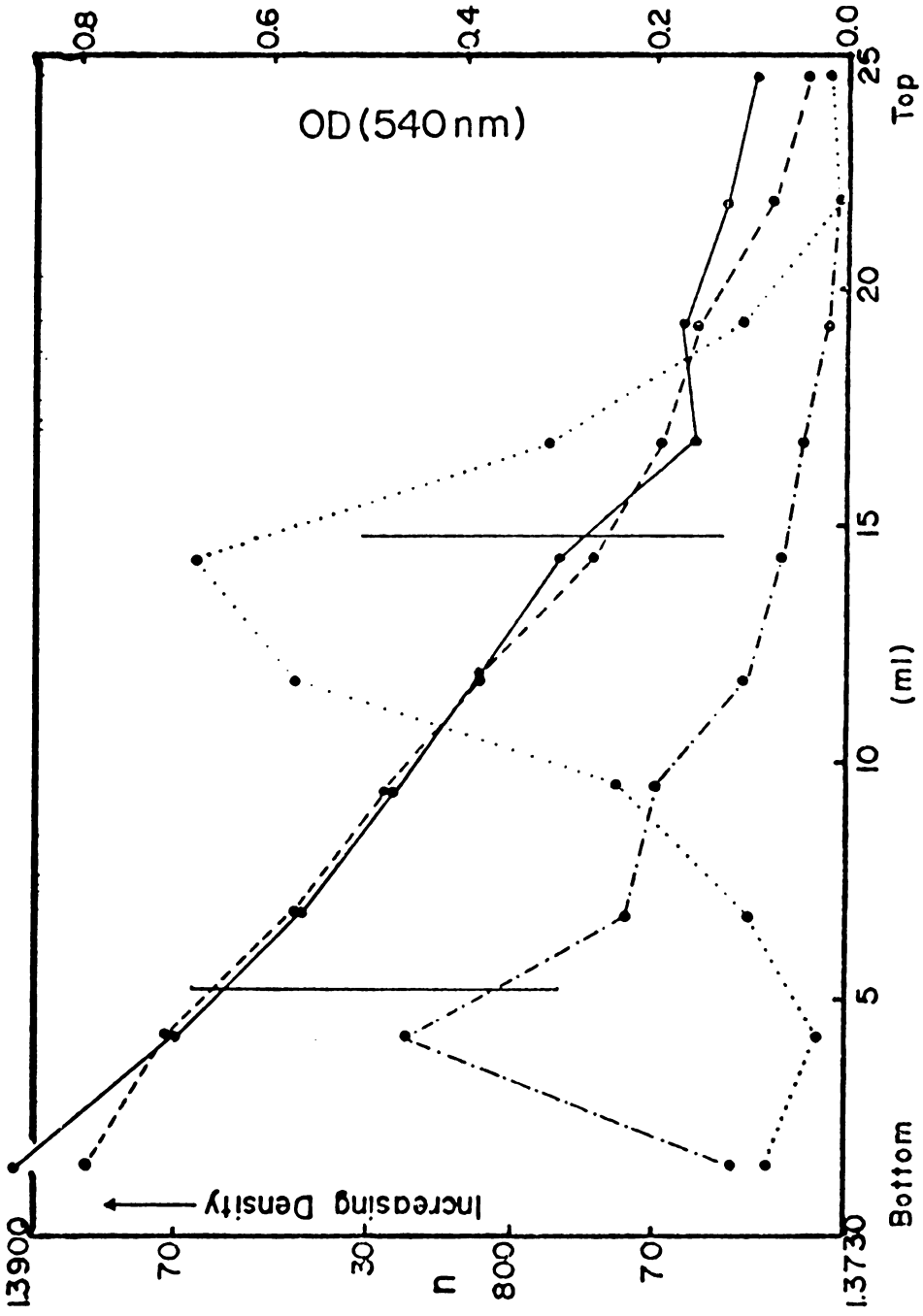

Figure 3. Relayering experiment (see text for details)

to age the ^{59}Fe activity was monitored. Samples of blood were removed from the sheep one day after injection and then weekly from that point on. The blood was run on the gradients and fractioned and these fractions were read on a liquid scintillation counter and the counts were corrected for changes in cell concentration by using OD measurements on the fractions. The results are shown in Figures 4 and 5.

The dashed line represents the absorbance peak of the red cell band on the gradient. The trailing edges to the bottom are the highest densities and those to the top are the lowest densities. One day after injection the label is in the high density portion of the gradient. These are the reticulocytes that have a lifetime of 24-48 hours and are more dense than the average red cell[38]. By day 7 after injection the reticulocytes have matured into young erythrocytes and appear at the very top of the gradient or the area of lowest density.

Figure 5 shows the progression from day 7 until day 57, by which time the radioactivity is reaching a point of being evenly distributed. It can be seen that the amount of activity is decreasing in the low density portion of the gradient and increasing in the high density portion. Since changing density is only a rough estimate of the changing age of the red cell, clear cut results are not evident. This does, however, show that the gradient is separating young and old cells.

II. Lysis Kinetics

The percent survivors of the red cells with time was determined for

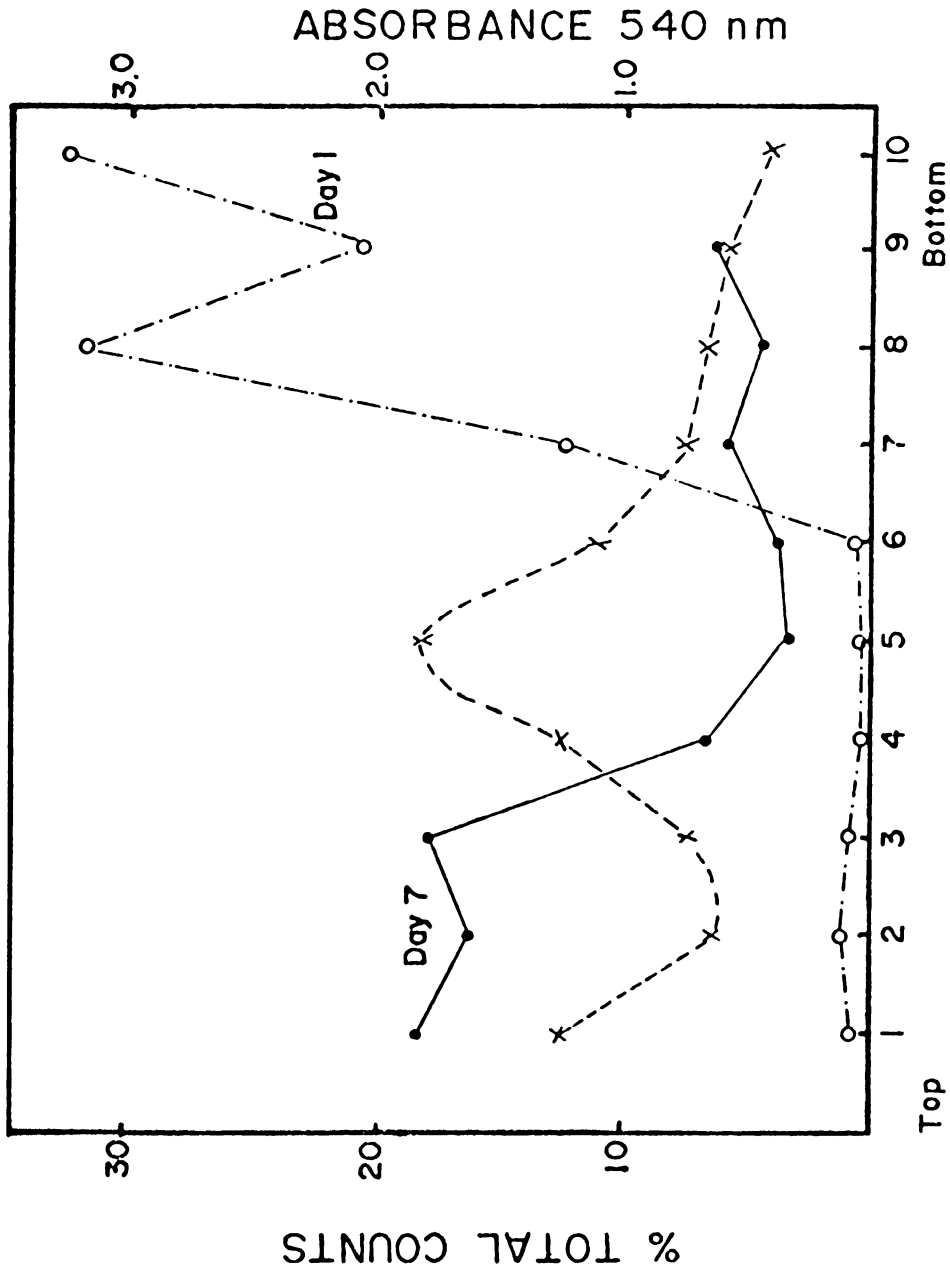

Figure 4. Location of radioactively labeled cells at day 1 and day 7

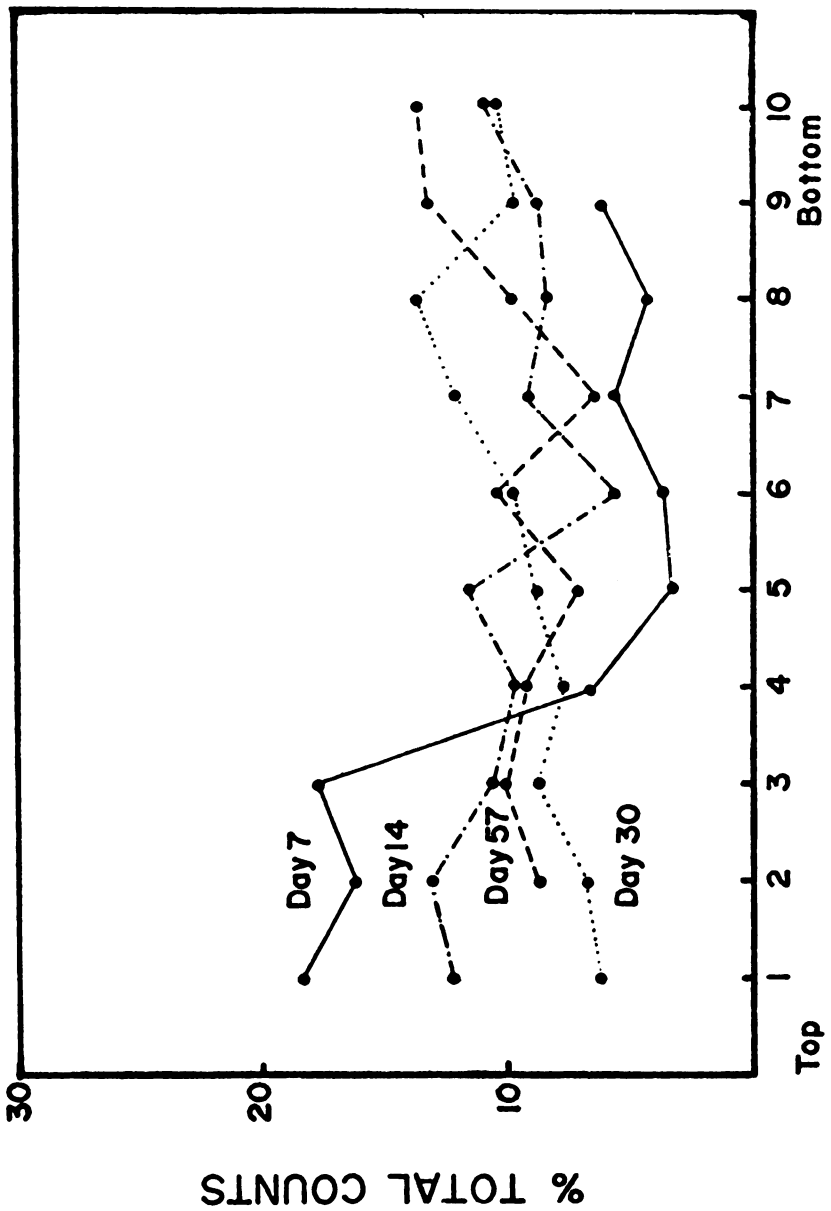

Figure 5. Continuation of figure 4 at days 14, 30, and 57

both young and old and for each temperature. The temperatures used were 42°, 44°, 46°, 48°, 50°, 52°, 54°, and 56°C.

Figure 6 shows a typical set of data for young cells at five different temperatures. It is plotted as log survivors versus time. If there had been large straight line portions in these curves, one could conclude that a simple first order process is occurring. These are not straight lines but resemble the curves for human survivors and fruit fly survivors of figures 1 and 2, suggesting a multistep process.

To show that this rate of decrease in population changes with the age of the cells, figure 7 contains young and old curves for two representative temperatures.

It was described in Chapter II that several functions of population decrease versus time can represent the kinetics seen in these multi-step processes. For the reasons given there the Weibull relation is the one of choice if it proves to represent the data well enough.

The Gompertz and the Weibull functions were both fit to the data. Figure 8 shows the typical Gompertz plot of the data for young cells. As can be seen, at large times, the curves tend to be straight lines with slopes that decrease with decreasing temperature. These slopes represent α and are changing dramatically with temperature. The ordinate intercept of these extrapolated lines, which are a function of R_o/α, are varying non uniformly, and as shown in Table 1, the R_o value is not constant with temperature. Although the Gompertz function does a good job in representing the survivor curves, it is a poor function to use when temperature is a factor in the study.

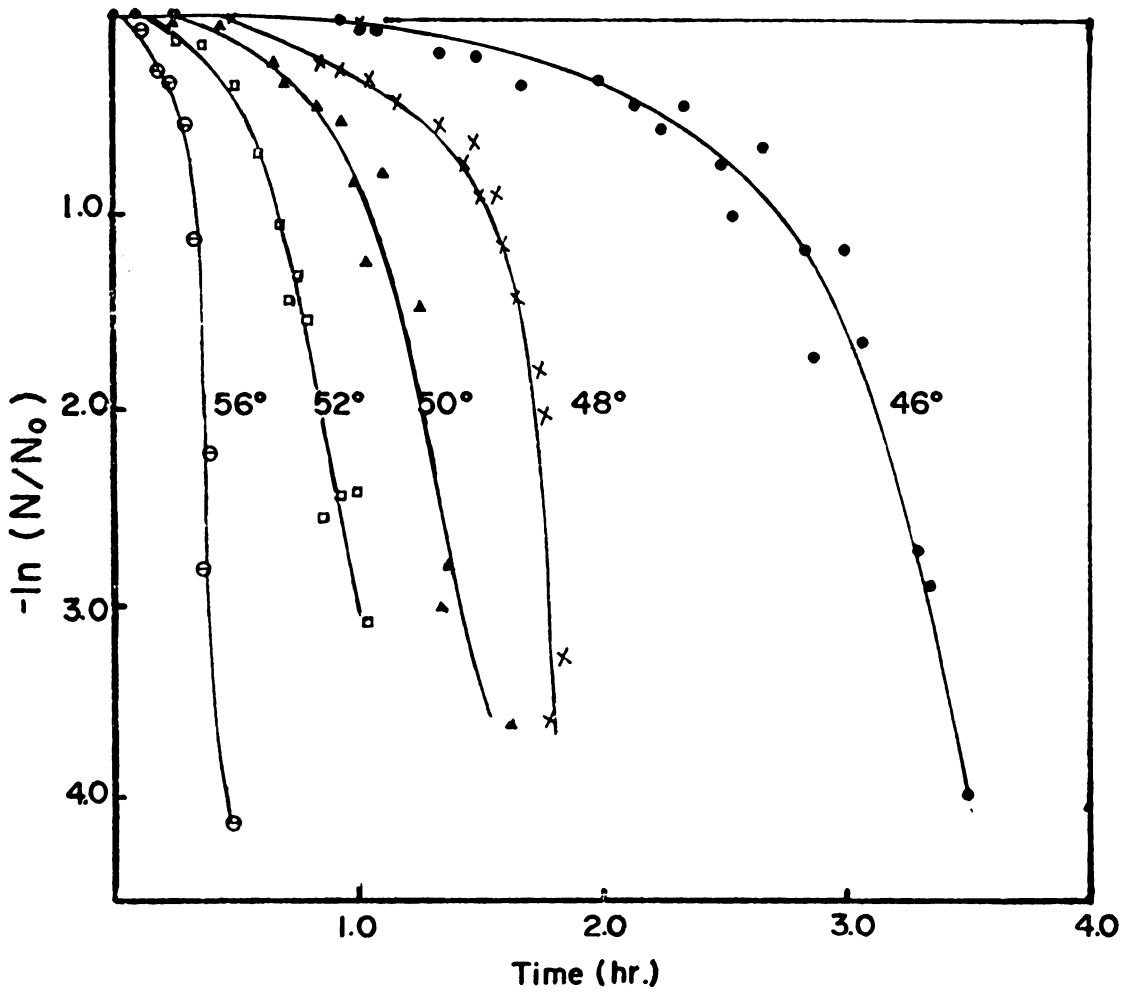

Figure 6. Log of the percent survivors plotted versus time in the water bath for young cells

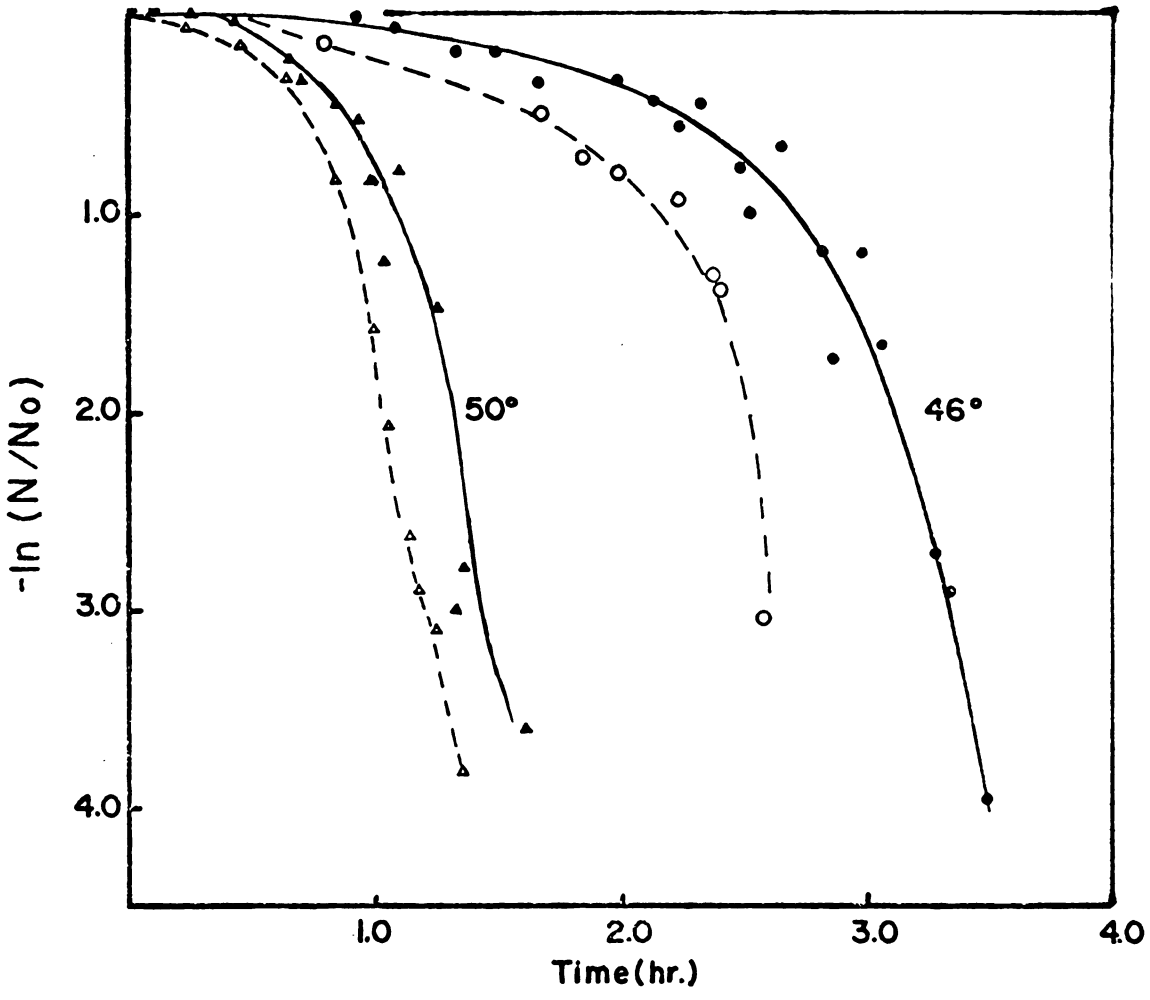

Figure 7. Solid curves repeated from figure 6, dashed curves represent old cells at the respective temperatures

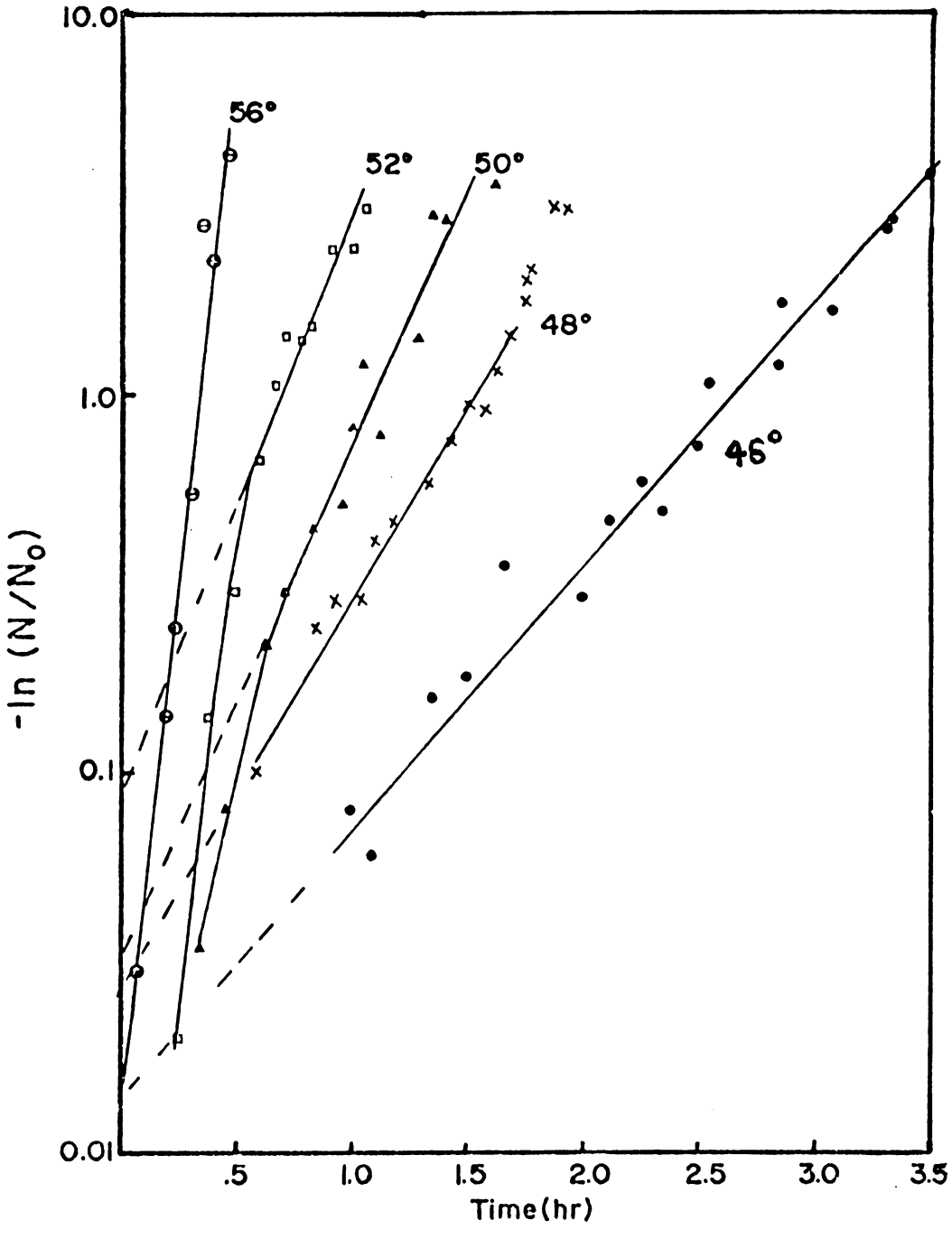

Figure 8. Gompertz representation of young cells

Table 1. Gompertz parameters R_o and α

GOMPERTZ PARAMETERS

R_o Value

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Young	.032	.033	.14	.067	.123	.424	.045	.307
Old	.103	.033	.18	.06	.228	.72	.979	1.35

α Value

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Young	.46	.706	.749	2.48	3.11	3.18	10.13	10.54
Old	.46	.976	.87	2.10	3.11	3.45	4.52	8.65

Both the α parameter and the R_o parameter are temperature dependent and that makes it very difficult to determine the effect of temperature on the system or to obtain any useful information about the activation enthalpy of the process.

Figures 9 and 10 show the Weibull representation of the data for young and old cells respectively. The "goodness of fit", as represented by the standard deviation of the residual, is given in Table 2 for both the Gompertz and the Weibull fit for young and old at each temperature. The Weibull fit is as good or better in describing the data as the Gompertz. It is, therefore, reasonable to use the Weibull function because, as is shown in Table 3, only the A value (or $1/\tau$ value) is temperature dependent, and the n value is approximately constant, and the variations present are fairly random.

Examination of Table 1 shows that the parameters of the Gompertz are both temperature dependent. This makes it impossible to use the Arrhenius relation for this analysis. The parameters of the Weibull relationship, however, as shown in Table 2 are not both temperature dependent. The n value is a stable value with respect to temperature and only the A value changes markedly. Therefore, the A value was tried in the Arrhenius equation to see if it resulted in a constant value for $\Delta H^{\ddagger}$. Figures 11 and 12 shown the Arrhenius plots for young and old cell populations respectively. A very good straight line is noticed in the young cell population; and for the old cells there appears to be a parallel set of lines with a discontinuity at about 50°C. The A parameter seems, therefore, to be related to the activation energy of the process of lysis.

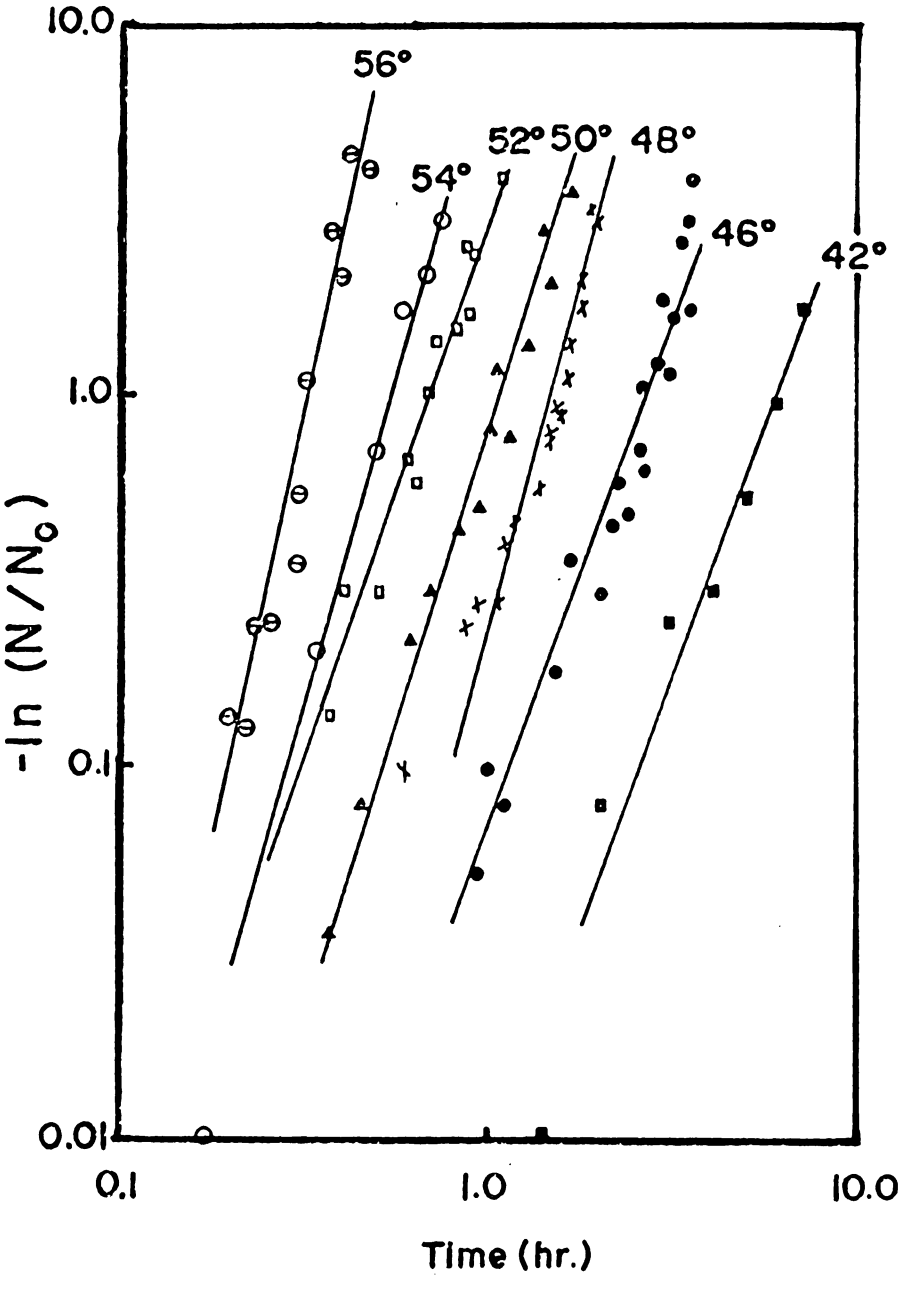

Figure 9. Weibull representation of young cells

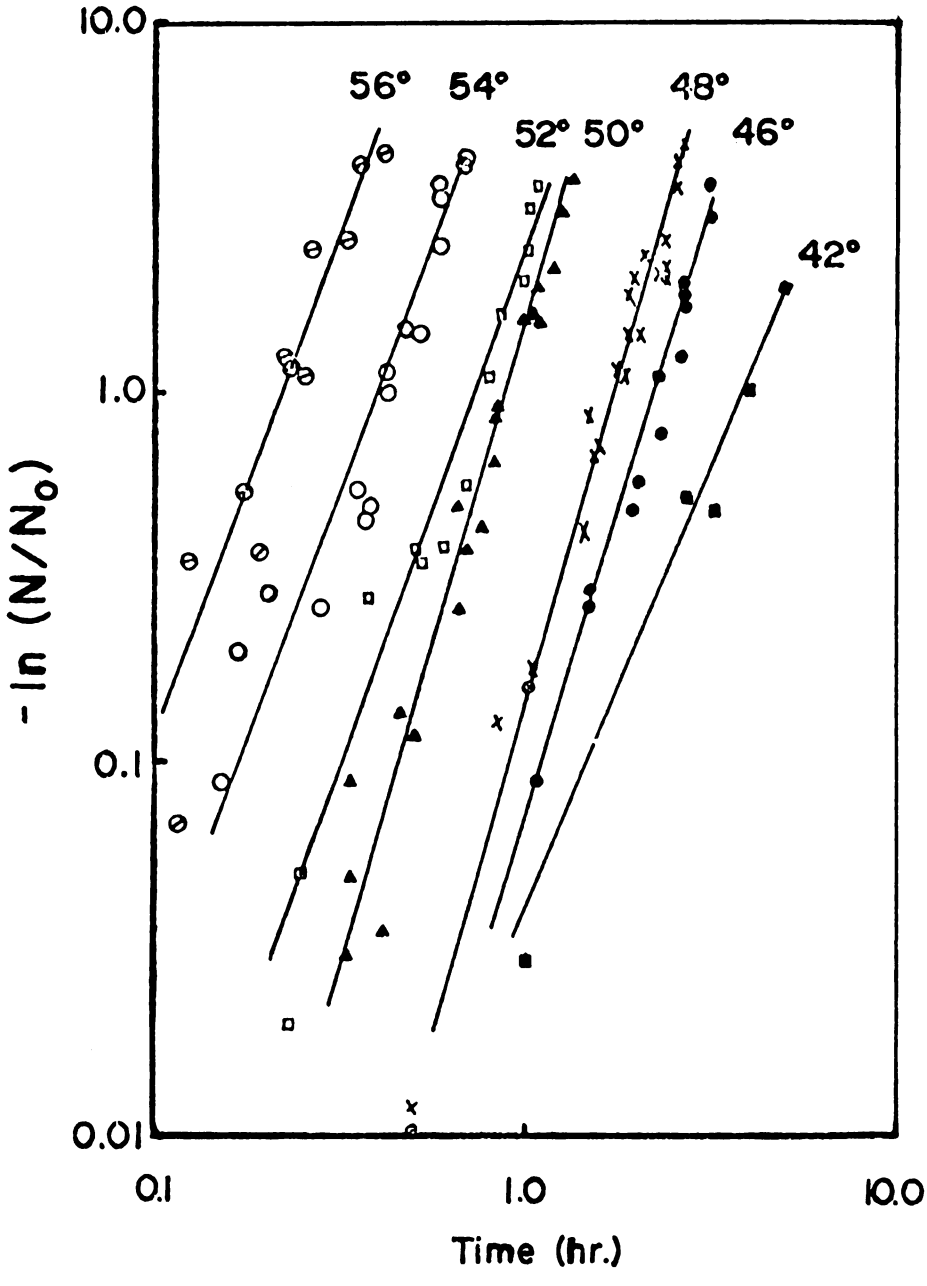

Figure 10. Weibull representation of old cells

Table 2. Standard deviations of the residuals for Gompertz and Weibull distributions

σ (RESID)

Young

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Gomp.	.065	.042	.11	.07	.089	.055	.105	.10
Weibull	.076	.04	.107	.07	.08	.073	.011	.027

Old

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Gomp.	.037	.047	.089	.055	.050	.075	.105	.109
Weibull	.038	.12	.08	.048	.034	.073	.06	.039

Table 3. Weibull parameters A, $1/\tau$, and n

WEIBULL PARAMETERS

A Value

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Young	.021	.044	.18	.85	2.9	6.7	32.9	128.4
Old	.095	.072	.23	.50	5.2	19.8	28.0	65.0

$1/\tau$ Value

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Young	.25	.34	.54	.96	1.37	2.08	2.7	4.35
Old	.37	.42	.63	.83	1.61	2.22	3.45	4.76

n Value

	42°C	44°C	46°C	48°C	50°C	52°C	54°C	56°C
Young	2.8	2.8	2.9	3.9	3.4	2.6	3.5	3.3
Old	2.4	3.0	3.2	3.7	3.5	3.8	2.7	2.7

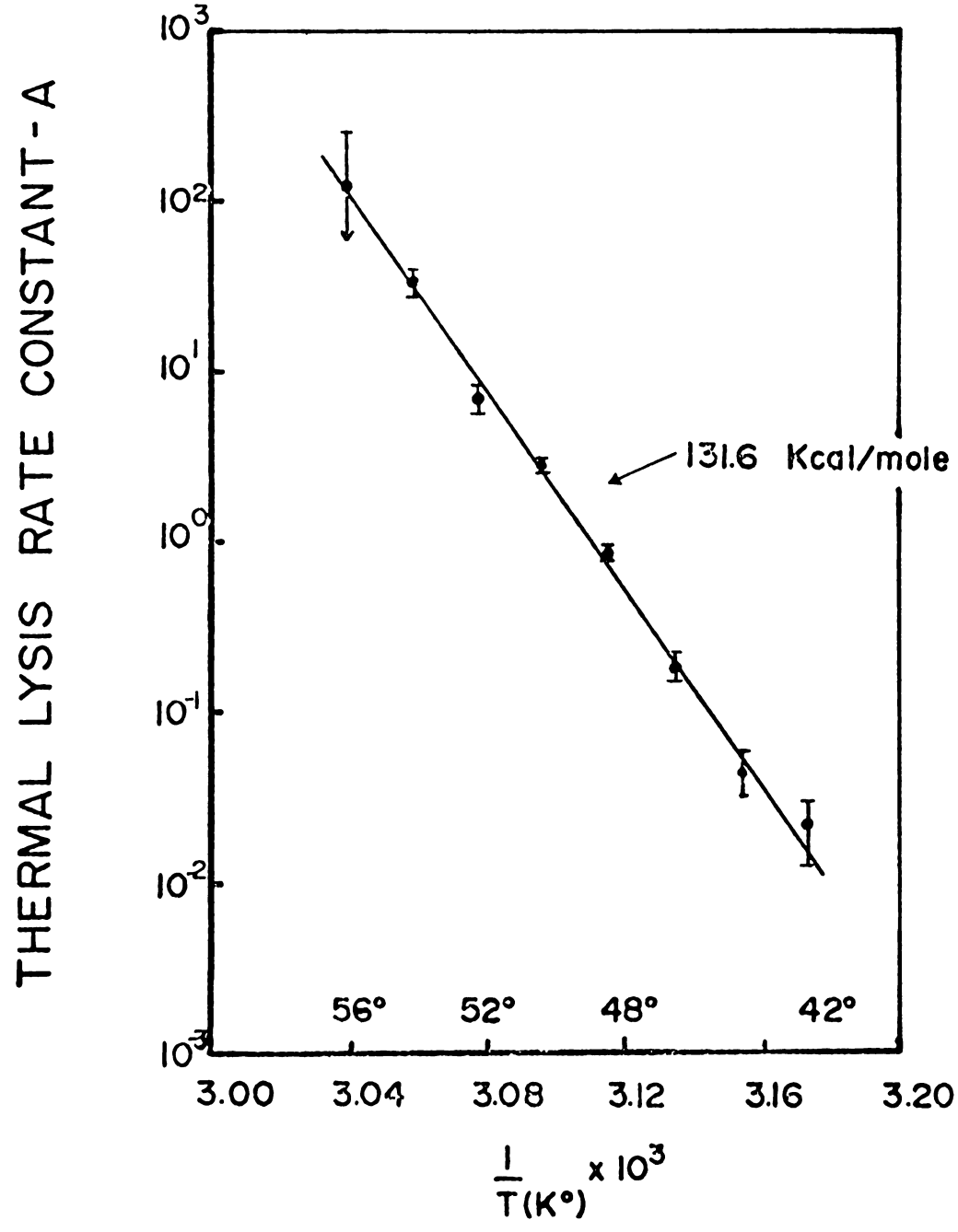

Figure 11. The Arrhenius plot for the young cells using the A parameter of the Weibull distribution

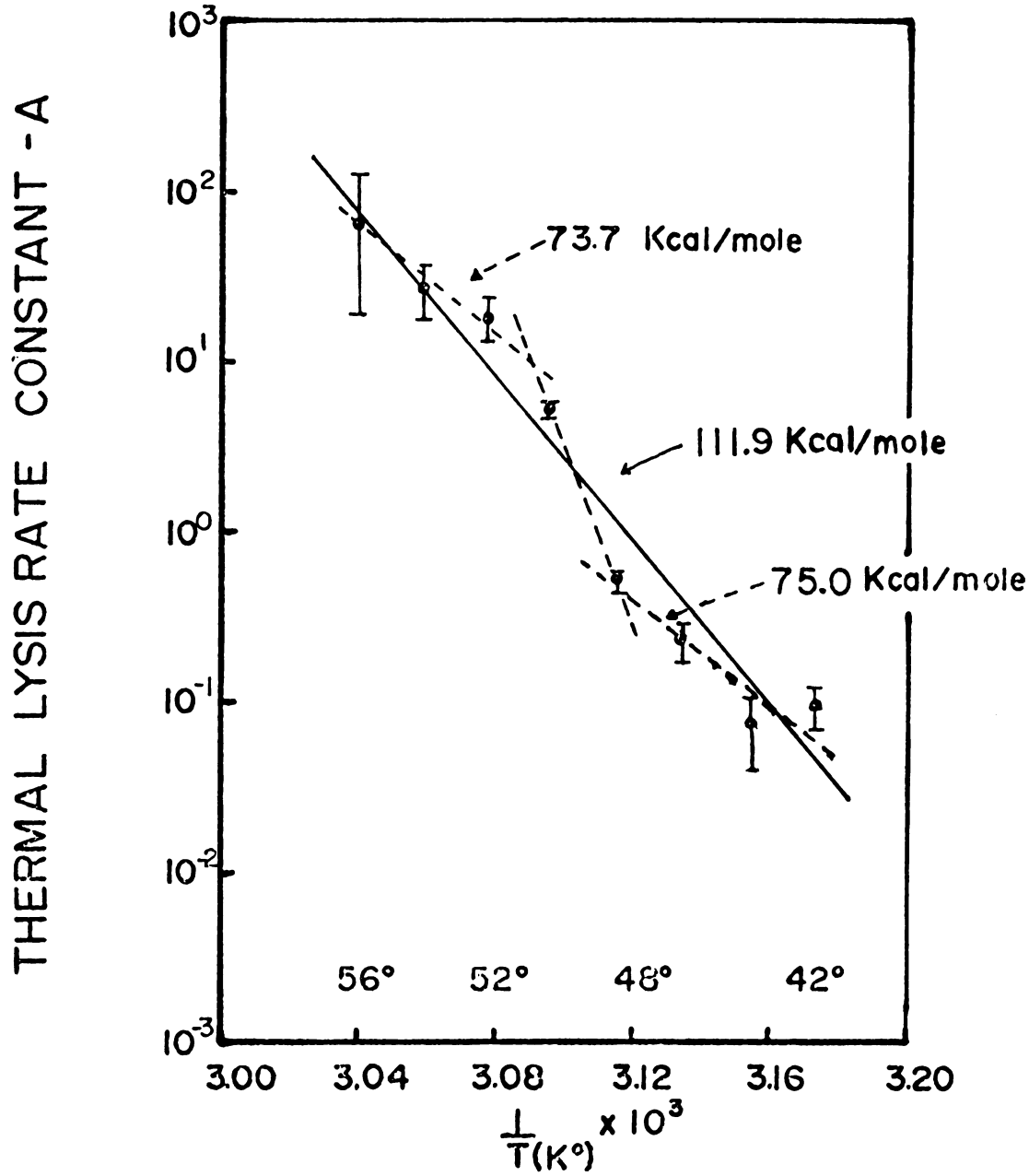

Figure 12. The Arrhenius plot for the old cells using the A parameter of the Weibull distribution

Table 4 lists the values of n and their corresponding marginal standard deviations as given by the computer program. These standard deviations would indicate that there is a significant difference of n values between certain temperatures. To arrive at these values, the data of several experiments was pooled at each temperature. In order to compare this to n values computed from unpooled data the three temperatures shown in Table 4 were chosen and n values computed for each experiment within the set and the n values were average and the standard deviations determined. This shows that there is more variance when one takes the experiments individually, suggesting that possible differences in n values are really within the experimental error. Even if one assumes the n values are different over the temperature range it is difficult to establish a relation for the n values as a function of temperature. They seem to vary in an unpredictable fashion, which also suggests that they are constant within experimental error.

The high value of the activation enthalpy obtained using the A value suggests that this might be a multistep process. In order to examine this possibility the Weibull function in the form of equation 12 can be used, where the value A has been replaced by $(1/\tau)^n$. Table 2 shows the values of $1/\tau$ at each temperature and age. Skurnick suggest that the value of n is an indication of the number of steps in a multistep process, so using the form of the Weibull in equation 12 effectively divides the value of n out of A. This results in a parameter which has the dimensions of rate which may possibly represent the rate constant of one of these steps.

Table 4. The values of $\Delta H^{\ddagger}$ computed by the shift in life expectancies $t_{\frac{1}{2}}(1)$ and $t_{\frac{1}{2}}(2)$ at the temperatures T_1 and T_2

$t_{\frac{1}{2}}(1)$	$t_{\frac{1}{2}}(2)$	T_1	T_2	Computed H (Kcal/mole)
.60	.29	52°	56°	38.9
1.40	.29	48°	56°	41.4
5.2	.29	42°	56°	42.7
5.2	1.40	42°	48°	44.2
5.2	.60	42°	52°	44.2
1.40	.60	48°	52°	44.2

As would be expected the value of $1/\tau$ follows the Arrhenius relation as does the value A, but since the n has been eliminated from A the parameter $1/\tau$ is less sensitive to variations in the value of n. Figure 13 shows the Arrhenius plots for young and old cells using $1/\tau$ as the rate parameter. It follows that the activation enthalpies would be about one third (three being the average value of n) of those shown in figures 11 and 12. The activation enthalpies for the young and old cells are approximately the same in figure 13 suggesting that a similar mechanism is responsible for lysis in both young and old cells. It is interesting to note that the variations in the plot for the old cell populations have diminished suggesting that the discontinuity seen in the plot of the A value is an artifact of the fluctuations in n.

III. Relationship of Lifespan to Activation Energy

When examining figure 6 it is apparent that the average lifespan for the red blood cell decreases with increasing temperatures. This directly follows from the Arrhenius equation and it is the value of $\Delta H^{\ddagger}$ that determines the amount of change in lifespan with change in temperature. By using the A parameter and the $1/\tau$ parameter two different activation enthalpies have been determined.

It can be shown that one would expect the $\Delta H^{\ddagger}$ associated with the $1/\tau$ parameter to predict this lifespan shift.

By taking the integrated form the Weibull function

$$\frac{N}{N_i} = \exp\left[-\frac{1}{n}\left(\frac{t}{\tau}\right)\right]^n \qquad (5)$$

and two hypothetic curves at temperatures T_1 and T_2 respectively, and

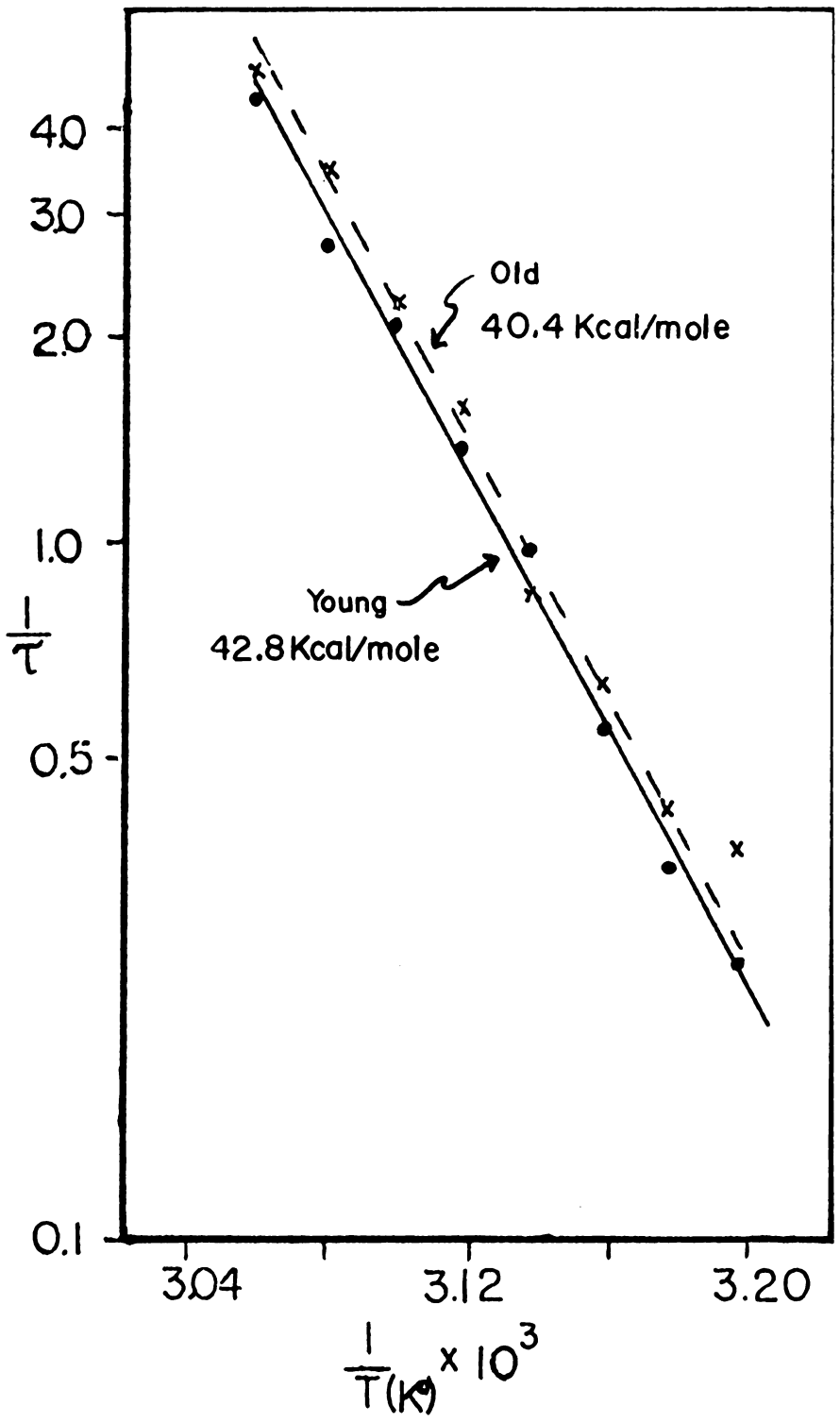

Figure 13. The Arrhenius plot for both young and old using the $1/\tau$ parameter

evaluating at the fractional survivorship $\frac{N}{N_i}$ = 50% for both, the result is

$$.5 = \frac{N}{N_i} = \exp\left[-\frac{1}{n}\ \frac{t_{\frac{1}{2}}(1)}{\tau_1}\right]^n = \exp\left[-\frac{1}{n}\ \frac{t_{\frac{1}{2}}(2)}{\tau_2}\right]^n$$

where $t_{\frac{1}{2}}(1)$ and $t_{\frac{1}{2}}(2)$ are the population lifespans associated with 50% survival for each temperature and $1/\tau_1$ and $1/\tau_2$ are the respective rate constants. This simplifies to

$$\frac{t_{\frac{1}{2}}(1)}{\tau_1} = \frac{t_{\frac{1}{2}}(2)}{\tau_2} .$$

Using the theory of rate processes (see equation 7) to substitute in for $1/\tau$ this then becomes with rearranging

$$\frac{t_{\frac{1}{2}}(1)}{t_{\frac{1}{2}}(2)} = \frac{C_2 \exp \frac{-\Delta H^{\ddagger}}{RT_2}}{C_1 \exp \frac{-\Delta H^{\ddagger}}{RT_1}} \simeq \exp \frac{-\Delta H}{RT}\left\{\frac{1}{T_2} - \frac{1}{T_1}\right\} \qquad (5)$$

This then relates lifespan to temperature and defines the constant $\Delta H^{\ddagger}$. Table 5 gives the value of $\Delta H^{\ddagger}$ for several values of T_1 and T_2. It shows clearly that the basic increase or decrease in lifespan with temperature is related to an activation enthalpy almost identical to that derived from the $1/\tau$ parameter of the Weibull distribution. This parameter, $1/\tau$, therefore represents a rate constant for the rate limiting step involved in cell lysis, and the A parameter must represent some combination of steps that affects the kinetic behavior of the population but not the thermodynamic behavior.

IV. Derivation of $n \cdot \Delta H^{\ddagger}$

Let us look more closely at this $\Delta H^{\ddagger}$ associated with the A

parameter. Evidence we have indicates that this activation enthalpy is equal to $n \cdot \Delta H^{\ddagger}$ where we are now talking about the activation enthalpy associated with $1/\tau$. The fact that the A parameter follows the Arrhenius equation and gives a $\Delta H^{\ddagger}$ which is the sum of the activation enthalpies of n steps possibly separated widely in space and time is solely the result of the statistics involved. Skurnick has shown in a general treatment of stochastic distributions of mortality that the Weibull distribution is one of the asymptotic distributions in the theory of extreme values of order statistics. He has proposed a model to show that the n parameter of the Weibull distribution represents the number of highly improbable, sequential, random events that lead to the death of one organism in the population. A result of this model is that the mortality rate, μ, of the population, as described by the Weibull function, contains the constant A which is related to n times the activation enthalpy associated with $1/\tau$. The simple derivation which follows shows this.

We assume a model that has M links, where M is very large, and the breakage of a link is the event that causes death. Damage to the links is random with time and the build up of n units of damage on any one link would cause it to fail. We can determine the distribution function of failure for the set of links and from that the mortality rate among a whole set of similar systems which would correspond to the mortality kinetics of a population.

Using simple first order kinetics, the distribution of links receiving no damage with time is given by the solution to

$$\frac{dM_o}{dt} = M_o k$$

where k is the rate constant representing the rate of incidence of "hits". The solution is $M_o/M_i=\exp(-kt)$, where M_i is the initial number of links. For a system receiving 1 unit of damage the distribution can be arrived at by considering the change in the number of links with one "hit" already minus those with zero "hits" receiving one. Therefore,

$$\frac{dM_1}{dt} = -(M_1 - M_o)\, k$$

Using the solution for M_o, the solution to this equation is therefore given by

$$\frac{M_1}{M_i} = kt \exp(-kt)$$

This can be continued until n units of damage are received at which point the system ceases to exist. The distribution of links with n units of damage is

$$\frac{M_n}{M_i} = \frac{(kt)^n}{n!} \cdot \exp(-kt)$$

By using a theorem demonstrated by Cox[40] we can obtain from equation (6) the distribution of systems that cease to exist due to the first link receiving n hits. This is what we want in describing a population of aging organisms each one dying when an average threshold of maximum damage is reached. Therefore, it can be derived from equation (6) that the fraction, N/N_o, of the number of systems surviving with time is given by

$$\frac{N}{N_o} \simeq \exp[kt]^n$$

The mortality rate, $\mu(t)=dN/Ndt$ is therefore given by

$$\mu(t) \sim k^n \cdot n \cdot t^{n-1}$$

(which is simply the Weibull function with $k=1/\tau$) or in taking the log,

$$\ln\{\mu(t)\} \sim n \ln k + \ln \{n \cdot t^{n-1}\}.$$

Since k is the only parameter on the right side of the equation that is a function of temperature we can use the theory of rate processes to give

$$\ln \{\mu(t)\} \simeq \frac{n \cdot \Delta H^{\ddagger}}{RT} + \ln \{n \cdot t^{n-1}\}$$

This, therefore, shows that, for fixed time, $\mu(t)$, and, therefore, the parameter A is proportional to $\exp \{-n \cdot \Delta H^{\ddagger}/RT\}$ which is the Arrhenius equation and the observed activation enthalpy is simple n times that for the process of receiving one unit of damage.

V. The Activation Entropy and Protein Denaturation

In order to determine an activation entropy we used the absolute rate theory equation (equation 1) which relates the rate of a process to the temperature at which it proceeds and the constants $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$:

$$K = \frac{kT}{h} \exp(\Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT),$$

where k, h, and R are Boltzman's constant, plank's constant, and the gas constant respectively. Since $1/\tau$ appears to be the more relevant parameter with respect to the thermodynamics, the values of $1/\tau$ and their corresponding $\Delta H^{\ddagger}$ and T's were used in equation 1 to determine the value of the activation entropy. The value of $\Delta S^{\ddagger}$ for young cells was 57.60 cal/mole/°K and for the old cells was 50.29 cal/mole/°K.

DISCUSSION AND CONCLUSIONS

I. Kinetics

The results show that the thermal induced lysis of the red blood cell does not follows simple first order kinetics but can be described by the Gompertz function and the Weibull (or power law) distribution. The Gompertz has been assumed by many authors of theories to be fundamental to the aging process. They have bent their theories and have designed their hypotheses such that they can derive the Gompertz function for the rate in decline in the population. Strehler[5] gives a review of such theories and himself assumes the Gompertz relation in an attempt to derive a major phenomena associated with aging, the linear decline in physiological function with age.

As was shown earlier both the Gompertz and the Weibull distributions are derivable from the asymptotic distribution of extremes. A model was presented by Skurnick which showed that a linear decline in vitality does result in both the Gompertz and the Weibull distribution in this asymptotic limit[29]. This appears to be the only way to derive these functions because the processes involved in aging and death involve a small number of events of a highly improbable nature. Choosing distributions for simple reactions and expecting them to describe these rare phenomena has been a mistake many have made. The gross phenomena that is observed when measuring the death rate in large populations is the probablistic result of these extreme occurrences in nature. Whether the cause of aging and death is the result of the denaturation of a protein or RNA or DNA or the melting of lipid, or due to action of a poison, or the build up or debris or

the specific action of a "death" related gene, the numbers and quantities involved make these events small in number and improbable in occurrence in comparison to the multitude of reactions of the normal cell processes. The reason that these can be called improbable events in the total number of events and not a whole separate class of occurrences is that they are simple molecular processes of the same exact nature as the other "normal" molecular reactions of the cell.

The Gompertz and the Weibull functions are the simplified descriptions of the behavior of extremes of other distributions. If these other distributions describe the processes of the cell in some way then the analysis of the extremes seems to be the only logical way to derive these functions and explain their relation to aging and death. No more simple method of deriving these functions has been shown that doesn't either assume the function itself in the first place or a simplified precursor in an ad hoc derivation.

Since these functions don't appear to be derivable from simple physical concepts, their parameters do not straight forwardly represent the physical world. These parameters, however, are the only possible attachments to reality and they do appear to have physical significance. The α and R_o parameters of the Gompertz have been shown by Strehler to be correlated, and different combinations of the two represent possible influences of the environment on the organism, ie. good or bad growth conditions. The fact that they are correlated means that they are both affected by external factors. This presents a problem when dealing with temperature. The Weibull

function on the other hand has only one parameter which depends heavily on the environment, so that this allows an attempt to evaluate the phenomena using the theory of rate processes which involves the relation of one parameter to temperature, energy, and entropy.

There have been no theories that have shown that the parameters of the Weibull distribution have physical meaning in biology. Rosenberg et al. have suggested that the parameter n represents the number of steps involved in a multistep process and that the A parameter is related via the Arrhenius relation to the activation enthalpy. This experiment has shown that both the A value and the $1/\tau$ value follow an Arrhenius relation suggesting a tie to the theory of absolute rate processes, and that n is fairly constant for this system independent of age. Comparing n with experiments on other organisms, n increases with increasing complexity of the system as would be expected if there are more steps responsible for death. Both of these facts suggest that the Weibull distribution, which can only be derived from asymptotic limits of order theory, has physically meaningful parameters describing the reactions rate and the possible number of processes occurring.

II. Activation Enthalpy and Entropy

A further result of this experiment is the activation enthalpies derived. An activation enthalpy for a lysis phenomena of 130 kcal/mole suggests a very large reaction involving many steps at once. The denaturation of protein has been shown to be this high due to its many hydrogen bonds and crosslinkings that can be broken. If we

assume a process of three equal steps as indicated by the number n, then the activation enthalpy of each step was shown from figure 13 to be about 40 Kcal/mole. If we further assume that $1/\tau$ represents a first order rate constant then an activation entropy of 57 cal/mole/°K can be calculated for the young cells. These individual steps are comparable to most protein denaturation values that have been measured in the past and, therefore, represent a possible denaturation process or, at the least, a degradative process. The high entropy suggests that it is an irreversible process.

The quantity in the exponential of equation 1 is also known as the free energy of activation, $\Delta F^{\ddagger}$, and is given by $\Delta H^{\ddagger} - T\Delta D^{\ddagger}$. The value of this quantity has been shown to be fairly consistantly equal to 25.0 Kcal/mole for heat induced protein denaturation and enzyme inactivation[22]. $\Delta F^{\ddagger}$ is also temperature dependent but over the range of this experiment the value is relatively constant. The value of $\Delta F^{\ddagger}$ for young cells at 50°C is 24.2 Kcal/mole and for old cells is 24.1 Kcal/mole. This directly suggests that the process involved in the lysis of the red cell is protein denaturation.

Further evidence for a protein denaturation process comes from the fact that the activation enthalpy and the activation entropy follow the compensation law. Rosenberg et al. showed that a compensation relation

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger}}{T_c} - b \qquad (7)$$

holds for the denaturation of many proteins and viruses, and the death of yeasts and bacteria. T_c is a compensation temperature which is a

constant approximately equal to 330°K and b is a constant equal to 64 - 66 cal/mole/°K. If the set $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ for each group of systems is plotted as $\Delta S^{\ddagger}$ vs. $\Delta H^{\ddagger}$ then a straight line is observed with intercept b and slope $1/T_c$. Protein denaturation and thermal death fall along this line suggesting that the rate limiting step in thermal death is protein denaturation.

In the experiment with the red blood cells, if the values of T_c and b which were derived from death in bacteria are used to determine $\Delta S^{\ddagger}$ using the known values of $\Delta H^{\ddagger}$ then equation 7 yields a value of 64.3 for $\Delta S^{\ddagger}$ for the young and a value of 56.9 cal/mole/°K for $\Delta S^{\ddagger}$ for the old. Both of these are within the error range of the regression line of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ given by Rosenberg et al.

This not only points to a possible protein denaturation as a mechanism in the lysis of the red cell but also gives more evidence that the parameter $1/\tau$ in the power law is indeed a first order rate constant. All the values compiled by Rosenberg et al. were from kinetics that showed a first order process. In fact, the way in which they were able to calculate a $\Delta S\dagger$ was by using

$$K = \left(\frac{\kappa T}{R}\right) e^{\frac{\Delta S\dagger}{R}} e^{-\frac{\Delta H\dagger}{RT}}$$

from the theory of rate processes knowing that the observed rate constant was a first order process. In this case, a first order process was assumed and a $\Delta S\dagger$ calculated. A good fit to the compensation law by this calculated $\Delta S\dagger$ suggests that the assumption was valid.

III. Young vs Old

One of the main purposes for this experiment was to evaluate the

kinetic and the thermodynamic differences between populations of young and old cells. Work of H. A. Johnson[20,39] has shown that fluctuations in thermal noise at physiological temperatures can cause macromolecular denaturation in sufficiently high numbers to explain the spontaneous mortality seen in cultured cells. He suggests that increasing the temperature simply speeds up this denaturation process by increasing the number of high energy fluctuations in the thermal environment. Therefore, the process of thermal death is not a special case but a real representation of the mortality process. By performing the high temperature experiments on young and old populations of cells the remaining portions of their aging process proceeds at rates fast enough to be observed in short times. The older cells have a shorter time remaining before deactivation or death than the younger cells and, therefore, their in vitro lifespans are shorter. This is observed in this experiment; the rate constant of lysis, $1/\tau$, is greater for the old cells characteristic of the shortened lifespan.

The thermodynamic parameters show little change between young and old populations but this is because small changes in these values can cause tremendous changes in the rate constants. A change in $\Delta F^{\ddagger}$ of 2 kcal/mole for example can change $1/\tau$ by a factor of 10. The important thermodynamic parameters are $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. The value of $\Delta F^{\ddagger}$ can remain unchanged while both the activation enthalpy and entropy vary widely. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ give the important clues to the mechanisms involved. High $\Delta S^{\ddagger}$ suggest increase in disorganization typical of denaturation of tertiary structure, and suggests the

breakage of many covalent or hydrogen bonds. Many combinations of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are possible including negative values. In this experiment the values of both of these parameters are reasonably high, but are approximately equal for both young and old. This implies that the same process is occurring in the lysis of the young and old cells and this process is associated with some macromolecular denaturation. The fact that $1/\tau$ is measured to be different between the two ages suggest that the same rate limiting process is occurring but that more of the degradative steps leading up to this rate limiting process have occurred in the old cells. The examination of the n value also supports this idea.

There is no apparent change in the n values of the Weibull distribution between young and old and this raises the question of the meaning of the parameter n. Many investigators have postulated that the shoulder seen in mortality kinetics of humans, fruit flies, and tissue culture cells is due to a series of steps constituting the rate limiting process of death[2,3,12,29]. Skurnick has shown that the n in the Weibull function represents the number of steps in a sequential process each with a rate constant $1/\tau$. He postulated that these steps occur during the aging process and thus the value of n would decrease with the age of the system. If these theories are assumed then the fact that the n value does not change suggest that the age differences between these populations of cells is not significant with respect to the process of lysis. This can be understood by considering that under normal conditions one would not expect the blood cells to be allowed to deteriorate to the point where they

were close to lysing before they were removed. It is to the organism's advantage to replace the red blood cells before they have reached the point where the proteins in the membrane are near denaturation in order to be pumping only highly active, highly efficient cells. So, in spite of the fact that this experiment has separated the youngest and oldest cells from the blood stream and has shown that the older cells have deteriorated somewhat, the analysis indicates that the older cells have not aged significantly towards the event that was chosen to represent the death event, or that the death event is unaltered regardless of age.

IV. Comparison to Normal Lifespan

If we extrapolate the line in figure 13 to 37°C to determine $1/\tau$ and then plug this into the integrated form of equation 12,

$$-\ln(N/N_o) = \frac{1}{n}\left(\frac{t}{\tau}\right)^n ,$$

with $n/N_o = .50$ and $n=3$, we can determine the lifespan at 50% survival ($t_{1/2}$). This results in a $t_{1/2}$ equal to approximately 30.6 hours. This is dramatically different than its 5 month lifespan in the organism. This is not unexpected, however, because it is known that red blood cells age rapidly in storage even at 2-6°C. Their "shelf life" at 4°C is only 21 days and at this temperature they metabolize glucose approximately 30 times more slowly than at 37°C[41]. This could put their lifespan at 37°C around the one day mark. The reason for this rapid aging in storage is unknown but is believed due to the highly foreign environment in which they are placed. This raises the question of how this in vitro deterioration effects the results given here.

This does not change the fact that the kinetics are best described by the Weibull distribution, that the possible number of steps involved is 3, that there exists an activation enthalpy for the process measured, and that this activation enthalpy is the same for both young and old even though the rate of deterioration is greater for the older cells. The question to be answered is: what is the process that is being measured? Most directly we are measuring the activation enthalpies for red blood cell deterioration in vitro. Does this invalidate or weaken the experiment?

The inability of the experiment to represent the red cell survivorship in vivo does not detract from the original idea of using the red blood cells as an aging model. The in vitro aging of the blood cell still represents a system which exhibits irreversible deterioration with time. The in vitro red cell is still an independent entity which does not reproduce or grow, but simply wears out with time. The increased rate at which this occurs in vitro is due to the environment which should not affect the activation enthalpy but rather the activation entropy. One would expect the lines given in figure 13 to be shifted downwards such that the activation enthalpies would be the same but the activation entropies would be greater. If one calculated the new activation entropies after shifting the lines in such a way that the lifespan at 37°C is 5 months, the result is 65.8 and 58.5 cal/mole/°K for the young and old respectively. If this is compared to the $\Delta S^{\ddagger}$'s computed using the compensation law, one sees that these values are even closer than those derived from the experiment. (see page 61 and 56) This suggests that the

activation enthalpies still validly represent the energy of activation for the catastophic, final event in this aging biological system.

SUMMARY

Briefly summarizing the results of the test we have seen that lysis kinetics of the red blood cell in an _in vitro_ system of heat induced lysis does not follow simple first order kinetics but rather kinetics with substantial shoulders. These are describable by the Gompertz and the Weibull distributions but only the Weibull parameters are suitable for further use in thermodynamic considerations. The A parameter of the Weibull follows the Arrhenius relation but this gives an activation enthalpy which appears to be n times higher than that which represents the rate limiting step. The $1/\tau$ parameter, when used in the Arrhenius relation, results in an activation enthalpy which correlates to that predicted by the shift in lifespan. This rate, $1/\tau$, also results in a $\Delta S^{\ddagger}$ from the rate theory equation which follows the compensation law for proteins and thermal death in simple organisms and in a $\Delta F^{\ddagger}$ which is closely comparable to those measured for thermal protein denaturation. This strongly implies that $1/\tau$ is a rate constant which is directly connected to the basic processes involved in the thermal lysis. The comparison of the n parameter and the thermodynamic parameters between the young and the old suggest that the only difference between the young and the old is an increased level of deterioration in the older cells but not a change in the basic process in lysis. Had a parameter been chosen which more appropriately describes the timing of the termination of red blood cell _in vivo_ then there might have been a noticeable change in the n parameter.

The connections that we can make to the aging and dying process

are that kinetics of mortality in living systems can be reproduced, in form, in a simple system where the mechanism of action is associated to protein denaturation. This is consistent with the mortality of Drosophila and human fibroblasts, which both suggest high activation energies typical of macromolecular denaturation. The fact that the red cell does not contain DNA and yet that its "mortality" is very similar to the mortality of other organisms and the activation enthalpies are also similar suggests that the cause of death in other organisms is associated with protein denaturation resulting from thermal fluctuations in the environment.

FURTHER WORK

This experiment along with many others has shown that temperature is a very important parameter in life. Slight changes in it can cause a speeding up or slowing down in many processes. The thermal bath regulates the system. I think it is clear that the causes of the rarely occurring low probability extremes that are assumed in the derivation of the power law and the Gompertz can be attributed to high energy fluctuations that occur with low probabilities. This idea fits well with this derivation of these functions and makes for a simple answer to the causes of aging. I suggest that the main contribution to these high energy fluctuations are due to the thermal bath. Others have suggested this[39], but so far the only method of changing the level of fluctuations in order to study it has been either to use very high energy X-rays or Gamma rays or to raise the temperature. The first lacks a basis in reality; its energies are too high. The second is lacking in that the whole thermal bath is being raised in order to increase the populations of the fluctuations. When the bath temperature is raised many, many reactions occur that are not normal either due to their speed or their type. More than just the affects of the fluctuations are accentuated. The mean heat level is increased which results in gross physical problems, such as membrane fluidity, heat dissipation, osmotic changes, etc. These are changes that are directly due to the mean thermal level and not to the distribution of the energies around the mean.

An experiment which might get around this problem would use a culture of bacteria or some other culturable organism that can be

suspended in media and followed for rate of proliferation. They would be treated in a constant temperature bath at physiological temperature with infrared radiation. This could be monitored for intensity and frequency resulting in specific quanta on the order of high thermal fluctuations at a density significant for affect. The bath would maintain a thermal mean equal to the best growing temperature but the radiation would be able to increase the probability of high energy fluctuations. A frequency of 1000 nm results in quanta of energy 30 kcal/mole which is high enough to break bonds. When everything is considered via quantum mechanics the thermal quanta in the form of infrared radiation and the thermal quanta being emitted and absorbed over and over within matter are identical. Therefore, this type of radiation is much more reasonable than the very high energies that are involved in X-rays or UV radiation.

The proliferation rate of the cells would be followed to see if they are "aging" faster than controls. The number of them unable to reproduce is a measure of their fight against degradation. If this increases with IR radiation, then this points to thermal fluctuations contributing heavily to the eventual death of all organisms.

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