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ERYTHROCYTE OSMOTIC FRAGILITY AND AGING

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Rose Marie Dougherty

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PATHOLOGY

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ERYTHROCYTE OSMOTIC FRAGILITY AND AGING

By

Rose Marie Dougherty

A THESIS

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

MASTER OF SCIENCE

Department of Pathology

1979

ABSTRACT

ERYTHROCYTE OSMOTIC FRAGILITY AND AGING

By

Rose Marie Dougherty

Erythrocyte osmotic fragility tests were performed on heparinized venous blood samples from 44 hematologically normal subjects who ranged in age from 18 to 82 years. Osmotic fragility measurements were refined over previous studies of this kind in that (1) an automatic pipetter was used to simultaneously dispense the red cell suspensions and NaCl solutions, thus reducing sample variability; (2) the NaCl solutions were adjusted to a pH of 7.40 without the use of buffers, which themselves may influence the integrity of the red cell membrane; and (3) the resultant sigmoid osmotic fragility curves of percentage of hemolysis versus NaCl osmolality were treated as linear transforms to permit statistical evaluation of the results.

The NaCl osmolalities (mOsm/kg) used were 300, 130, 125, 120, 115, 110, 105, and 0 (distilled, deionized water). The hemoglobin content of the supernatant fluids was assayed spectrophotometrically to provide a measure of hemolysis, and controls for hemolysis due to mechanical damage during sample processing were employed.

The NaCl osmolality at which 50% hemolysis occurred (H_{50}) was determined for each subject from the linear regression of transformed hemolysis against osmolality and plotted against the subject's age in years. The slopes of the linearized osmotic fragility curves were also determined and plotted against subject age.

Statistically significant ($p<0.05$) positive correlation ($r = +0.35$) was demonstrated between erythrocyte osmotic fragility and age in years. Fifty percent hemolysis occurred at osmolalities varying from 116 mOsm/kg to 136 mOsm/kg.

Red blood cell variability, as expressed by the slopes of the osmotic fragility curves, also increased significantly with age ($r = +0.50$, $p<0.01$).

These results are discussed with reference to (1) factors known to influence erythrocyte osmotic fragility and (2) various theories of aging.

TO

ROSEMARY D. WOOD: my mother

DR. G. MARIAN KINGET

ELWOOD N. WHITBECK

ACKNOWLEDGEMENTS

I wish to acknowledge the assistance of the members of my graduate advisory committee: Dr. Anthony J. Bowdler of the Department of Medicine, Mrs. Martha T. Thomas and Dr. John F. Dunkel of the Department of Pathology; Dr. Robert W. Leader, Chairman of the Department of Pathology; Dr. Garson H. Tishkoff of the American Red Cross; the staff and senior citizens at the Senior Citizens' Centers of Lansing and Ann Arbor, Michigan; Noelle Bowdler; Louise Mueller and April A. Whitbeck.

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Aging

Definition

The terms "aging" and "senescence" are familiar, but unfortunately are rather imprecise (103): there appears to be no general agreement either in general usage or among gerontologists about how they should be used. "Senescent" and "senescence" are used when referring to changes which occur during the period of obvious functional decline in the later years of an animal's life-span. Some gerontologists use the term "aging" for the same processes and period. Others use it in a much more general way, with "aging" meaning simply "growing older", and aging changes being any changes related to age, regardless of when in the life-span they occur. There are advantages in using aging in this general way, but it is difficult to do so, because in common language aging implies something more than simply getting older. Most biologists have accepted these connotations and think of aging as occurring only after maturity has been reached. It is evident from some of the definitions given below that the terms "aging" and "senescence" are frequently used interchangeably.

There have been many formal definitions of "aging" and "senescence." Medawar (120) defines "senescence" as follows:

> "Senescence. . .may be defined as that change of the bodily faculties and sensibilities and energies which accompanies aging, and which renders the individual progressively more likely to die from accidental causes of random incidence. Strictly speaking, the word 'accidental' is redundant, for all deaths are in some degree accidental. No death is wholly 'natural'; no one dies merely of the burden of the years."

Comfort, whose view is similar, has stated his definition in the following terms (33):

> "Senescence is a deteriorative process. What is being measured, when we measure it, is a decrease in viability and an increase in vulnerability. Other definitions are possible but they tend to ignore the *raison d'être* of human and scientific concern with age processes. Senescence shows itself as an increasing probability of death with increasing chronological age. The study of senescence is the study of the group of processes, different in different organisms, which lead to this increase in vulnerability."

Shock (170) defines aging thus: "Aging is the sum total of changes during an individual's life span, which are common to all members of his species or strain." Finch and Hayflick (59) state:

> "Aging will be considered as any time-dependent change which occurs after maturity of size, form or function is reached and which is distinct from daily, seasonal, and other biological rhythms."

According to Strehler (187),

> "Senescence is defined as the changes which occur (1) generally in the postreproductive period and (2) which result in a decreased survival capacity on the part of the individual organism. It must be clear from this definition that different evolutionary lines might very well decline in their survival capacities for entirely different immediate reasons. It may also be, however, that there are one or more dominant mechanisms of aging, common to all higher forms of life. Such common pathways of aging might well reflect similarities in the developmental or evolutionary history of various kinds of animals and plants."

Criteria of Age Changes

It should be emphasized that aging is a property of the organism and not of its environment. Age changes due to the basic aging process should occur even in the most propitious environments in order to be considered as true age changes. It is reasonable that changes ascribable to an aging process should occur in all individuals within a species rather than in occasional instances; for in occasional instances the age-associated changes might be consequences of accidents or of unusual genotypes. A primary objective of aging research is the description of the nature of the basic underlying changes in structure and function which lead to the gradual dysfunction and, finally, to the death of the individual organism. Much of the confusion which has surrounded the biological concept of aging in the past is due to a failure to establish a usable definition of aging and objective criteria through which the results of specific research on animals at several different ages can be evaluated.

Strehler (186) has suggested four criteria which are in essence general, arbitrary definitions of what constitutes or is meant by biological aging, as tentative guideposts in the search for basic mechanisms of the aging process. It was proposed that the criteria which any age-associated change should meet before being considered a part of any basic aging process are four in number and include the following concepts: (1) universality, (2) intrinsicality, (3) progressiveness, and (4) deleteriousness.

The basic postulate underlying these criteria is that there exist gradual changes in the structure of organisms which are not

due to preventable diseases or other gross accidents and which eventually lead to the increased probability of death of the individual as he grows older with the passage of time. Such changes are consistent with the implied criteria stated by Medawar (120) and Comfort (33). Strehler chose these criteria for the following reasons.

(1) Universality - If a given observed phenomenon is part of a natural aging process, it should occur in all older members of the species. This criterion eliminates many specific, hereditary aberrations as well as diseases which are dependent on a given environment. True, underlying basic aging processes will occur in all older individuals of a species whether they express themselves as recognizable disease lesions or not.

(2) Intrinsicality - Like universality, this criterion is designed to distinguish aging from age-correlated changes which are due to the operation of factors outside of the organism. It is included as an additional criterion because certain age changes may be universal but still be a consequence of environmental effects on living systems.

(3) Progressiveness - Aging is usually considered to be a process rather than a sudden event. It follows, therefore, that its onset should be a gradual and cumulative occurrence. Predisposing factors are very likely a part of the aging process, and it is for this reason that the study of aging mechanisms is a key to a greatly neglected area of biomedical research. Generally processes are gradual in their occurrence because they are due to changes in small subunits of structure, e.g., in cells or even in subcellular organelles or molecules. The individual events at the molecular

level leading to dysfunction at the cellular level and ultimately reaching up to the behavior of the organism do occur in a very short time, but the large number of events necessary to produce gross change tends to smooth out the statistical fluctuations occurring at the micro level and to give the processes an appearance of smooth-flowing uniformity.

(4) Deleteriousness - This final criterion is based on the fact that the most characteristic change occurring during the aging process is the decline in functional capacity which is reflected in an increased mortality rate. Universal, intrinsic, and gradual changes must also contribute to the increased probability of death if they are to be considered as part of the basic biological aging process. Some age-correlated changes which meet the first three criteria may thus be eliminated because they may simply be the final stages in adaptive processes and actually result in an improvement in the organism's survival capacity. As such, they would be classed as part of the developmental process rather than of aging. Not all developmental processes, however, are necessarily eliminated, for certain of them may become deadaptive in their final or extreme expression. Thus, while certain aspects of development may indeed have great relevance to the basic biology of aging, it is equally apparent that this is only true of those developmental changes whose ultimate expressions in time decrease the organism's capacity to survive or which constitute a mixture of adaptive and deadaptive effects.

Categories of Age Changes

It is possible to classify the kinds of changes leading to the decreased functional capacity of living systems. Generally, they are divisible into two general categories: those which are the result of the normal aging process and those which are the result of environmental factors. Medawar (120) discussed this division into the two kinds of causes in the following terms:

> "Senescence, as it is measured by increase of vulnerability, or the likelihood of an individual's dying, is therefore of at least twofold origin. There is (a) the innate or ingrained senescence, which is, in a general sense, developmental or the effect of nature; and (b) the senescence comprised of the accumulated sum of the events of recurrent stress or injury or infection. The latter is environmental in origin and thus, in a paradoxically technical sense, the effect of 'nurture.'" (180)

Strehler (187) uses somewhat different terminology. He calls those processes which are an intrinsic portion of the life history of a species determinate processes; those which are the results of unpredictable environmental influences he terms ancillary or stochastic processes. Determinate (genetic) processes are those which occur with certainty in all animals of a given species regardless of their environment and experience. This definition includes the definition of normal aging and such processes which are considered to be a consequence of any combination of general features of the physical world [Tables 1 and 3 (187)]. Ancillary (stochastic) processes lead to the decreased vitality of old organisms and are considered as consequences of gross accidental events which are not a result of the structural organization of an organism per se. That is, ancillary processes are a consequence of deleterious agents in the environment and are generally subject to modification, whereas determinate processes by their very nature are an inherent part of the organism.

Tables 1, 2 and 3 illustrate the origins and relationships of the various categories of age changes (184,186,187). These tables illustrate that the change of most general interest, the decreased survival capacity of individual animals with age, is based on a hierarchy of events proceeding from elementary interactions of molecules at the cellular and subcellular level to the very complex interactions of the whole organisms, societies, and their environments. Thus, it becomes evident that each of the higher levels of function or organization is dependent on less complex phenomena, structures, or processes.

Molecular Stochastic Disruptions

Stochastic processes at the micro level, that is, those involving the reactions of small molecules or aggregates of molecules, may also result in deleterious changes with the passage of time. The rate of disruptive reactions is determined by two fundamental factors. In order for a reaction to occur the atoms making up the reactive molecules must become appropriately sited and the environment must furnish sufficient energy to overcome the activation barrier to the reaction. Any structure will have a certain inherent instability if it is subject to local energy fluctuations that exceed the bond energies maintaining structure. The sources of those potentially disruptive energy fluxes arise only from three types of events. These are (1) the redistribution of heat energy, (2) the absorption of radiant energy, or (3) the occurrence of chemical or nuclear reactions which result in sudden local concentrations of heat energy. A brief description of each type of event follows.

TABLE 1.

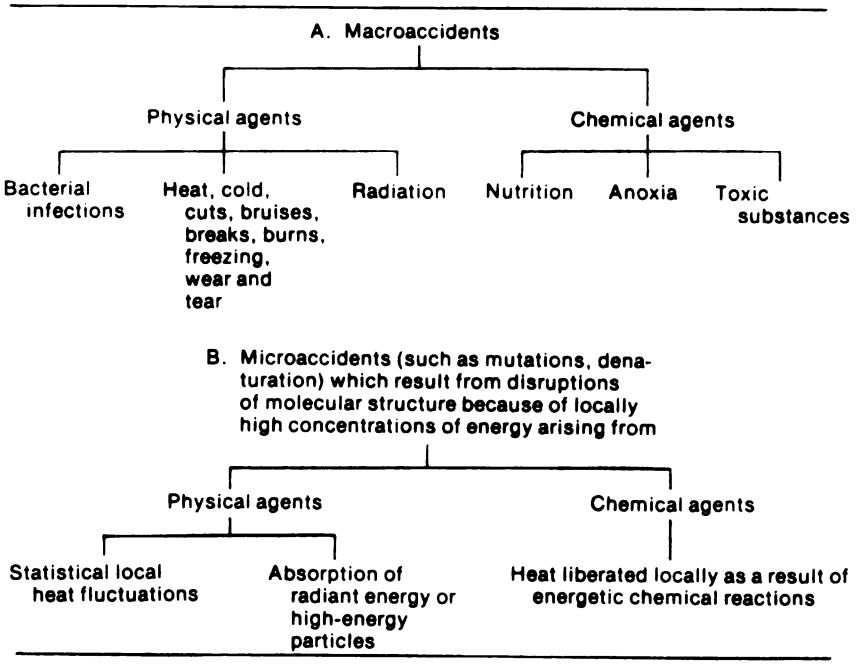
Origins, Levels, and Categories of Age Changes*

Level of organization	Manifestation	Measure
Population	Increased probability of death (aging) derives from ↓	Mortality rates
Individual	Decreased adaptation which, in turn, reflects ↓	General performance, morbidity
Integrated function (organ systems)	Decreased ability to perform specific functions resulting from ↓	Physiological tests
Tissue	Changes in tissues and/or ↓	Histochemical, electron microscopic and chemical measurements
Cell	Death of or changes in cells consequent to ↓	Histology, histopathology, cell physiology
Subcellular elements: organelles and molecules	Changes in subcellular elements or extracellular environments (e.g., nuclei, DNA, polysomes, membranes, mitochondria, Golgi, lysosomes, collagen, basement membranes, solutes, stroma) which, in turn, arise from ↓ Changes in synthetic rates, rates of utilization, diffusion, transport, storage (formation of precipitates, exhaustion of stored materials), all of which are the results of failure of design (deficiencies in genetic properties) to take into account the deleterious effects of ↓	Histochemistry, immunochemistry, biochemistry molecular biology
	Accidents (stochastic processes)	Genetics and development (genetic processes)
	Inadequacy of design (inability to repair and/or avoid gross damage)	Omission of design (inability to repair effects of microaccidents)
	Errors in design (presence of slow, but harmfull side-reactions, catalyzed and uncatalyzed)	Contradictions in design (opposing effects of same design feature at different times, in different cells, in different environments)

* Reprinted from (187).

TABLE 2.

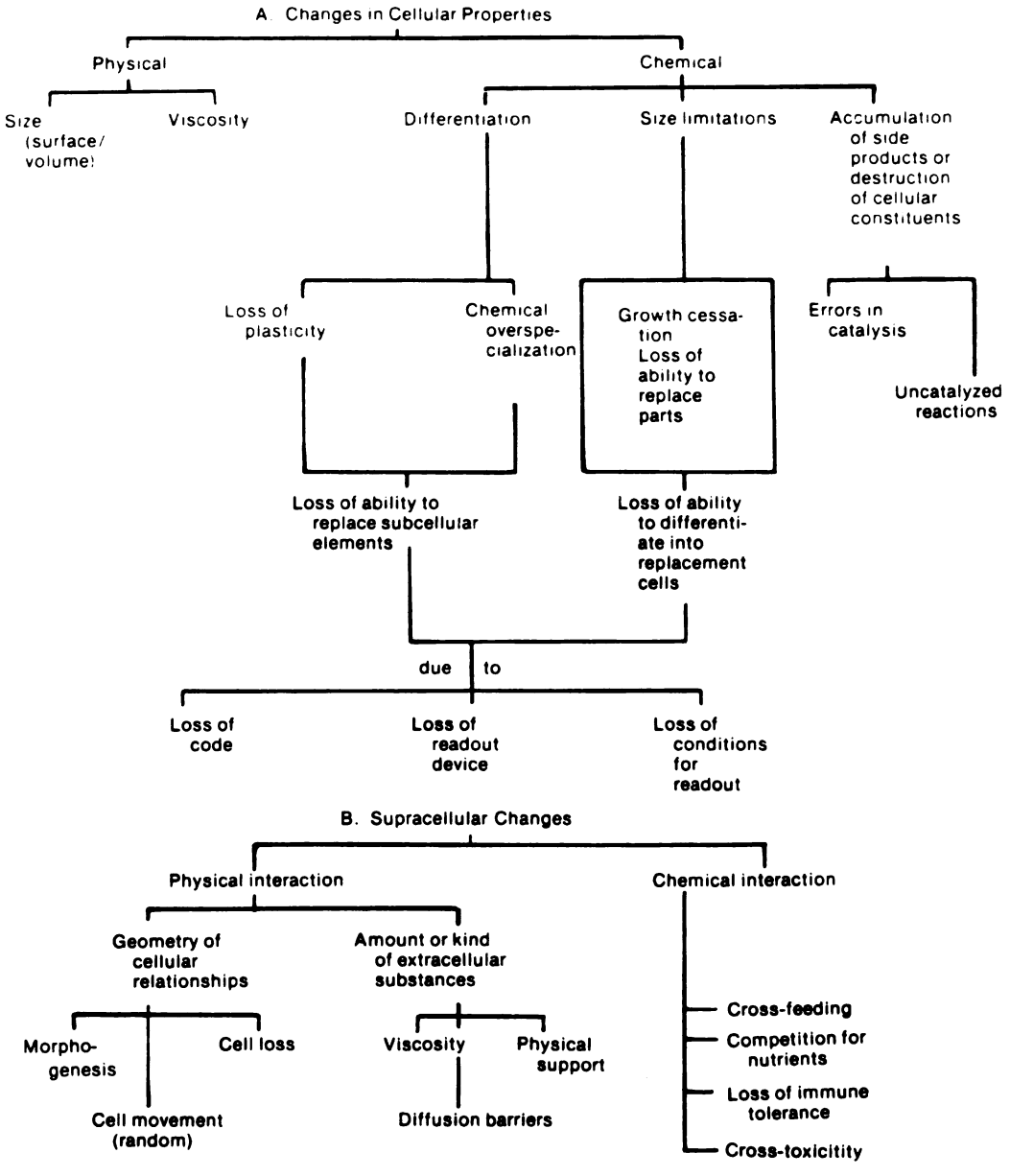
Stochastic Causes of Aging*



*Reprinted from (187).

TABLE 3

Genetic Causes of Aging*



*Reprinted from (187).

(a) Heat - Random local fluctuations in energy about some average value will occasionally result in local energy concentrations sufficiently high to permit the alteration of structures in the vicinity.

(b) Radiation - A second path by which molecules or polymolecular structures may become unstable is through the localization of energy as a result of the absorption of radiant energy. For a short time after the absorption of a quantum of light an absorbing system exists in a so-called excited state. Energy stored in this temporary form is shortly dissipated in one of four ways: (1) through the reemission of light in the form of fluorescence or phosphorescence; (2) through a chemical reaction between the unstable (excited state) and some other molecule in the environment; (3) by the breaking of a bond in the excited molecule before the excitation energy can be dissipated; or (4) by the transfer of the energy to some other molecule, resulting either in reaction or in further redistribution of the excitation energy as heat energy. The last three possibilities are capable of promoting disruptive chemical reactions (61,187).

(c) Chemical reaction - The third major source of energy capable of producing molecular disruption is the liberation of a locally high concentration of thermal energy as a result of the occurrence of chemical reactions. The energy available locally at the end of a reaction may be considerably greater than the free energy liberated by a reaction. This is a consequence of the fact that a molecule must absorb thermal energy before it can react. A catalyst will increase the total rate of reaction, and it will decrease the amount of the total energy liberated at the instant

of reaction by the amount that it decreases the activation energy. Thus, even though an intermediate in a reaction sequence might be transformed noncatalytically into its product, there is an advantage, so far as the long-term survival of the catalytic system is concerned, to inserting a catalyst which will decrease the energy liberated locally at the end of the reaction. Since catalyzed reactions are characterized by lower activation energies, it also follows that the energy possessed by the product at the moment of its formation will be less for catalyzed reactions than for uncatalyzed ones. Enzymes thus function to decrease the probability of damage per reaction as well as to speed up the rate of reaction. It is not possible, at the present time, to evaluate the extent to which uncatalyzed chemical reactions contribute to the overall decline in function characteristic of senescence. However, it is known that certain kinds of age change can be accelerated either by raising the temperature or by exposing tissue to visible, ultraviolet, or ionizing radiation (61,187).

(d) The sources of gene-determined damage (aging) - In addition to the destructive effects of macroaccidents (Table 2) and the slow degradation of living systems as a result of chemical reactions not governed by genetically controlled catalysts, there is a third broad category of age changes that is a part of the aging process (Table 3). This consists of the changes in cells and in multicellular structures, tissues, and organs, which occur as a result of the operation of genetically determined factors. These changes are either the direct or indirect result of the presence of catalysts.

Aging as non-steady state - In a general sense, the chemical constitution of cells will change if the rate at which each substance appears is not equal to the rate of its disappearance due to transport out of the cell or through its utilization. In this context, aging may be viewed as change in constitution due to the inequality of the rates of appearance and disappearance of specific chemical structures, changes which are not directed toward the production of a more effectively adapted organism.

Sources of non-steady state - At the cellular level, those changes which are genetic in origin are detailed in Table 3. These changes are produced by changes in one or more of the following factors so as to yield a steady state which is incompatible with continued survival: (a) rate of synthesis, (b) rate of utilization, (c) rate of transport, (d) rate of storage or depletion of stores, and (e) rate of entropy change, rearrangement, disorganization, or organization.

The various possible theories of the mechanism of aging are expressions of the above hypothesis in various degrees of refinement and sophistication.

(a) Surface/Volume - Of particular importance in determining the steady state are such factors as cell surface-to-volume ratio, cytoplasmic viscosity, and the geometric arrangement of cells. These factors would appear to be extremely important in determining the ingress and egress of diffusible constituents. Intracellular chemical changes also include by-products of catalyzed or uncatalyzed reactions as well as continuations or extensions of the normal differentiation process.

(b) Differentiation - The hypothesis of differentiation as a prime causative factor in cellular aging has received support from biologists. The possibility that senescence may actually be a consequence of cellular overdifferentiation has been explored by Strehler (183). Pearl (40) particularly favored the teleological concept that senescence is the price which organisms pay for the luxury of differentiation.

(c) Growth cessation - Comfort (33) has discussed in detail the fact that many organisms attain a finite adult size, and the consequences of this limitation, as regards repair processes and the maintenance of chemical constancy.

(d) Damage to genome - During recent years a number of theories (40) have been suggested which are based on the idea of a deterioration of the genetic code or information content during the aging of organisms. Such losses of code would be extremely difficult to repair once they occurred within a cell. However, they might be circumvented by simply discarding damaged cells as they are formed.

(e) Nongenetic damage - Another category of chemical changes in cellular properties which are directly attributable to the presence of genetically controlled catalysts is the occurrence, at low rates, of improbable chemical reactions between components. Even though such "side reactions" may be extremely slow compared to catalyzed reactions, their cumulative effects are probably very large. Since catalysts usually change the steady-state concentrations of intermediates, they will indirectly promote the occurrence of uncatalyzed reactions purely through the operation of the law of mass action. Such reactions might either involve the destruction

or alteration of cytoplasmic components or they might produce insoluble or toxic products which cannot be removed efficiently from the cells. "Unprogrammed" reactions might also occur because of errors in catalysis, that is, because of nonabsolute specificity of catalysts' activities or because of the occurrence of non-catalyzed reactions between highly reactive intermediates produced metabolically. Such changes are to be expected in practically any organism whether it be single-celled or composed of many cells. But, in addition, multicellular organisms, which possess certain advantages as a consequence of their multicellular specialized organization, also acquire certain disadvantageous instabilities as by-products of multicellularity. Such instabilities result either from changes in chemical or in physical relationships (interactions) between cells. Physical and chemical interactions between cells are moderated by the nature of the nonliving matter between cells, as well by the cells' geometrical relationships. The geometrical factors which result in coherent cell function will be altered by changes in relative cell size, by the elimination of certain cells through death, by the random movement of cells with relation to each other, or through a postdevelopmental continuation of various morphogenetic processes. Of great potential importance in regulating the interactions between cells are the gels, membranes, fibers, and particles which make up intercellular substances. Because of their location between living cells, such structures may well influence the rate of diffusion of metabolites and waste products into and out of cells and between the cells making up a tissue, organ system, or organism.

(f) Altered cell interactions - Chemical interactions between the cells of a multicellular organism largely depend on the kind and

concentration of diffusible substances passing from one cell to another (3,16). These interactions involve inhibition or stimulation of cellular function by cross-feeding. They involve competition for nutrients. They involve reaction to toxic or regulatory substances produced by one cell and utilized or detoxified by another. They may involve autoimmune reactions between different cells of the same organism.

It is not yet possible to decide which of these many alternatives are the major contributing factors to the normal aging process. However, it is clear that practically all these possibilities must take place at some rate during aging. Therefore, the major general scientific objective of aging research is the assessment of the relative contributions of each of these processes and the definition of detailed underlying mechanisms (187).

Theories of Aging

Theories play two major roles in science. The first is to systematize a range of discrete observations into a single conceptual pattern that will make possible a smaller number of generalizations. The second role of theories is to develop rational explanations for the mechanisms which will explain how and why a general phenomenon occurs. Theories are important to a science since they will have a marked influence on the nature of experiments that will be performed (168). The following will outline in general terms current theories which attempt to explain the biological and physiological changes which occur with aging.

Biological Theories. Ultimately all biological theories of aging have a genetic basis. The fundamental assumption is that the life-span of any animal species is ultimately determined by a program which resides in the genes of the species and ultimately in the information contained in the DNA molecules of the gene. One can divide these theories into two categories. In the first category the primary focus of the theories is on the way that information is transferred from DNA molecules through a variety of steps to the ultimate formation of proteins (enzymes) which are critical for the continued function of specific cells in the animal. The second category of theories, which are termed nongenetic, places emphasis on the changes that may occur with the passage of time in the cellular proteins after they have been formed. These theories place primary emphasis on changes that occur at the cellular and tissue level.

Physiological theories, on the other hand, place major emphasis on the interaction between cells, tissues and organ systems in attempting to explain age changes in overall response of the intact animal. Physiologists are aware of the fact that the ultimate explanation of the phenomena with which they are concerned may well be at the cellular level. However, their emphasis lies in investigating the interrelationships between the performance of different organ systems in maintaining the overall performance of the animal (171).

Genetic Theories

General genetic theory. There are marked differences in average age of death or life-span between different species of

animals (33). It is therefore probable that there is some genetic program which sets the upper limit of life-spans. Maynard Smith's (117) experiments have shown that inbred strains of a species usually have shorter life-spans than the original wild strain. It has therefore been concluded that long life is related to many different genes and a large gene pool is advantageous for long life. In humans, females live about eight years longer on the average than males. The same sex difference in favor of females also appears in a number of other species. Since the primary factor that determines sex in the animal is the presence of XY chromosomes in males and XX chromosomes in females, it has been postulated that the sex difference in longevity is due to the greater amount of X chromatin in females. Support for theories of preprogrammed aging has also been provided by studies which indicate that at least certain cells of the body when maintained in tissue cultures are capable of only a limited number of cell divisions (76). Furthermore, cells taken from old animals can undergo fewer cell divisions in contrast to cell divisions possible in cells taken from young animals. It is important to note that although these studies involving cell division provide a potential model for studying aging of cells under defined conditions, they may not reflect the aging characteristics of cells within the intact animal.

The present evidence indicates that (1) there may be a genetic program which sets the upper limits of life-span in a species, (2) that there is some familial characteristic that influences differences in life-span among individuals of a single species, and (3) the expression of the basic genetic program can be altered by environmental factors (168).

Cellular genetic theories - DNA damage. Identification of the DNA molecules as the ultimate source of all information required by the cell for the production of the essential proteins required to maintain life has led to the theory that death occurs in the cell because of damage to the cellular DNA. The information for formation of specific enzymes and other proteins depends on the precise molecular structure of different parts of the very large DNA molecules (197). Breaks in the chain of the DNA molecule or exchange of positions of parts of the chain would alter the message and result in the inability of the cell to manufacture essential enzymes. This could result ultimately in death of the cell (168).

Somatic mutation - radiation. Exposure to nonlethal doses of radiation substantially shortens life-span. These experimental observations led to the development of the somatic mutation theory of aging (40). According to this theory, exposure to radiation accelerates aging. It was postulated that even in the absence of radiation exposure some cells undergo mutations, most of which are deleterious.

The "hit theory of aging", proposed by Szilard, is a variation of the somatic theory. Szilard postulated that over the lifetime of the animal "hits" occur at random which render a gene or its DNA molecules ineffective in providing the information necessary for the formation of critical proteins in the cell. According to Szilard's conception, a cell becomes ineffective when the two similar chromosomes of a pair have each suffered a "hit" in the same place or when one of the pair has been "hit" and the other carries an inherited "fault" in this locus. A "fault" is regarded as a recessive gene

which renders the cell inviable or incapable of performing necessary cell functions in the adult organism only if it occurs in both genes of a pair. Death of the animal occurs when some critical fraction of the cells initially present is rendered ineffective.

The somatic mutation theory has been abandoned for three reasons. In the first place, the effects of radiation and mutations occur primarily in dividing cells, such as red cell precursors. Effects are seen after cell division when mutations occur. In contrast, age effects are primarily centered in cell lines that no longer divide in the adult animal, as for example neurons and muscle cells. The second reason for abandonment of the theory is that calculations on the rate at which mutations occur in the absence of exposure to radiation indicate that the number of cells which are apt to go through mutations in mammals is too small to account for overall age changes (118). In the third place, recent studies have shown that most cells contain mechanisms for the repair of damaged DNA molecules (210). Consequently, it is doubtful whether damage to the DNA molecule itself plays much of a role in cellular senescence.

Error theory of aging - cellular. In any cell the information stored in the genetic code, as determined by the structure of the DNA molecule, must be transcribed and translated in various steps until the final protein or enzyme molecules required by the cell finally appear. The actual assembly of appropriate amino acids to form specific proteins occurs at another location in the cell, namely, on the ribosome. In order for this to occur, information from DNA must be transferred to an appropriate location on the ribosome. This transfer occurs by the assembly of RNA molecules at specific points

on the DNA molecule which then migrate to the ribosomes. Protein formed depends upon what part of the very large DNA molecule is decoded with the formation of an appropriate mRNA.

This general theory of biology has been transferred to gerontology as the error theory of aging (121). According to this theory, aging and death of the cell are the result of errors which may occur at any step in this sequence of information transfer resulting in the formation of a protein or enzyme which is not an exact copy and therefore is unable to carry out its function properly. The error may be simply the incorporation of the wrong amino acid in the protein or a slight error in the normal sequence.

Unlike DNA molecules, which are highly stable and are maintained throughout the life span of a cell, RNA molecules have a half-life of only eight or nine days. Consequently, new RNA molecules are being formed continuously to provide replacement of enzyme molecules. Orgel (142) has argued that the ability of a cell to produce functional proteins, such as enzymes, depends not only on the correct genetic information contained in the DNA molecules, but also on the competence of the protein synthetic apparatus, i.e., the enzymes necessary for the translation and transcription of the information from the DNA to the final protein product. According to this theory, aging is the result of errors in enzymes involved in translation and transcription. Orgel claims that although the initial frequency of errors in a cell may be low, in the absence of cell division as in an adult animal there would be an exponential increase in the frequency of errors in the proteins synthesized with the passage of time which would lead to an "error catastrophe" and the death of the cell.

Although the error hypothesis is an attractive one, it has a number of limitations. In the first place, there are many steps between the information source (DNA) and the final protein product and errors can conceivably occur at many points. Thus, the theory is relatively non-specific (78,154). Furthermore, the details of these transfer processes are still unknown so that the nature of the error cannot be specified (168).

Nongenetic Cellular Theories. At the cellular level non-genetic theories of aging presume that, with the passage of time, changes take place in molecules and structural elements of cells which impair their effectiveness.

Wear and tear theory. This theory is based on the assumption that living organisms behave like machines. That is, from repeated use parts wear out, defects occur, and the machinery finally comes to a halt. However, the man-machine analogy is not a very good one. Unlike machines which cannot repair themselves, living organisms have developed many mechanisms which permit self-repair. In some tissues, such as skin, the lining of the gastrointestinal tract, and red blood cells, new cells are continuously formed to replace old ones so that the effects of aging are minimal. In other cell systems, such as nerve cells and muscle cells, which no longer divide in the adult, repair mechanisms are present and there is a constant turnover among many of the molecules within a cell.

Increasing the environmental temperature shortens the life-span of cold-blooded animals. At lower environmental temperatures life-span is lengthened. Since a rise in temperature increases metabolic rate in cold-blooded animals, these experiments have been interpreted

as evidence to support the "wear and tear" or "rate of living" hypothesis about the mechanism of aging. In contrast to cold-blooded animals which show a reduction in metabolism as environmental temperature is lowered, warm-blooded animals increase their metabolism when exposed to cold temperature. These experiments were also interpreted as support for the "wear and tear" theory. However, all of the experiments on the effects of temperature on life-span can be interpreted as evidence to support any theory of aging which involves chemical reactions, as for example, the cross-linking theory, or even error theories, since the rate of all chemical reactions is influenced by changes in temperature (168).

Deprivation theories - diffusion. Other nongenetic theories assume that aging is due to inadequate delivery of essential nutrients and oxygen to the cells of the body. These theories point to the increasing prevalence of atherosclerosis with advancing age and the well known effects of vascular degeneration on cellular and tissue functions for their primary support. However, when the blood supply is shut off from a tissue by vascular disease, all of the cells in the area supplied die. This is in sharp contrast to the more or less chance distribution of cells which drop out or disappear from a tissue or organ with advancing age. Furthermore, there is no evidence for a systematic reduction in oxygen content of the blood with advancing age. Fasting blood glucose levels (174) and amounts of other nutrients present in the blood do not fall with advancing age. It seems plausible that even in advanced old age adequate supplies of oxygen and nutrients are available to cells and tissues of the body except under circumstances associated with diseases of the blood vessels.

Carpenter (28), however, has speculated that with advancing age, changes occur in cell membranes which limit the diffusion of oxygen and other nutrients from the blood to the inside of the living cell. However, there is little experimental data which would indicate that this is the case. It is, therefore, extremely doubtful that senescence can be ascribed to cellular deprivation of essential materials (168).

Accumulation theories. Cellular aging and senescence have also been ascribed to the accumulation of deleterious substances in the aging animals. A number of investigators have reported that highly insoluble lipofuscin particles accumulate in cells of various tissues, such as neurons and muscle fibers of the heart, with advancing age (185). It has been argued that the presence of these insoluble particles interferes with the normal cellular metabolism and ultimately results in cell death. However, at present no evidence of impaired physiological function associated with the presence of these age pigments has been provided. The accumulation of these large molecules has been ascribed by Carpenter (29) to impair diffusing capacity of cell membranes with advancing age. Since the age pigments consist of varying proportions of lipids and protein molecules, it has been assumed that they represent an accumulation of non-metabolized molecules. The accumulation theory is a reasonable one to pursue, but the toxic substances, present in old cells but absent in young ones, must be isolated and identified. Furthermore, physiological experiments are required to show that the presence of age pigments impairs the function of the cell (168).

Free radical theory. Harman (74) has proposed that aging and cell death stem from the damaging effects of the formation

of free radicals. Free radicals are chemical compounds which contain oxygen in a highly activated state. Hence, they are very unstable and tend to react quickly with other molecules in their vicinity. As a result of these reactions enzymes and other proteins may be altered in structure and function. The rate of formation of free radicals is accelerated by radiation and inhibited by the presence of antioxidants. Harman offers as evidence for his theory the increase in average life-span observed in mice which have been fed antioxidants. Unfortunately, the experiments reported by Harman have a number of limitations so that the theory is still unproved (168).

Cross-linking theories. Many macromolecules of biological importance develop, with the passage of time, cross-linkages or bonds either between component parts of the same molecule or between molecules themselves. The formation of these cross-links alters the physical and chemical properties of the molecules involved so that they no longer function the same way as before. Since these cross-links are very stable they are apt to accumulate with the passage of time. The cross-linking theory first proposed by Bjorksten (17) holds that the accumulation of these cross-link molecules is a primary cause of aging.

Most of the experimental evidence supporting this theory has been derived from studies on extracellular proteins, namely, elastin and collagen (17,194). It is well established that with aging, cross-links develop between the two intertwined strands of the collagen molecule and between adjacent strands. Formation of these cross-links results in the well known loss of elasticity with advancing age in many tissues of the body, as for example, the skin and blood

vessels. It is also known that the body contains many substances, primarily aldehydes, which are effective in accelerating the rate of formation of cross-links. From these observed facts with respect to extracellular protein, it has been assumed that similar changes occur in intracellular proteins such as enzymes and also in the DNA molecule (194). Although cross-linking between different parts of the DNA molecule has been observed *in vitro*, no evidence has yet appeared which indicates these changes also occur *in vivo*. The cross-linking theory is, however, a viable one which may well explain molecular changes which form the basis for known changes in the characteristics of structural proteins at least. The theory leads to the search for methods whereby cross-links once formed may be broken down but, perhaps more importantly, to methods which would reduce their rate of formation in the animal and thus influence the rate of aging.

Starting with the observations that (1) collagen becomes stiffer with age and (2) collagen constitutes 25 to 30 percent of the total body protein and surrounds blood vessels and cells, Kohn (95) has hypothesized that age changes in collagen may have far reaching effects on other physiological functions. Although this hypothesis relates molecular changes in collagen to more general physiological processes, it is highly speculative and has not been experimentally tested (168).

Physiological Theories of Aging. Physiological theories of aging attempt to explain aging and the life-span of various species of animals either on the basis of a breakdown in the performance of

a single organ system or more reasonably in terms of impairments in physiological control mechanisms.

Single Organ System Hypotheses

Cardiovascular system. In the past, physiological theories of aging have focused attention on a single organ system to explain the total effects of aging. Since failure of the heart and blood vessels represents a primary cause of death, especially among the aged, failure of the cardiovascular system has long been regarded as the primary cause of aging. According to this hypothesis, aging results primarily from the progressive deterioration of blood vessels as a result of the development of atherosclerosis. However, atherosclerosis is a disease process which occurs in the young and increases progressively with advancing age. Death from this disease may occur at any age. Furthermore, aging occurs in many lower forms of animal species where no blood vessels are present. Hence, the hypothesis that aging is basically related to reduction in blood supply does not have general biological significance, even though it is an important cause of death among humans. Another theory has the hypothesis that aging is primarily the result of lowered delivery of oxygen to critical tissues such as the brain. However, experimental evidence fails to support this hypothesis. The oxygen content of arterial blood does not fall with advancing age and blood flow to the brain is only slightly diminished after maturity (94,168).

Thyroid gland. Another hypothesis assumed that aging was due primarily to slowing of metabolic processes at the cellular level. Since the rate of cellular metabolism is regulated by the

thyroid gland, it was assumed that aging was due to the inability of the thyroid gland to supply adequate amounts of its hormone. This hypothesis was also supported by some of the superficial similarities between the symptoms of patients with hypothyroidism and senescent subjects, as for example, loss of hair, slower movements, lengthened reaction times, drying and wrinkling of the skin, and the presumed reduction in basal oxygen consumption with advancing age. However, more detailed studies have shown that the age related fall in basal oxygen uptake is a reflection of tissue loss with advancing age and that the oxygen consumption of individual cells does not change significantly (169). Other experiments have shown that with the exogenous administration of thyroid stimulating hormones the thyroid of the older individual responds as well as that of the young with respect to increasing the output of thyroxin, as evidenced by increased basal metabolism, heart rate, and uptake of iodine by the gland (9). Hence, the theory that aging is due to failure of the thyroid gland can no longer be considered as very probable (168).

Sex glands. Similarly, failure of the sex glands has been looked upon as a primary cause of aging. In fact, this hypothesis has led to the administration of sex hormones in an attempt to induce rejuvenation (119). However, even with a highly purified male sex hormone, the side effects, as for example, the possibility of developing prostatic cancer, and the ephemeral results reported have indicated the futility of this approach to influence overall aging.

Chronic administration of female sex hormones to postmenopausal women has been urged by some endocrinologists to minimize the overall effects of aging (69). This recommendation is based on the

presumption that aging in women is related to the level of sex hormones present in the body. Although there is evidence that some of the complications of the menopause can be alleviated by the administration of female sex hormones, the possibility of inducing or activating cancer in the female genital tract by prolonged administration of the hormone has led most clinicians to utilize the treatment on the basis of relatively short-term therapy and to avoid chronic administration (80,161). There is little evidence that aging as a biological phenomenon can be ascribed to diminished sex hormone production (168).

Pituitary gland-neurohypophysis. Since the pituitary gland plays a central role in the modulation of activity of the adrenal gland, the thyroid, and the gonads, aging has been ascribed to a failure of this gland to carry out its appropriate functions (163). However, methods for determining blood levels of the regulatory hormones produced by the pituitary and the hypothalamus are not yet available. Since the possibility that the pituitary plays a key role in aging cannot be ruled out at this time, recent advances in utilizing radioactive immunological techniques, which are more sensitive than the usual biochemical methods, may resolve this problem in the near future (168).

Stress theory. The stress theory of aging, developed largely by Selye (163) and his collaborators, holds that aging is the result of the accumulation over time of the effects of living. According to Selye, any stress which requires adaptation by the organism leaves a residuum of impairment from which the animal does not completely recover. These residuals, although they may be quite

small with respect to specific events, accumulate over the life-span so that ultimately the reserve capacities of the animal are exhausted. The basic assumption of this theory is that there is always residual damage which persists and accumulates. However, this is not supported by experimental data (168).

Immunological theories. The immune system protects the body not only against invading microorganisms but also against atypical mutant cells which may form in the body. The immune system carries out this protective function both by generating antibodies which react to foreign organisms, proteins, etc., but also by the formation of special cells which engulf and digest foreign cells and substances. Aging has a marked impact on the capabilities of the immune system. The production of antibodies reaches a peak during adolescence and then declines.

One assumption is that the basic impairment in the immune system is a loss in the ability to recognize slight deviations in molecular structure and cellular characteristics so that cells which have undergone mutation and would ordinarily be destroyed by the immune system are no longer recognized and are permitted to grow and develop to the detriment of the animal. By the same token, impairment of cell recognition capacity might also result in the development of antibodies which inactivate normal cells in various tissues. It may also be hypothesized that even though recognition occurs, the immune system of the senescent animal is unable to produce an adequate supply of antibodies or phagocytic cells. Experiments have shown that in senescent animals the supply of stem cells from which the phagocytic cells are derived, as well as the antibody producing cells,

is reduced as a result of a fall in the rate of cell division in the stem cells (78).

The autoimmune theory of aging (19,196) proposes that aging results from the development of antibodies which react even with normal cells in the body and destroy them. This might occur either because of failure to recognize normal cells as normal or the development of errors in the formation of antibodies so that they now react with normal cells as well as the deviate ones which stimulated the immune cells to form antibodies in the first place. This hypothesis gains its greatest support from the age-related increase of autoimmune antibodies found in the blood, and the similarity between many of the so-called immune diseases (some types of anemia, rheumatoid arthritis, arteritis, maturity onset diabetes, thyroiditis, and amyloidosis) and the phenomena of aging.

Although evidence for the key role of the immune system in aging is still sketchy, the general hypothesis is an attractive one which can be experimentally tested. It has added merit in that, if true, it can lead to the development of methods which could potentially influence aging in the human (168).

Aging and Physiological Controls

The overall performance of an animal is closely related to the effectiveness of a variety of control mechanisms which regulate the interplay of different organs and tissues (63). Frolkis has advanced the hypothesis that aging and death of an animal are the result of failure of adaptive mechanisms. Since adaptation to environmental stresses involves the appropriate functioning of control mechanisms,

the adaptation hypothesis can be considered as a special case of the broader theory of control mechanism failure.

With advancing age many control mechanisms lose their effectiveness. Any control mechanism must have a system which will detect changes for which adjustments must be made. In addition to the sensing device, there must also be a mechanism which can carry out the proper adjustment. In the human body, there are many sensing devices which activate effective processes to carry out readjustments of body temperature, blood glucose, blood pressure, heart rate, etc. In developing theories of aging based on control mechanisms, it is necessary to distinguish between alterations in the sensitivity of detecting devices and the effectiveness of the organ system or cells which initiate the changes necessary to maintain stable conditions.

In the mammal the primary control mechanisms operate either through the endocrine system or the nervous system (168).

Endocrine Control System. Many metabolic processes are regulated by hormones which are produced in a gland and are then transported to the target organs through the blood stream. Although the detailed mechanisms whereby the hormone produces its effect within target organ cells are still unknown, recent studies have shown that tissues of senescent animals show a reduced binding capacity for many of the steroid hormones (124) so that the ultimate site of action of a breakdown of the regulatory mechanisms through endocrine control may well be at the cellular level. There are, however, a number of examples of the breakdown of endocrine control in terms of overall physiological effects. For example, the response of the kidney to a standard amount of antidiuretic hormone is significantly less in the aged

subject than in the young (124) and also with advancing age, the rate at which excess sugar is removed from the blood is significantly reduced (174). The removal of the excess glucose is regulated primarily by the release of insulin from the pancreas. Extensive experiments have shown that the age deficit is due to the reduced sensitivity of the beta cells of the pancreas to a slight rise in blood sugar level (152).

Nervous Control Mechanisms. The nervous system also plays an important role in regulating many physiological activities. Performances which require coordinated movements mediated through the nervous system show greater decrements with age than do component parts of the total performance. For example, the maximum breathing capacity shows a significantly greater age decrement than does vital capacity itself (138). The maximum breathing capacity requires the coordinated movement of the chest cage and the diaphragm for its performance. In contrast, vital capacity involves only a single maximum inspiration followed by an expiration, which does not involve a coordinated rate-adjusted performance.

In mammals, body temperature is normally regulated within fairly narrow limits. Experimental studies in which subjects of different ages were exposed to different environmental temperatures have shown that young subjects can maintain body temperature even when environmental temperatures are either increased or decreased, whereas old subjects show a significant change in body temperature (96).

The ability to perform muscular work is a function of the integrated activity of the cardiovascular, respiratory, and neuromuscular systems. Studies have shown that perhaps the greatest decrements

shown with age lie in the area of exercise and maximum work performance. Although decrements can be identified within each of the organ systems involved, they are less than the decrements in total performance. In both physiological and psychological performances, the primary finding is that with increasing age the rate of adjustment or performance diminishes. The slower response observed in many organ systems in the elderly may well be a reflection of impaired integrative functions both at the neural and endocrine levels (168).

Summary of Theories of Aging

A brief review of the theories of aging shows that, although some specific experimental evidence may be found which will support each of them, it is clear that some are more probable than others. In some cases, critical tests of an hypothesis cannot be made at present because of technological limitations. This is especially true of the error hypothesis which, although it is still diffuse, is currently having considerable impact on the direction of research on aging. Methods which will permit the identification of molecules which have an error in their assembly will undoubtedly be available in the near future. When the nature of the error and the point at which it occurs have been identified, further research can be conducted to develop methods either for reducing the occurrence of error or for correcting the existing errors.

The cross-linking theory also offers promise for the future. The first step is to determine whether cross-linking occurs in intracellular proteins as it does in extracellular proteins such as collagen and connective tissue. It will be possible, with future research, to identify methods to both reduce the rate of formation

of cross-links and even to eliminate them once they are formed.

The autoimmune theory of aging also holds great promise for the future. Although a great deal of experimental work remains to be done, the rapid developments in the field provide new methods and techniques which will make it possible to test many of the specific aspects, especially at the cellular level.

Although the ultimate cause of aging may lie at the cellular or even molecular level, current theories in this area fail to take into account the important consideration of interrelationships between cells, tissues, and organs. It is in this area that theories which focus on the role of regulatory and control mechanisms offer promise for the future. Such theories are currently less elegant than the cellular and molecular theories but they do address themselves to the important problem of explaining aging in the total animal.

It seems doubtful whether a single theory can adequately explain all aspects of aging because aging is a highly complex phenomenon which may require different explanatory principles for different aspects of the process (168).

Effects of Aging in Body Systems

In Table 4 (adapted from 2), which follows, the first column is a list of the morphological changes usually attributed to aging for different body systems. The second column comprises findings on clinical examination of old people which are usually accepted as "normal" or inevitable changes imposed by aging. They may induce the aging individual to seek medical help and appropriate advice but seldom for investigation or treatment as clinical problems. The

TABLE 4.

Effects of Aging in Body Systems*

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Skin		
Increased pigmentation	Skin discoloured, thin, wrinkled, dry, and fragile. Tendency to senile purpura, Campbell de Morgan spots. Deformed or atrophic nails, greying and recession of hair (12, 32, 41,127)	Seborrheic keratosis Abrasions and infections Pruritis Heat loss—hypothermia Onychogryphosis; Paronychia Pressure sores Basal-cell epithelioma Keratoses—squamous epithelioma
Atrophy { epidermis, sweat glands, hair follicles		
Degeneration { collagen, elastin		
Thickened blood vessels		
Somatic mutations		
Diminished subcutaneous fat (125, 139, 146)		
Central nervous system: special senses		
Eye		
Loss of orbital fat (139)	Sunken appearance of eye Laxity of eyelids Senile ptosis	Entropion Ectropion Trichiasis (ingrowing lashes) Basal-cell carcinoma of eyelid
Stenosis of lacrimal duct	Epiphora	Dacryocystitis Lacrimal abscess
Lipid deposits in cornea (4)	Arcus senilis	
Conjunctivitis sicca (52)	Reduced tears and dry cornea	Necrotizing sclerokeratitis, corneal ulcers
Shallow anterior chamber	Reduced filtration angle	Glaucoma
Loss of elasticity and nuclear sclerosis in lens	Presbyopia	Cataract
Degenerative changes in muscles of accommodation, iris, vitreous, retina, and choroid (51)	Contracted pupils, slowed reflex Impaired visual acuity and tolerance of glare Reduced fields of vision Defective color vision Slowing of dark adaptation Muscae volentes (objects floating in field of vision)	Retinal detachment Occlusive vascular disease
Degeneration in cortical neurones relating to vision (occipital lobes) and of intrinsic or extrinsic ocular muscles	Visuo-spatial perception and discrimination less accurate Impaired accommodation Limitation of upward gaze	Confusional states caused by sensory deprivation

* (Adapted from 2.)

TABLE 4—cont.

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Ear		
Degeneration of organ of Corti (loss of hair cells) Loss of neurons in cochlea (ganglion cells) and temporal cortex Impaired elasticity affecting vibration of basilar membrane	Presbyacusis—impaired: (i) Sensitivity to tone (high frequency) (ii) Perception (especially against background noise) (iii) Sound localization (iv) Cortical sound discrimination	Psychological effects of deafness (isolation; suspicion; depression)
Otosclerosis of ossiculuar chain in middle ear (206) Excessive wax accumulation		Conductive deafness
Atrophy of striae vascularis (impaired endolymph production) Degeneration of hair cells in semicircular canals (206)	Impaired reflex postural control Uncertainty and unreliability in moving about in darkness	Ménière's syndrome
Nose, throat & tongue		
Atrophic changes in mucosae (128) Neuronal degeneration (taste buds reduced 64 per cent by age 75) (7)	Impaired sense of taste and of smell Diminished responsiveness of reflex cough and swallowing	Anorexia Food fads Malnutrition
Atrophy and loss of elasticity in laryngeal muscles and cartilages	Vocal folds slack, voice pitch raised; power and range reduced	Avitaminosis Risks of gas or food poisoning
Central nervous system: Brain and spinal cord		
Macroscopic changes:		
Meningeal thickening, cerebral atrophy (brain weight down 10 per cent between ages 30 and 70) (25)	Diminished intellectual responsiveness, perception, mental agility and efficiency Impaired memory and learning ability	Reduced intellectual reserves predisposing to acute confusional states Sustained mental, behavioural, and motor changes of dementia

TABLE 4—cont.

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Cellular changes:		
In all cells—deposits of lipofuscin ('wear-and-tear pigment' formed in degenerating cytoplasm probably from lysosomes or mitochondria) (181)	Less resilient, more rigid in outlook	Depression
	More self-centered, withdrawn, and introverted	Persecutory symptoms of paraphrenia
In neurones—(i) loss of RNA, mitochondria, and enzymes in cytoplasm; decline in function; death of cell (65,110)	Sensori-motor performance slower to achieve accuracy	Defective appreciation or localization of pain
	Impaired sensory awareness (pain, touch, heat and cold, joint-position sense)	Predisposition to falls and injuries
		Transient ischaemic cerebral episodes
(ii) Hyaline and eosinophilic inclusions and Lewy bodies	Impaired mechanisms controlling posture, anti-gravity support, balance, and moving equipoise (nerve conduction velocity reduced 10 per cent by age 75)	Impending, progressing and completed strokes
(iii) Neurofibrillary tangles and senile plaques—different degenerative changes occurring with increasing frequency in people over 60 years of age, but not directly related with each other		
Corpora amylacea—occur anywhere in brain tissue (62)		
Vascular changes:		
Intimal and medial fibrosis: Siderosis, amyloid, and hyaline degeneration		
Atheroma—increasing in extent with age, but pathogenesis is multifactorial (97,160)		
Locomotor system		
Muscles—atrophy affecting both number and size of fibres conditioned by metabolic disorder and 'functional denervation', weakness attributable to biochemical or hormone deficiencies	Loss of muscle bulk	Bunions, subluxation of small joints in hands and feet
	Degenerative joint changes	Painful feet (and other chiropody problems)
	Decline in physical strength	Paget's disease
	Limitation of range and speed of movements	Muscular wasting, especially distal
	Disability—combined effects of muscular weakness, joint stiffness, and	

TABLE 4—cont.

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Atrophy of bones: {osteoporosis / osteomalacia Joints— loss of resilience and elasticity in ligaments, cartilage, and periarticular tissues Degeneration, erosion, and calcification in cartilage and capsule (14, 72)	impaired central mechanisms for sensori-motor performance: (i) Less precision in fine movements and in rapid alternating movements (ii) Irregular timing of action, loss of smooth flow of one form of action into another (iii) Slowing down to avoid outcome of one action before planning the next Confidence and reliability of activity reduced Difficulty with intricate tasks (especially if complicated by uncompensated visual defect) Stooped posture, loss of height, and other distortions owing to atrophy and effects of weakness in skeleton and major muscle groups responsible for posture and antigravity support.	extremities Arthritis: ankylosis and contractures Fractures, spontaneous and traumatic Herniae, extra- and intra-abdominal
Gastro-intestinal system		
Dental caries; gingival recession (38, 81)	Problems of adaptation to dentures and altered alignment of bite	Retained carious stumps Cysts Dental sepsis Temporo-mandibular arthritis
	Capricious appetite	Anorexia Malnutrition

TABLE 4—cont.

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Atrophic changes in jaw, mucosae, intestinal glands, and muscularis (111)	Asymptomatic alterations in secretion, motility, and absorption occur in 'normal' aging	Achlorhydria (incidence increases over age 60): related to defective absorption of iron and vitamins, and to pernicious anaemia Dysphagia (pseudo-bulbar palsy: oesophageal reflux, pouches, and carcinoma) Constipation Peptic ulcer Diverticulosis
No significant age changes described in liver, and there are as yet no tests of function sufficiently refined to detect the impairment sometimes suspected in older patients		
Respiratory system		
Coalescence of alveoli (atrophy and loss of elasticity in septa)(8) Sclerosis of bronchi and supporting tissues Degeneration of bronchial epithelium and mucous glands (79)	Total lung volume unchanged but vital capacity is diminished, O_2 diffusion impaired and respiratory efficiency reduced; as are sensitivity and efficiency of self-cleansing mechanisms	Increased susceptibility to pneumonia Pulmonary tuberculosis—(reactivation of 'healed' tuberculosis) Carcinoma of bronchus Pulmonary embolism
Osteoporosis (130) { thoracic vertebrae / rib cage	Kyphosis and increasing rigidity of chest wall	Concurrent respiratory disease and cardiac failure
Reduced elasticity and calcification of costal cartilage Weakness of intercostal and accessory muscles of respiration (92,93, 179)	Functional reserve respiratory capacity is therefore impaired in old age, but clinical evidence is minimal unless evoked by illness. Compliance changes little because the rise to be expected from diminished elastic recoil is offset by increased lung stiffness (fibrosis) and loss of flexibility in chest wall	

TABLE 4—cont.

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Cardiovascular system		
Aorta—loss of elasticity in media and intimal hyperplasia (68)	Aorta dilated and unfolded (may obstruct venous return in left side of neck)	Arrythmias
Cusps of heart valves degenerate—less resilient with nodular sclerosis and sometimes calcification which may extend into interventricular septum	Apex beat difficult to locate if chest rigid or distorted by kyphoscoliosis Stiffened valves cause murmurs—aortic systolic, mitral regurgitant; not necessarily significant	Conduction defects—bundle branch block probably indicates significant heart disease Aortic stenosis Pulmonary heart disease (pulmonary embolism)
Myocardial changes—lipofuscin deposits; myocardial fibrosis, and amyloidosis (182) Atrophy and fibrosis of media, and intimal hyperplasia in coronary arteries Brown atrophy only in association with debilitating states—malnutrition, cancer, pernicious anaemia, etc. (heart weight correlates with body weight) (107)	No specific age-determined changes or degeneration in the heart (i.e. no 'senile heart disease') can be correlated convincingly with impaired cardiac function in old age but it is accepted that; (i) Cardiac output declines owing to reduced stroke volume. (ii) Therefore capacity for physical work is limited. (iii) A given amount of exercise raises heart rate and blood pressure more in old age than in youth.	Blood-pressure changes—difficult to define hypertension over age 65. B.P. readings alone do not constitute a diagnosis (it requires evidence of adverse effects on eye, heart, brain, and kidney). Hypotension often more sinister in old age.
Atheroma—incidence increases with age, probably promoted by hypertension and cigarettes (178)	Mental confusion and profound weariness should raise suspicion of heart disease in old people. They are often more prominent than anginal pain or even breathlessness (because of restricted activity)	Ischaemic heart disease is the most common cause of heart failure in geriatric medical practice

TABLE 4—cont.

Morphological age changes in various systems	Related 'normal' age changes	Clinical pathological trends
Genito-urinary system		
Thickening of basement membrane of Bowman's capsule and impaired permeability Degenerative changes in tubules Atrophy and reduced numbers of nephrons Vascular changes affect vessels at all levels from intimal thickening of the smallest, to arteriolar hyalinization and intimal hyperplasia in large arteries Prostatic atrophy—acini and muscle with focal areas of hyperplasia Benign nodular hyperplasia present in 75 per cent of males over 80 years of age Histological (latent) prostatic carcinoma demonstrable in most males aged over 90 years (clinical carcinoma very much less) (5, 44,90)	Renal efficiency in waste-disposal impaired: (i) The number of nephrons is halved in an average life span (ii) Renal blood flow is also halved by age 75. (iii) Glomerular filtration rate and maximum excretory capacity reduced by same proportion. The aging kidney can still maintain normal homeostatic mechanisms and waste disposal within limits, but it is less efficient, needs more time, and its reserves may be minimal. Therefore relatively minor degrees of dehydration, infection, or impaired cardiac output may precipitate failure	Renal calculi Renal infections—pyelonephritis, cystitis Prostatic disease Gynaecological disorders Retention Incontinence

TABLE 4—cont.

Other systems

Endocrine

Failure is not a consequence of 'normal' aging but, as in other systems, poverty of reserve may precipitate evidence of deficiency.

(i) Secretory capacity of the pancreatic β cells diminishes, and diabetic abnormality of glucose tolerance increases with age. The efficiency of insulin in dealing with excess glucose apparently declines, and aging is the most conspicuous factor predisposing to clinical diabetes

(ii) Functional thyroid activity decreases with age; B.M.R. and radioactive iodine uptake fall, myxoedema is three or four times more common than thyrotoxicosis in older people.

(iii) Pituitary activity appears to be retained at normal levels with age, but adrenal activity is impaired.

Homeostasis

Particularly vulnerable in old age to plasma or blood loss, dehydration, potassium depletion, and metabolic acidosis. At rest the normal old person can maintain a constant internal environment, but capacity to react to stress, even the demands of daily living, is markedly lessened owing to two key characteristics of aging:

(i) Poverty of reserve which impairs the ability to restore systemic equilibrium quickly when it is upset.

(ii) Breakdown in co-ordination because different organs age at different rates, and functions dependent on the performance of several systems are therefore impaired.

Central autonomic dysfunction

May contribute to postural hypotension, impaired temperature control and the risk of hypothermia, loss of appreciation of visceral pain, and defective alimentary motility.

third column shows the more common pathological predispositions of aging individuals which necessitate medical intervention.

A Model for Studying Aging: The Red Blood Cell

The death of a cell may be considered as a progressive phenomenon, a kind of telescoped death of a higher organism in miniature. The senescence of a cell may be considered similarly. Investigations of the mechanism and characteristics of cellular aging represent, therefore, a larger scope than the aging of a minute part of an organ of the body. The choice of a model for such a study may be directed either by consideration of the importance of the cell to be investigated, or by its relative simplicity in terms of anatomy, biochemistry, and physiology and, therefore, the probability that it offers to elucidate some basic problems in the process of cellular aging. From this point of view, the mammalian red blood cell is a suitable model for the study of aging at the cellular level (43). Aging, in a general sense, as exmplified by the various theories of the mechanism of aging, can be viewed as any change from steady state. One of the sources of a change in steady state, at the cellular level, is a change in cell surface-to-volume ratio. The erythrocyte osmotic fragility test provides a way of evaluating the surface area/volume (SA/V) relationship of normal and abnormal red blood cells (201). Therefore, the erythrocyte osmotic fragility test was utilized to assess changes in human red blood cells with aging. These changes in surface-to-volume ratio of human red blood cells, as measured by the osmotic fragility test, would thus reflect a change from steady state indicative of aging at the cellular level.

Osmotic Fragility

Changes in the fragility of red cells are closely related to changes in their osmotic behavior, and although the fragility is often spoken of as if it were a simple property, it depends in a complex way on a number of variables. To determine it, red cells are placed in a series of relatively large volumes of media of decreasing tonicity separated by intervals of tonicity, and the tonicity in which there is just commencing hemolysis determines the fragility. Some investigators prefer to use tonicity in which there is complete hemolysis, and some plot the whole curve for the percentage of complete hemolysis as a function of tonicity (149).

The classical model of osmotic swelling and lysis of the red cell postulates a balloon-like cell in which (1) constant intracellular contents are present with no significant anomalies of osmotic coefficient, (2) the membrane is virtually inelastic and offers no significant resistance to deformation from disc to sphere, (3) the membrane is virtually impermeable except with respect to water molecules, and (4) the membrane shows minimal resistance to rupture when the cell reaches the maximal volume which can be encompassed by its surface area. Each of these postulates can be regarded as inadequate or to have been incompletely demonstrated, although the design of most osmotic fragility studies avoids serious errors due to deviations from these expectations. For example, cation interchanges occur slowly across the membrane, the intracellular protein is at a concentration in which the osmotic coefficient may vary significantly with changes in protein concentration (1,47,189,205), and in some mammalian cells membrane elasticity may show significant variations (129). Likewise, in some animal cells

cation permeability may show considerable variation with changes in cell volume (145,164), possibly due to a conformational change in a membrane receptor site (158), and there is some evidence of a similar relationship between cell volume and potassium flux in human red cells (153). The significance of these findings to the lysis of the cell swollen by a hypotonic environment needs to be elaborated; aspects of red cell osmotic lysis and departures from the precise expectations of classical theory have recently been reviewed by Wessels and his colleagues (207,208,209). It was observed by Ponder that the red cell does not act as a perfect osmometer even when the relationship between the volume changes and osmolality refers only to the volume of the intracellular water space (148). It was found that there were significant differences in the relationship of volume to osmolality, depending on whether the cells were taken from defibrinated blood or from oxalated blood. Another observation made at this time by Ponder was that the osmolality of plasma was approximately equal to that of 0.15 M sodium chloride solution, whereas the red cell had to be suspended in slightly higher concentrations (1% or 0.172 M chloride) to be isovolumic with cells in plasma. Other evidence that the osmotic properties of the red cell are affected by the composition of the suspending medium for the red cells is given by Ponder (151), who studied the relationship between red cell volume and tonicity of suspending solutions of barium, magnesium, strontium and calcium chlorides. The effect on hemolysis of the addition of low concentrations of calcium and magnesium salts to hypotonic buffered saline were studied: concentrations of calcium chloride as low as 10^{-4} M significantly diminished hemolysis of red cells in 145 mOsm saline, whereas magnesium chloride

produced no effect below a concentration of 10^{-2} M. Both the fast and slow phases of hemolysis were diminished (Figure 1). The calcium treated cells were less osmotically fragile than the red cells suspended in sodium chloride alone. The osmotic fragility curve is highly dependent on the specific solution used as the suspending medium (22).

Clinical Significance of Osmotic Fragility Measurements

Increased osmotic fragility, or decreased resistance to hemolysis, is a feature of spherocytes of all types and may therefore indicate congenital spherocytosis, idiopathic acquired hemolytic anemia, isoimmune hemolytic disease of the newborn infant (more common in A-B-O incompatibility than in Rh sensitization), and other hemolytic anemias. Normal osmotic fragility (Figure 2) (122) is found in symptomatic hemolytic anemia and in paroxysmal nocturnal hemoglobinuria. Decreased osmotic fragility is seen in sickle cell anemia and in hemolytic anemias characterized by abnormally flat target cells, such as occurs in some of the hemoglobinopathies (Figure 3) (123). Any history of biophysical studies of the red cell membrane in disease states should certainly credit the earliest studies of mechanical and osmotic fragility. Although these laboratory tests were not definitively interpretable at the time they were first found useful for evaluation and diagnosis of clinical disease, their physiologic significance has now become much more apparent (201). Stated in its simplest form, the osmotic fragility (O.F.) test provides a way of evaluating the surface area/volume (SA/V) relationship of normal and abnormal cells (213). Thus, increased fragility is generally interpretable as indicative of a decrease in SA/V or

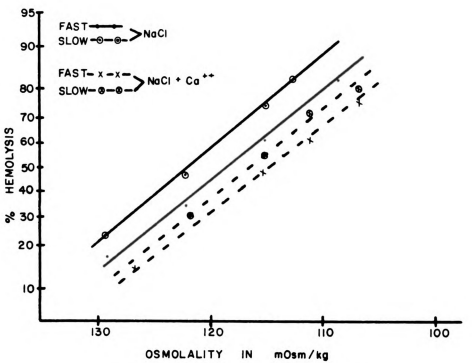


FIGURE I. EFFECT OF IONIC COMPOSITION OF SUSPENDING MEDIUM ON OSMOTIC FRAGILITY OF HUMAN RED BLOOD CELLS. Shown are the fast and slow phases of hemolysis with NaCl as the suspending medium, and the fast and slow phases of hemolysis with NaCl plus Ca^{++} (1.25 mM) as the suspending medium. Ca^{++} decreased the osmotic fragility of the red blood cells at the same medium osmolality.

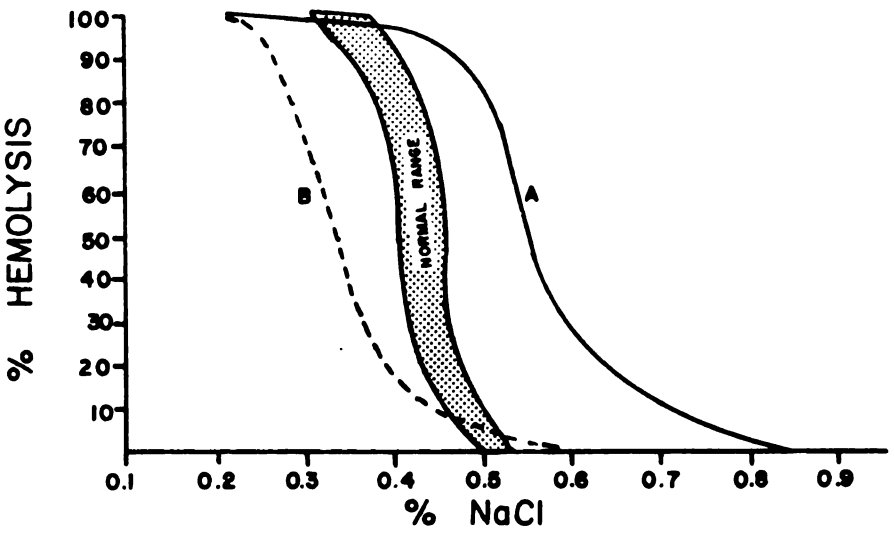


FIGURE 2. NORMAL AND ABNORMAL OSMOTIC FRAGILITY CURVES. A, Increased osmotic fragility. B, Decreased osmotic fragility (122).

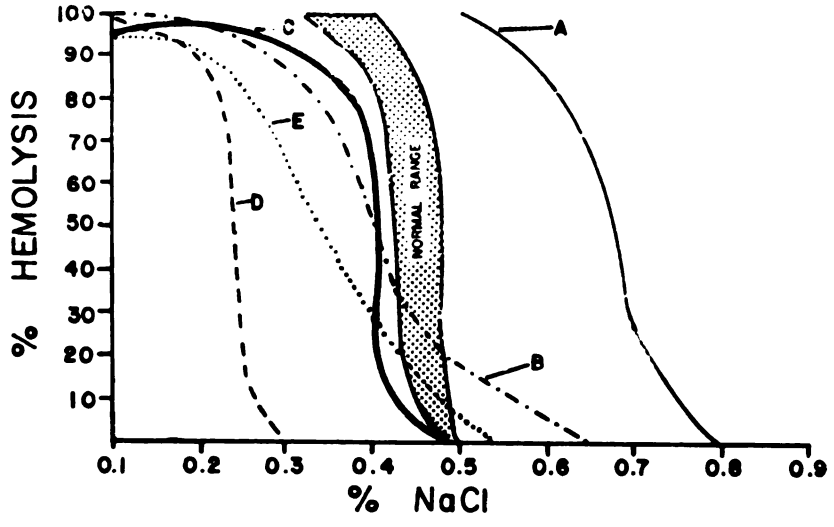


FIGURE 3. OSMOTIC FRAGILITY IN DISEASE STATES. A, Hereditary spherocytosis. B, Thalassemia major. C, Thalassemia minor. D, Hb - E disease. E, Hb - E - thalassemia (123).

sphering. Conversely, decreased O.F. (increased resistance to lysis in hypotonic salt solutions) implies either an increase in surface area or a decrease in cation content or volume in an isotonic medium. Both hereditary spherocytosis and spherocytosis seen in immune hemolytic disease are associated with increased O.F., while the increase in membrane lipid content seen in a variety of disorders is associated with enhanced osmotic resistance (37). However, in spite of broadened insights into the pathophysiologic alterations in red cells implied by altered O.F., it is still essential to recall the fundamental assumptions upon which the test is based. First, as a way of estimating red cell surface area, the O.F. test assumes a normal cell volume in plasma, reflecting primarily a normal total cation content which should not change in prelytic hypotonic salt solutions. Inferences regarding decreased surface area from an increased O.F. must exclude any increase in cell volume. Interpretation of increased surface area based on decreased O.F. must also be based on normal volumes in plasma and must exclude any excessive prelytic loss of cation from partially swollen cells, as demonstrated by Ponder (150) in cells affected by certain detergents and surface active agents. Finally, interpretation of O.F. as an expression of SA/V assumes that the intrinsic properties of the membrane have not changed. In hereditary spherocytosis, however, the membrane itself becomes very rigid and shows an anomalously low swelling prior to lysis and shrinkage after lysis (99). Mechanical fragility, on the other hand, is related also to the shape factor, i.e., decreased SA/V (sphering) makes the cells less able to tolerate deforming stresses and, therefore, more mechanically fragile. In addition, however, mechanical fragility may reflect either a cell with rigid

contents, e.g., sickle cell, or cells which have lost their normal intrinsic membrane deformability, such as metabolically depleted cells (202). Thus, rather than being a non-specific indicator of abnormal cells, the fact that mechanical fragility is abnormal in so many disorders strongly suggests that loss of cellular deformability for any reason, whether it be shape change or development of a rigid interior or membrane, is a final common pathway leading to premature cellular destruction (201).

The Time Course of Osmotic Hemolysis

A further dimension to osmotic fragility studies has been the time course of the hemolytic process. According to Bowdler and Chan (20), the degree of lysis of erythrocytes in hypotonic solutions is time-dependent. They showed: (a) Red cells are lost from suspension in hypotonic electrolyte solutions in two phases. An early rapid phase, believed to be due to water influx resulting from the higher chemical potential of water external to the cell, is followed by a second, smaller phase of cell loss with much longer half-time. (b) The second phase is most prominent in the middle of the range of partial hemolysis and is much less at the extremes of the range of hemolysis. (c) The second phase is prevented or terminated by inclusion, or addition, of sucrose to a concentration of 20 mM in the system, and it is slowed by the presence of 0.005% tannic acid, without alteration of magnitude of the second phase. (d) The magnitude of the second phase is dependent on the dominant cation of the suspending medium; it becomes progressively greater through the series Mg^{2+}, Na^{+}, Li^{+}, K^{+} and Rb^{+}. These findings were interpreted as being due to increased passive cation permeability in cells

undergoing swelling to a volume close to that critical for hemolysis, with water influx resulting from the unopposed colloid osmotic pressure of the intracellular protein. Wessels and Veerkamp (209) have similarly shown that osmotic resistance in NaCl solutions is strongly time dependent. Though the time of lysis depends in some way on the permeability, it is clear that the rate of hemolysis may also be affected by the following factors: (a) the surface/volume ratio, which varies with the shape of red blood cells of various mammalian species (86,211), (b) differences in membrane elasticity, though not observed with human erythrocytes (129), (c) the loss of osmotic material from the cells during the permeation experiments (50,98), (d) variations in initial intracellular tonicity (141), (e) changes in membrane properties induced by the penetrating substances. According to Jay and Rowlands (88), when an erythrocyte is placed in a sufficiently hypotonic environment it swells to a sphere and hemolyzes, releasing its hemoglobin into the suspending medium (Figure 4) (30). The cell becomes a ghost cell, having lost all or most of its hemoglobin. The rate at which the cell volume increases depends on one or a combination of a driving force, resulting from an osmotic or a concentration gradient, and the permeability of the cell membrane to water and to the permeating solute, of which there is a concentration gradient across the membrane. Jay et al. (88) state that hemolysis is a multistage process. The stages are swelling, popping, reduction in volume accompanied by ion leakage and, finally, hemoglobin leakage (Figure 5). The classical hemolysis time (T_H) is made up of a swelling time (T_{SW}) and a stress time (T_{ST}). Stress time is not negligible and with the faster permeants it may occupy more than 75% of the hemolysis time. The stress time can also be

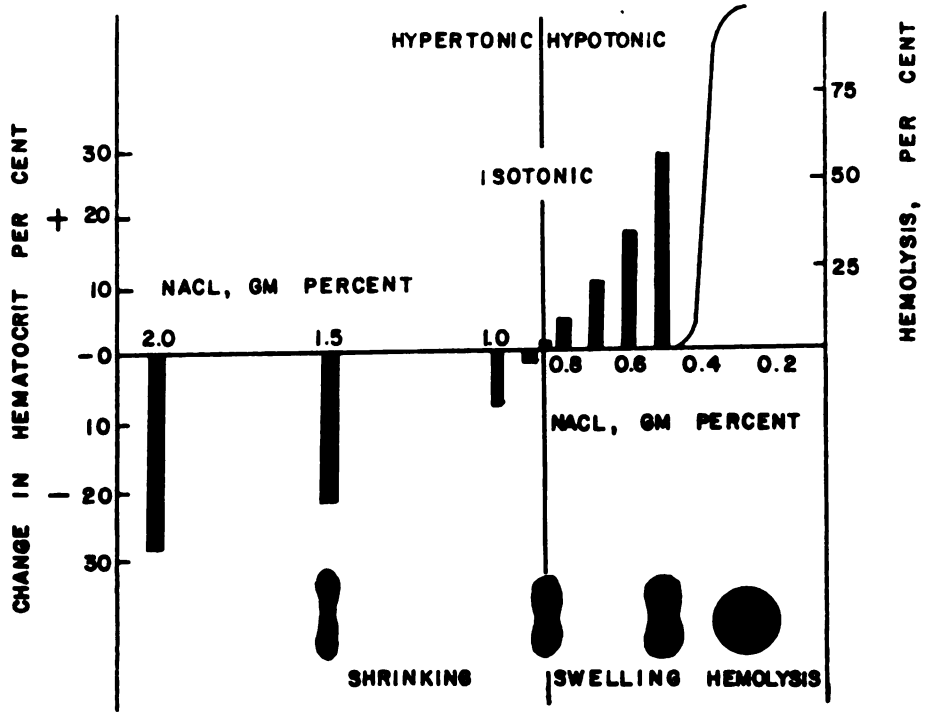


FIGURE 4. BEHAVIOR OF NORMAL CELL IN HYPERTONIC AND HYPOTONIC MEDIA (30).

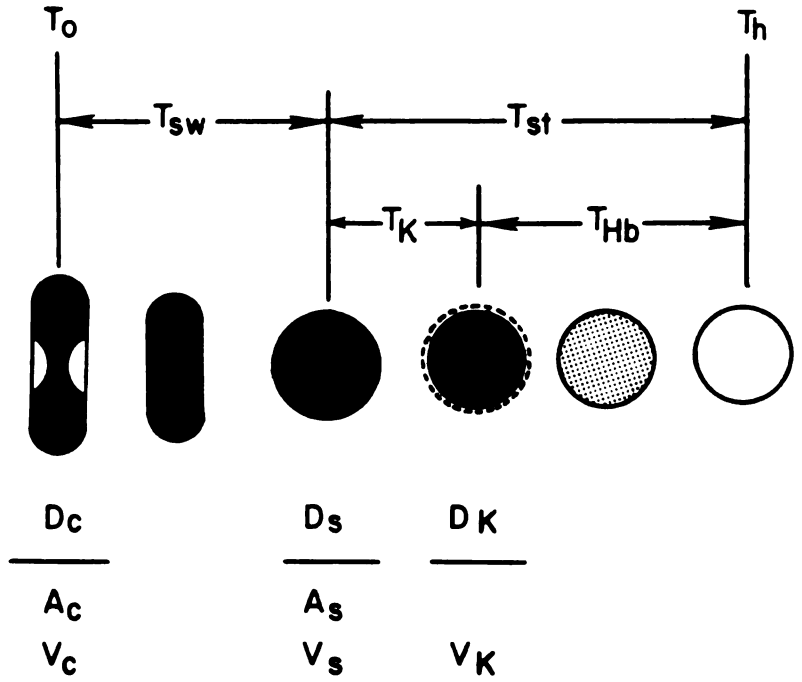


FIGURE 5. DIAGRAM OF THE SEQUENCE OF EVENTS IN THE PROCESS OF OSMOTIC HEMOLYSIS. T_o, initial time $T = O$; T_h, total hemolysis time; T_{sw}, swelling time; T_{st}, stress time; T_K, potassium leakage time, and T_{Hb}, hemoglobin leakage time. The diameters of the cell D_C, D_S and D_K can be measured at the various times and the corresponding areas and volumes can be calculated. V_o, the initial cell volume can also be calculated from D_s, the diameter of the cell at maximum swelling (88).

divided into two parts: a K^+ leak time (T_K) during which the cell shrinks and a time (T_{HB}) during which hemoglobin is leaving the cell. The T_{HB} occupies a substantial part of T_H, from 25 to 65%, and is relatively longer in fast hemolysis.

Red Blood Cell Membrane Components and Suggested Models

Biconcavity and deformability are two unique properties of the human erythrocyte. It is generally accepted that the biochemical composition of the red cell membrane underlies these biophysical features (99,201,212). Approximately 44% of the dry weight of the red cell membrane is lipid, while 49% of the dry weight is protein and only 8% of the mass is made up of carbohydrate (71). Enzymes and calcium are also present.

The lipids in erythrocytic membranes are of three types: neutral lipids, phospholipids, and glycolipids. The neutral lipids of the red cell membrane consist almost entirely of free, nonesterified cholesterol (136). Cholesterol accounts for 29% of the dry weight of membrane lipids (37) and for 43% of the molar concentration of membrane lipids (172,198). Knowledge about the location of cholesterol in the erythrocyte membrane is extremely limited. Murphy (132) observed that cholesterol was concentrated around the periphery of the biconcave disc. The results of these autoradiographic studies have been neither confirmed nor denied (27). It has been generally assumed that on a molecular basis cholesterol is associated with other lipid molecules. It is not known, however, whether cholesterol is distributed equally between the outer and inner regions of the lipid core of the erythrocyte membrane (162,191). Reticulocytes can synthesize cholesterol from acetate (10), but mature red cells lose this

capacity (116). Red cell membrane cholesterol is in an equilibrium exchange with the free cholsterol bound to serum lipoproteins (87). Complete equilibrium between plasma and cell free cholesterol occurred in approximately eight hours, both *in vitro* and *in vivo* (131). Sterols other than cholesterol are capable of exchanging with cholesterol in the red cell membrane (26). The action of steroid hormones may be related to their direct incorporation into membranes, and this may be true for progesterone (46). There are changes in red cell lipids during storage. Normal red cells stored at refrigerator temperatures in acid-citrate-dextrose (ACD) undergo a progressive loss of both cholesterol and phospholipid amounting to approximately 25% in seven weeks (73,193). A decrease in critical hemolytic volume (an indirect measure of surface area) accompanies this lipid loss. In contrast to this symmetrical loss of lipid, saline-washed red cells frozen in saline containing EDTA undergo a progressive, but selective, loss of glycerophosphatides during storage (199). This alteration results in a decrease of total lipid phosphorus by more than 30% after eight weeks of storage. There is little or no change in the red cell content of sphingomyelin or cholesterol (37). There are also changes in red cell lipids *in vitro*. The selective loss of cholesterol from red cells in incubated serum results from the esterification of free cholesterol in serum by the serum enzyme lecithin:cholesterol acyltransferase (LCAT) (87). This loss of cholesterol from membranes is associated with a proportional loss of surface area, as measured by osmotic fragility (31). When incubated under conditions leading to metabolic depletion, red cells undergo a loss of both cholesterol and phospholipid. This change occurs prior to the onset of measurable hemolysis (36). Similar lipid loss has

been observed with red cell ghosts (104). Protein is not lost from ghosts under these conditions, suggesting that lipid loss occurs, at least in part, because of an altered membrane affinity for lipid. The late loss of lipid from intact red cells, however, is accompanied by a loss of membrane protein. Associated with this lipid loss is a proportional decresae in membrane surface area, as estimated from critical hemolytic volume (202). In contrast to the selective loss of cholesterol, the loss of lipid under these conditions is irreversible (36,37). There are changes in red cell lipids in disease, e.g., hereditary spherocytosis (37). After splenectomy in patients without HS, target cells with increased surface area occur and, parallel to their appearance, there is an increase in osmotic resistance. Both evolve to a maximum over several weeks to several months. The red cell content of lipid is increased 15 to 20% in patients without HS who have undergone splenectomy, as compared with normal subjects with intact spleens (35). Comparison of red cells from patients with and without HS, all of whom have undergone splenectomy, reveals, therefore, a 15 to 20% deficiency of both cholesterol and phospholipid in the HS cells. However, HS cells after splenectomy have a lipid content which is, nonetheless, similar to that of red cells from normal subjects whose spleens are intact. Despite this similarity, red cells from HS patients without spleens are more osmotically fragile than red cells from normal subjects with spleens. This difference indicates that factors in addition to lipid deficiency per se are responsible for the deficiency of surface area in HS red cells. Data indicate that an abnormal interaction between ATP, divalent cations, and the membrane may exist in HS red cells, and that this abnormality may account for the cells' leakiness to sodium

and instability upon ATP depletion (100). Thus, despite considerable data regarding membrane lipids in HS and the role of lipids in various membrane phenomena which appear abnormal in HS, the nature of the membrane defect in this disorder is still unknown. However, the acquisition of lipid and surface area by HS red cells *in vivo* appears to circumvent this defect and to allow a marked prolongation of cell survival (37). More recently, Shinitzky, Moses and Livne (167) have reported that erythrocytes affected by HS, obtained from splenectomized patients, showed a varying degree of elevated osmotic fragility. The increased microviscosity of the membrane lipid core correlated with the severity of HS. The data supported the proposition that the defect in HS-affected cells is associated, at least in part, with alterations in the membrane lipids. Farnsworth et al. (58) have demonstrated that modifications of the lipid composition of the cellular and subcellular membranes of rat erythrocytes are associated with changes in their permeability. Modifications of the lipids are associated with a significant increase in the osmotic fragility of the erythrocytes and an indication of a more homogeneous red-cell population in comparison to the controls. Patients with various forms of liver disease, including hepatitis, cirrhosis, and obstructive jaundice, have red cells with an increased content of cholesterol and phospholipid (34,135). Increases in membrane cholesterol content correlate with increases in membrane surface area, as measured by osmotic fragility. Using Cr^{51}, it has been found that normal red cells progressively acquire surface area and become resistant to lysis in the circulation of patients with obstructive jaundice over the course of two to three days, and that osmotically resistant target cells lose surface area and become osmotically normal over a

similar time course in the circulation of normal subjects (34). The phospholipids of the erythrocyte membrane account for 69% of the dry weight of membrane lipids and for 54% of the molar concentration of membrane lipids. The four major classes of phospholipids, arranged in order of decreasing concentrations, are: phosphatidyl choline (lecithin), phosphatidyl ethanolamine (cephalin), sphingomyelin, and phosphatidyl serine (37). Recent studies appear to support the concept of a nonrandom distribution of phospholipids between the inner and outer regions of the membrane (91). Lecithin and sphingomyelin are predominantly located in the outer part of the lipid layer and phosphatidyl ethanolamine and phosphatidyl serine on the inner part of the lipid layer (162,191). About 50% of the fatty acids in red cell membrane phospholipids are saturated and 50% are unsaturated. Reticulocytes, but not mature red cells, can synthesize fatty acid *de novo* (10). Plasma free fatty acid and plasma phospholipids can exchange with their respective counterparts in red cell membranes (172). Glycolipids account for a small fraction of the total membrane lipids of erythrocytes (2% by weight and 3% by molar concentration). The most abundant erythrocyte glycolipid is globoside (37). Unlike cholesterol and phospholipids, which are widely distributed in membranes, globoside seems to be restricted to plasma membranes. Other red cell membrane glycolipids have antigenic activity corresponding to the Lewis and ABH blood groups (114). It is of interest that neuraminic acid (sialic acid) is confined to membrane glycoproteins and is not a component of membrane glycolipids. Erythrocyte membrane carbohydrate is found primarily as glycoprotein and glycolipid complexes (135). As a group, the glycoproteins of the erythrocyte membrane all appear to be integral membrane proteins and

are hydrophobically bound to the lipid bilayer of the membrane (188). Red cell membrane proteins are not as well characterized as the lipids, although about half the mass of these membranes is composed of protein. At least seven to nine major proteins can be defined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by gel filtration technique (109,147,204). Two membrane proteins are more or less well characterized. The first is referred to as spectrin, because of its initial identification in experiments using red cell ghosts, or as erythrocyte actomyosin because it bears a strong resemblance to muscle actomyosin (71,144). Spectrin accounts for about 20 to 25% of stromal protein and the monomeric form has a molecular weight of about 240,000 (57,190). Spectrin is soluble in the presence of chelating agents, contains no lipids or carbohydrate groups, is rich in glutamic acid, contains no cysteine, and has been purified to apparent homogeneity as judged by electrophoresis, immunodiffusion, and ultracentrifugation (71). It has a tendency to form insoluble fibers or filaments in the presence of divalent cations, especially calcium (71). It is believed that spectrin is located on the inner surface of the red cell membrane (24). Erythrocyte glycophorin is the other well studied red cell membrane protein. It is the principal glycoprotein of erythrocyte stroma, accounts for about 10% of total membrane protein, has a molecular weight of about 55,000, and is transmembranous with its carbohydrate moiety located on the external surface of the red cell (113). About 60% of glycophorin in membrane is sialic acid. This, in turn, accounts for the characteristic negative charge of the red cell that regulates interaction with neighboring cells and the surrounding medium. Moreover, this protein carries the A, B, M, and N specific blood group antigens

as well as receptors for phytohemagglutinin, wheat-germ agglutinin, and influenza viruses (113). Several enzymes seem to be tightly bound to the red cell membrane: glyceraldehyde-3-phosphate dehydrogenase, aldolase, 3-phosphoglycerate kinase, adenylate kinase, cyclic AMP-dependent protein kinase, adenosine triphosphatases (Mg^{2+}-activated ATPase, Ca^{2+}-activated ATPase, [Ca^{2+} + Mg^{2+}]-activated ATPase, [Na^{+} + K^{+} + Mg^{2+}]-activated ATPase [195]), and cholinesterase (11). Evidence strongly indicates that the bulk of the erythrocyte calcium, and perhaps the whole of it, is present in the membrane (53,75,195).

Recent evidence suggests that the primary defect in hereditary spherocytosis is not restricted to the lipids but may also involve the membrane proteins. Johnsson (89) proposes that the alterations in red cell lipid components in spherocytes of varying origin may represent a phenomenon secondary to the abnormal red cell shape rather than being related to a specific etiological factor. Release of cholesterol and phospholipid, particularly under conditions of metabolic depletion (35), increased cation pumping activity, and increased phospholipid turnover, could all be secondary to altered lipid-protein interactions (191). Although the exact nature of the erythrocyte defect has not yet been fully clarified, many lines of evidence indicate that the primary defect resides in the red cell membrane protein. These include (1) a partial or complete deficiency or malfunction of a spectrin-like membrane protein component of the erythrocyte (66,105); (2) the presence of a genetically defective membrane microfilament (83,84,85); (3) reduced levels of membrane spectrin phosphorylation probably secondary to a defective membrane kinase enzyme (70,126); (4) an excess of negative charges of membrane

proteins (55); (5) a defect in shape regulation which results from an alteration of any of the proteins in the shape change complex and/or accessory regulatory proteins (11,166,214). The net outcome of these biochemical, physiologic, and structural aberrations results in the formation of microspherocytes, cells that are rigid, with decreased surface area to volume ratio, increased mean corpuscular hemoglobin concentration, increased osmotic fragility, decreased deformability, and reduced life-span (11,82). The biconcave shape of the red cell and other properties of it such as its capacity to transform between the shapes of disc and sphere despite the uniformity of the physical properties demonstrable over the surface, result at present in the lack of a completely adequate model. However, there are some early membrane models: (a) Gorter and Grendel (67) suggested a simple lipid bilayer. They extracted the erythrocyte with acetone and measured the monomolecular film formed from the extraction product with Langmuir's device. They found a total film area of about 200 square microns per cell, about twice their estimate for the surface area of the erythrocyte. Thus, they concluded that there is just enough lipid in each cell to cover it twice, and that the lipids are associated in a bilayer. The fact of the matter is that erythrocytes do not always behave as if they had a lipid exterior. For example, their surface tension, which is a measure of the tenacity with which molecules at the surface of a liquid cling to their own kind, is far too low. Fats and oils in an aqueous environment have very large surface tensions because of hydrophobic bonding; lipids extracted from erythrocytes do not. (b) Later, Danielli and Davson (42) extended the model to include a coating of protein. The interior, in their model, was an unspecified "lipoid" region. Danielli suggested that

the anomalously low surface tension of erythrocyte lipids is the result of contamination by protein, which would naturally seek the surface of a lipid droplet and thereby change its character. The hydrophilic behavior of intact cells could also be explained by this assumption, which led Danielli and Davson to propose the first complete membrane model in 1935. Danielli envisioned a membrane with a lipoid center, coated on either side with protein. The phospholipids in his model are oriented in two monomolecular layers, with their hydrophobic tails toward the inside of the structure and their hydrophilic phosphates on the surface, contacting the layers of protein. The fundamentals of this structure are still accepted today, although there have been numerous proposals for modification. (c) The unit membrane, first proposed by Robertson (157) in the 1950's, explained the trilaminate appearance of many membranes in the electron microscope by including an extended layer of protein on either side of the bilayer. In the middle 1950's Robertson found a way to verify the basic features of the Danielli model with the electron microscope. He devised staining techniques that resolve most membranes into two distinguishable lines on micrographs, whereas both electron and light microscopists had hitherto seen only single lines. It was later demonstrated by others that partial extraction of membrane lipids with acetone leaves the double lines intact, suggesting that the stain was revealing protein layers on the two surfaces of the membranes. Robertson's membrane model has rather thin protein layers on the two surfaces, more consistent with an extended β-structure than with the compact globular proteins suggested by Danielli. Furthermore, Robertson's model is not necessarily symmetrical--whereas the internal surface in the model is

coated with protein, the outer surface could be either a mucoprotein or a mucopolysaccharide. These features, along with the single lipid bilayer leaflet 40 to 65 Å thick, is known as the unit membrane model, a name that implies a homogeneity in structure. There are some modern membrane models. The size and solubility properties of membrane proteins suggest that they are not confined to the surface but are interspersed among the lipids: (a) The protein crystal model is a regular arrangement of protein and bilayer. A number of workers have published electron micrographs in which the membranes, prepared by freeze-etching, seem to be composed of regular, repeating units, or subunits. This concept represents a considerable departure from a continuous lipid layer. David E. Green (192) would discard both the Danielli and the unit membrane hypotheses for an alternate model for the protein-lipid interactions in membranes called the protein crystal model. The presence of protein within membranes, rather than merely on their surface, not only explains the hydrophobic interaction between protein and lipid, but also helps to explain certain aspects of membrane permeability. In addition, it provides enough room to allow the proteins to assume a globular form like that of most enzymes, which frequently have diameters of 40 Å or more. Various physical measurements carried out on membrane proteins suggest that they are indeed globular, and, therefore, inconsistent with the space allotted them in the unit membrane. (b) A more irregular structure is known as the fluid mosaic model. This fluid mosaic model introduced by Singer (176) features the same intimate contact between lipid and protein, but a less rigid arrangement and stoichiometry. Rather than being discretely arranged, proteins float freely in a phospholipid bilayer (54). Blank (18) presents

even another model. The measured two dimensional yields of protein monolayers are high and parallel the rigidity of erythrocyte membranes with regard to the dependence on divalent cations in the aqueous phase. Although the surface properties of membrane proteins have not been measured, it is likely that this protein layer would also be far from fluid, and could be the basis for the mechanical stability of the membrane. The rheological properties of protein monolayers suggest that the membrane proteins normally present could account for the mechanical stability of the membrane and for some of the observed rheological properties of erythrocyte membranes. However, a membrane having this structure would also be thicker and considerably less fluid, at least in parts, than has been suggested in recent membrane models. Skalak (177) reports that the mechanical behavior of the red cell membrane is consistent with the fluid mosaic model of the structure of the membrane. Electron microscope studies on red cell membrane have provided some ultrastructural information on membrane structure. It has been reported that examining the red cell ghost membranes by electron microscopy and metal shadowing revealed that the membrane consists of short cylinders (or plaques) with heights of approximately 30 Å and diameters of 100 to 500 Å. Cylindrical particles approximately 100 Å in diameter and normal to cell surface have been demonstrated in the membrane interior by the use of freeze-cleaving technique. A model of the red cell membrane composed of cylindrical apoprotein molecules bound at their central belts by lipids has also been suggested (203). However, the information based on metal-shadowed membranes loses much of its appeal when the artifacts and limitations of the technique are considered. Many problems also remain in transferring the

information from freeze-cleavage replicas to a detailed model of the architecture of the erythrocyte membrane. It is not known whether the intramembranal particles represent intact proteins or only hydrophobic regions of proteins. Nor is it possible to assess the extent of heterogeneity of the particles in the A fracture face and their physical and chemical relationship to the particles in the B fracture face (188).

RBC Shape:Deformability:Surface Area/Volume Relationship

In the biconcave disc shape, it appears likely that the shape is a net result of the equilibrium between surface tension, ratio of cell radius to membrane thickness, pressure differential across the membrane, and extensional stiffness (177) of the membrane, assuming the erythrocyte membrane is in fact elastic. Rakow et al. concluded that the elasticity of the red cell membrane is attributable to one or more of the membrane proteins (155). Evidence has accumulated that indicates that the conformation of spectrin (and probably actin) determines red cell shape and deformability (85). Shrinkage of human red cell ghosts is controlled by membrane "contractile" proteins possessing Ca^{++}-ATPase activity (144) and phosphorylation-dependent shape changes in erythrocytes are related to the association of spectrin per se (190). Mohandas et al. (126) have demonstrated that red cells can be shown to sphere suddenly when heated to a specific temperature, between 48 and 50°C. This is the temperature at which spectrin, and only spectrin, denatures or melts. It has also been demonstrated that the distortion of the red blood cell cytoskeleton induces a redistribution of integral membrane proteins which modifies the surface properties of the cell (115). Sheetz

postulates that red cell membrane shape change is dependent on a protein shape change complex and/or accessory regulatory proteins (166). Lin has reported that divalent cation, such as calcium, causes fusion of lipids in the membrane with resultant shape and stability changes (106). Sheetz states that there are two different categories of shape change, one physical and one enzymatic, each having a different molecular basis (165). Investigators (60,108,143) have reported that cross-linking of membrane proteins is related to shape and deformability changes in the red blood cell and that this cross-linking has an enzymatic basis (173). Beck (13) reports that cell shape changes result from monolayer condensation and/or expansion of plasma membranes when clustering of intrinsic proteins occurs. Shape, intrinsic membrane deformability, and the fluidity of cell contents, the major determinants of erythrocyte deformability, are interdependent. The extreme flexibility of the cell as a function of its biconcave shape is dependent on the intrinsic deformability of the membrane and fluidity of the intracellular hemoglobin solution. Fluidity of contents in a shear field requires a deformable membrane which transmits force to the cell interior. Shape may be a function of cell content (e.g., sickle hemoglobin at low oxygen tension) and also of the membrane (e.g., spherical shape at low intracellular ATP concentration) (202). Shape is an important parameter, for a sphere is recognized to be a geometric form of considerable rigidity (101). ATP is widely recognized as the source of high energy phosphate requisite to the maintenance of normal intracellular cation composition in the erythrocyte by means of an ATPase-related active cation transport mechanism. ATP is also essential for the preservation of the normal biconcave disc shape

(134). Reduction of the intracellular ATP results in the classical disc-to-sphere shape transformation (149). It has been demonstrated that the post-transfusion survival of erythrocytes (133) is directly dependent on erythrocyte ATP levels, an observation which clearly relates cell survival to its metabolic integrity. More recently, it has been observed that ATP and Ca^{++} were linked to contractility of erythrocyte ghosts, a finding which suggested that ATP-related shape change is a function of ATP-Ca^{++}-erythrocyte membrane interaction (144). Studies have been interpreted to indicate that a sensitive ATP-Ca^{++}-Mg^{++} intracellular relationship may regulate the physical state of hemoglobin and non-hemoglobin protein at the inner membrane surface. Ca^{++} accumulation favors a protein gel formation at the inner cell surface, whereas ATP and Mg^{++} move the equilibrium toward the sol state. Gel formation would result in increase of membrane thickness, and gel-induced stiffening of the membrane could increase the viscosity (49,195). Another Ca^{++}-modulated system in the erythrocyte membrane might be a Ca^{++}-dependent contractile protein whose presence in the membrane is strongly suggested by: the ATP-dependent shape change (134), the ATP-Ca^{++}-linked contraction of ghost membranes (144), the demonstration of actomyosin-like protein (112), and the evidence shown (159) for Ca^{++}-dependent ATPase activity of certain erythrocyte fibrillar proteins. Ca^{++} may induce membrane rigidity and shape change by producing a denser thickened gel structure at the inner membrane surface and by stimulation of the membrane protein to a more dense, less compliant contracted state. Most mechanisms causing decreased erythrocyte deformability have their effect either by reducing membrane flexibility, by reducing fluidity of cell contents, and/or by causing increased sphericity, i.e.,

reduction of the membrane surface area-to-volume relationship (101). Mechanisms altering the erythrocyte surface area-to-volume relationship are (a) Heinz body formation, (b) fragmentation, (c) partial phagocytosis, and (d) aging. Heinz body formation in disorders of unstable hemoglobin, defects of the pentose phosphate pathway, or disorders of unbalanced globin synthesis causes increased viscosity of the cell contents; because of membrane attachment of Heinz bodies, the overlying membrane is made rigid. If the entire cell is not eliminated, the splenic pitting mechanism is thought to tear the rigid Heinz body containing portions from such cells, with a relatively excessive loss of surface area as compared to volume. The surviving cell may be spherical and hence more rigid than before passage through the splenic cord-sinus circulation (39). Erythrocyte fragmentation also results in increasing sphericity causing sufficient cell rigidity to make the cell susceptible to eventual mechanical trapping and destruction by the spleen. Partial phagocytosis of erythrocytes is a special case of fragmentation, and the result is increasing sphericity (200). Apparently, phagocytic fragmentation occurs when sensitized cells contact phagocytes, and/or the release of erythrocyte contents as a result of mechanical injury stimulates phagocytosis. In addition to fragmentation as a mechanism for the termination of erythrocyte life-span, many investigators have inquired whether decreasing metabolic capacity resulting from a reduced amount of enzymes or substrates could prejudice survival (156). Studies indicate that significant differences exist in ATP concentration between the youngest and oldest erythrocytes separated by centrifugation. These data and the preceding discussion of ATP as a determinant of erythrocyte deformability, strongly suggest that

eventual erythrocyte destruction may be a consequence of age-related decrease in ATP/Ca^{++} ratio due both to ATP depletion and calcium accumulation (101). Recent analysis of various models of membrane structure by Bull and Brailsford (27) suggests that a protein chain meshwork, interacting to provide a skeletal network, would provide mechanical properties corresponding to those observed in the erythrocyte. Skalak et al. (177) and Evans (56) have developed general two-dimensional elastic material models to describe the continuum mechanical behavior. Shape changes of the erythrocyte are significant in their potential adverse modification of the rheologic properties of the erythrocyte, and thus the survival of cells, as well as the potential compromise of exchange processes at the capillary level. An understanding of the mechanisms of shape change has importance for the insights into structural organization and dynamics of the membrane protein and lipid components and environmental factors, which affect membrane properties and integrity. However, there is no satisfactory model to account for the biconcave shape of the red cell and its capacity to transform between the shapes of disc to sphere despite the uniformity of the physical properties demonstrable over the surface (56,64,102).

MATERIALS AND METHODS

Approximately ten milliliters of blood were drawn by venipuncture from each of 44 subjects and from a control subject on the day of testing. Thirty of these subjects were male blood donors obtained from the American Red Cross and ranging in age from 18 to 59 years. The remaining 14 subjects (seven male and seven female) were from the Lansing and Ann Arbor, Michigan, Senior Citizens' Centers and ranged in age from 60 to 82 years. Heparinization was achieved by drawing the blood into Vacutainer tubes containing premeasured amounts of sodium heparin (143 IU). Heparin was the anticoagulant of choice because its effect on erythrocyte osmotic response is most similar to that of defibrination (23). Packed cell volumes were determined using the microhematocrit method. Hemoglobin (cyanmethemoglobin method) and red blood cell (RBC) counts were determined using the Coulter Counter, Model Z_F.[a] From these values, red cell indices (MCV, MCH, MCHC) were calculated. Only hematologically normal subjects (MCV = 76-96 cuμ; MCH = 27-32 μμg; MCHC = 32-36% [175]) were included in the study. Each blood sample was centrifuged at 2,500 rpm for 10 minutes in an International Centrifuge (IEC) Universal Model UV,[b] the plasma was removed and

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[a] Coulter Electronics, Inc., Hialeah, FL.

[b] International Equipment Co., Needham Heights, MA.

the volume measured. A volume of each subject's red blood cells was added to its respective plasma specimen to yield a packed cell volume (PCV) of approximately nine percent. (This PCV value insured that the optical density of the experimental supernatants would be within the range of the spectrophotometer used.) The RBC osmotic fragility test was then performed on each of the PCV-adjusted cell/plasma suspensions by the following procedure. Four 16 x 100 mm glass culture tubes were prepared for each of the following osmolalities (mOsm/kg) of NaCl: 300, 130, 125, 120, 115, 110, 105 and 0 (distilled deionized water). The osmolality of each solution was determined to within $\pm$ 1.0 mOsm/kg by the use of an Advanced Instruments Model 3L Laboratory Osomometer.[c] The pH of each solution was adjusted to 7.40 with NaOH using a Beckman Model SS-2 pH Meter.[d] Reagents were analytical grade and distilled deionized water was used for all solutions. A Fisher Model 241 Automatic Pipetter[e] was used to simultaneously expel 50 microliters of the previously prepared RBC/plasma suspension and 5 milliliters of the appropriate NaCl solution into an appropriately labeled test tube. Each test tube was incubated for seven minutes at room temperature (22°C) and then the hypotonic solution was returned to isotonicity with 200 microliters of 4 M NaCl added with an Eppendorf pipette. The quadruplicate samples were then centrifuged at 2,500 rpm for 10 minutes and the supernatants were transferred to a duplicate set of test tubes. The optical density of each supernatant was analyzed by using a

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[c]Advanced Instruments, Inc., Newton Heighlands, MA.

[d]Beckman Instruments, Inc., Fullerton, CA.

[e]Fisher Scientific Co., Pittsburgh, PA.

Gilford Model 240 Spectrophotometer[f] at a wavelength of 414 nm. The percentage of cells hemolyzed was determined as a proportion of the total cells in the suspension as follows:

$$\frac{\text{O.D. of supernatant solution}}{\text{O.D. of RBC in distilled water (100\% hemolysis)}}$$

The O.D. of the supernatant from RBC prepared in the same manner but suspended in isotonic saline was subtracted from each O.D. value obtained; this correction allowed for the small number of cells traumatically lost by mechanical damage during the procedures.

Analysis of the osmotic fragility data was performed using a Wang 700B programmable calculator, for which the regression analysis was adapted (48). Curves of fractional hemolysis (H) versus osmolality of the test medium were linearized using the transform $h = \text{In } \frac{1-H}{H}$. Regression lines were then calculated for curves of h versus osmolality. From these results, the osmolality at which 50% of the erythrocytes in the suspension hemolyzed (H_{50}) was determined using the equation

$$H_{50} = - \frac{\text{y-intercept}}{\text{slope of regression line}}$$

Correlation coefficients were then calculated for H_{50} versus age and slope of the regression line versus age.

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[f]Gilford Instrument Laboratories, Inc., Oberlin, OH.

RESULTS

Statistically significant positive correlation was demonstrated between osmotic fragility and age in healthy subjects between the ages of 18 and 82 years ($r = +0.35$, $p<0.05$). Fifty percent hemolysis (H_{50}) occurred at osmolalities varying from 116 mOsm/kg to 136 mOsm/kg (Figure 6 and Table 5).

Red cell variability, as expressed by the slopes of the osmotic fragility curves, increased significantly with age ($r = +0.50$, $p<0.01$) (Figure 7 and Table 5).

Figure 6. H_{50} versus Age. Relationship between H_{50} (the osmolality of saline solution at which 50% of the red cells in the suspension hemolyzed) and age of hematologically normal subjects.

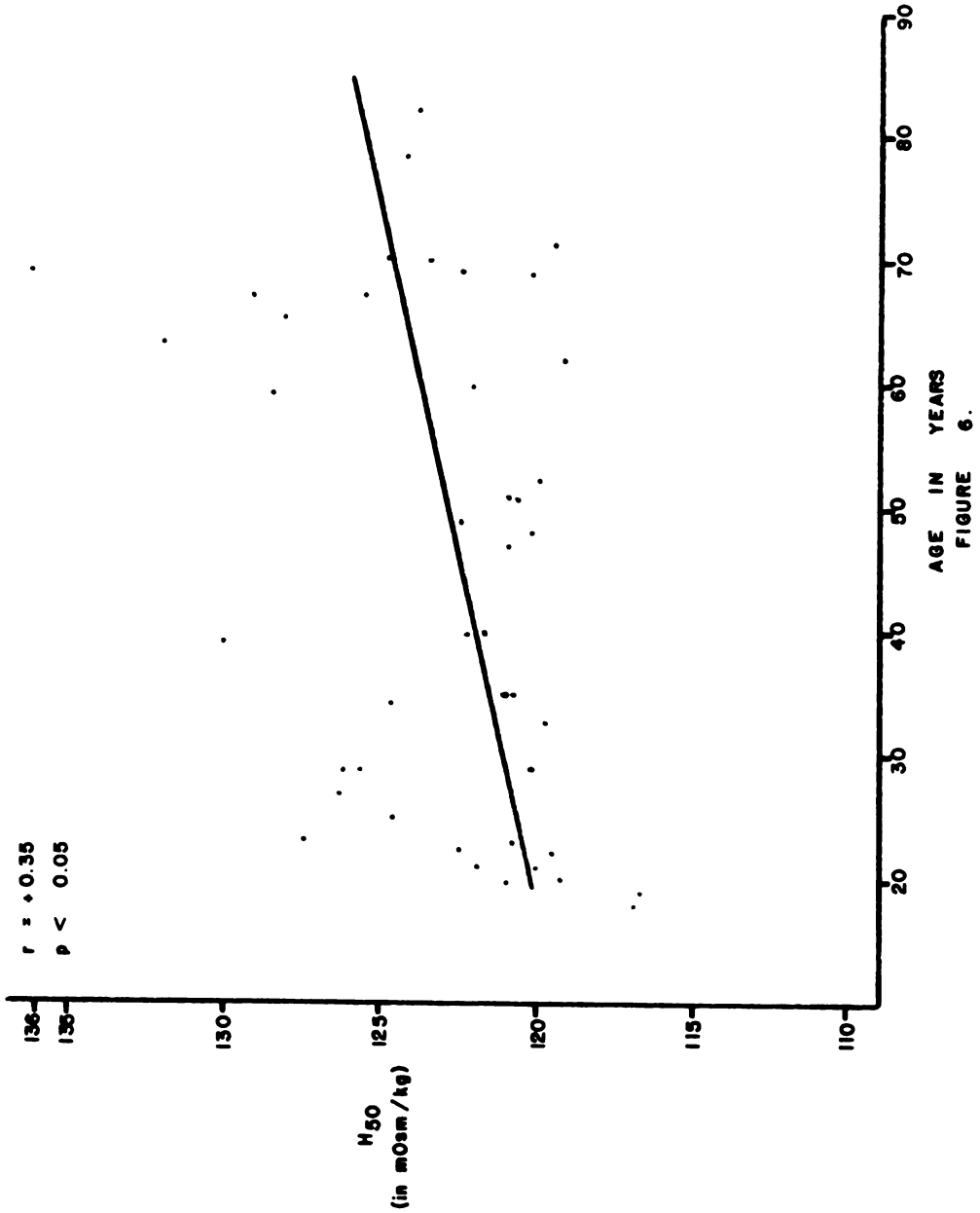
r = +0.35
p < 0.05
135
130
125
120
115
110
H_{50}
(in mOsm/kg)
20
30
40
50
60
70
80
90
AGE IN YEARS

FIGURE 6.

Figure 7. Slope of Osmotic Fragility Curve versus Age. Relationship between the slope of the osmotic fragility curve (indicative of red cell variability) and age of hematologically normal subjects.

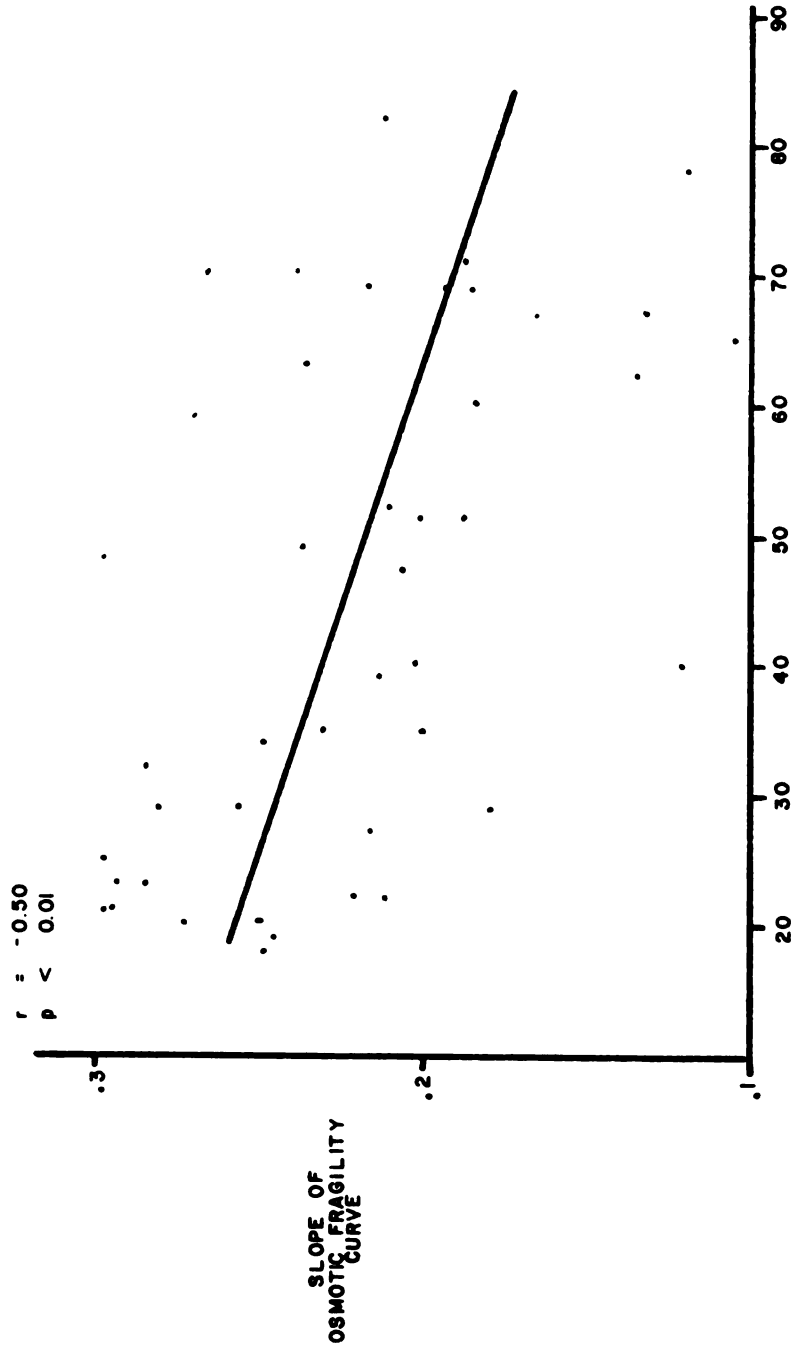
r = -0.50
p < 0.01
.3
.2
.1
SLOPE OF
OSMOTIC FRAGILITY
CURVE
20
30
40
50
60
70
80
90
AGE IN YEARS

FIGURE 7.

TABLE 5.

COMPARISON OF AGE
WITH H_{50} AND SLOPE
OF OSMOTIC FRAGILITY CURVE

		r	p
H_{50}	vs. AGE	+0.35	$p < 0.05$
SLOPE	vs. AGE	-0.50	$p < 0.01$

DISCUSSION

In 1943, Newman and Gitlow (137) found that in old age the osmotic fragility of erythrocytes showed hardly any change; however, a mixture of ammonium oxalate and potassium oxalate was used as the anticoagulant and only heparinized and defibrinated blood are suitable for osmotic fragility studies (213). In 1948, Olbrich (140) succeeded with the aid of a more refined technique in demonstrating a slight increase in fragility (15). In 1974, Detraglia et al. found differences in osmotic fragility of erythrocytes between an elderly and a young population. Age-related changes in erythrocyte osmotic fragility were identified in the present study. These experiments expanded the earlier work of Detraglia and this study refines osmotic fragility measurements by the following methods: (1) sample variability was reduced by automatic pipetting; (2) pH-adjusted but unbuffered saline solutions diminished buffer-induced aberration; (3) the apparently sigmoid osmotic fragility curves were treated as linear transforms to permit statistical treatment of results.

Since the dimensions of the cells with respect to volume and hemoglobin concentration were unaffected by age, the increased osmotic fragility could arise from (a) a reduction in mean cell surface area, resulting in partial sphering, or (b) diminished capacity of the membrane to withstand tensile stress. Classic osmotic fragility theory denied the latter; however, the work of

Bowdler and Chan (20) showed prelytic changes to occur in the membrane attributable to tensile stress less than that required for lysis. A recent study supports the former. Hegner et al. (77) report a decrease in erythrocyte membrane phospholipid content in an older donor group compared with a younger donor group. This reported decrease in membrane lipid would lead to a decrease in the surface area/volume and would be consistent with increased osmotic fragility (201). Both the decrease in membrane lipid and the increase in osmotic fragility reflect a change from steady state indicative of aging at the cellular level.

Red cell variability, as expressed by the slopes of the osmotic fragility curves, is increased in red cells in older subjects; thus, the number of distinct types of erythrocytes in a given population from an aging person has a greater variety of types of cells (45,135). It is well known that the immune system undergoes drastic deteriorative changes with age (187). The spleen, like most lymphoid tissues, becomes smaller with age (15,21,59) concomitant with vascular architectural changes (6,187) manifest in a resultant decline in efficiency. Conceivably the filtration function of the spleen becomes less efficient. If this is the case, the spleen may not trap and remove red blood cells as efficiently and may allow more cells that are spheres (reflecting loss of membrane) to enter the general circulation. This decline in the efficiency of the spleen with age, as evidenced by a decrease in filtration ability and vascular architectural changes, would lend credence to the wear and tear theory of aging. The results of this study could also be interpreted as support for the immunologic theory of aging, in that one assumption of this theory seems to be supported. The assumption is that the

basic impairment in the immune system is a loss in the ability (e.g., of the spleen) to recognize slight deviations in molecular structure and cellular characteristics (reflected by increased osmotic fragility) so that cells which have undergone change (e.g., spherocytes) and would ordinarily be destroyed by the immune system are permitted to remain to the detriment of the animal. Indeed, these rigid spheres may give rise to the initial insult to vascular walls and this insult may lead to the formation of atheromatous plaques and subsequently to atherosclerosis. If this is the case, it would be support for the cardiovascular theory of aging.

Bjorksten (17) proposes that the accumulation of cross-link molecules is a primary cause of aging. Recent observations on the self-association of spectrin (190) and cross-linking of membrane proteins in the red blood cell tend to suggest that the human red blood cell membrane may serve as a model for the investigation of the cross-linking theory of aging, and indeed may prove to be a very suitable model of aging at the cellular level (43).

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