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AGE-RELATED OXIDATIVE CHANGES IN THE HAMSTER UTERUS

by

RAYMOND FRANKLIN PEAT

A DISSERTATION

**Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy**

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INTRODUCTION

Sex and Aging

Theories of aging are historically closely associated with ideas about sexuality, and it is only in the twentieth century that the two have been habitually separated. The earliest tradition that was explicitly concerned with the nature of aging is that of the Taoist culture of China.

In China, experimental science was generally closely related to the Taoists' concept of natural balance and their desire for long life or immortality of the body. Deterioration of the body with age was considered to result from the imbalance of various factors, including masculinity and femininity, and attempts were made to find techniques or materials to cure disease, impotence, sterility, and aging itself. Ko Hung (a Taoist experimentalist) believed in the early fourth century A.D. that plant drugs would prolong life, and a compound of minerals and metals would give immortality. While other Taoists experimented on criminals, Ko Hung used plants and animals to study aging (Needham, 1956). Ginseng, which is rich in water soluble "anabolic" steroids (Bykhovtsova, 1970; Claus, Tyler, and Brady, 1970) is the best known of the Chinese herbs used for prolonging sexual vigor and

lengthening the life span. Recent studies indicate that ginseng promotes resistance to disease and stress, and increases the life span of rats (Yudkin, 1970; Grinevich and Brekhman, 1970).

Eventually, in the Middle Ages, sophisticated methods were developed for extracting highly purified steroids from human urine, and these were used in treating senescence (Needham and Lu Gwei-Djen, 1968).

In the late nineteenth century, Brown-Sequard in France believed that testicular extracts could restore sexual, physical and mental vigor to old men. At the time of his announcement he was ridiculed and said to be deluded (Steinach, 1940). Up to the present time his claim is not generally accepted (Turner, 1966; Connors, 1969) because of the supposed insolubility of androgens in water, though testosterone is routinely used for postponing some of the symptoms of senescence in men. However, Brown-Sequard's claim seems reasonable enough, considering the abundance of fatty acids, lipo-proteins, and soluble simple proteins in the testes that would be able to make steroids soluble in water. Even the slightly polar nature of the androgens themselves (their hydroxyl and carbonyl groups) gives them a certain small degree of solubility in water.

The gonadal theory of aging (i.e., that degeneration of the gonads precedes and causes the degeneration of all other organs: Steinach, 1940) was generally discarded by 1940, but by the same time it was widely accepted that aging does involve changes in hormone controlled processes, and that hormonal insufficiency

or imbalance is likely to be intimately involved with senescence. Increasing biochemical sophistication was leading most investigators to look for subtler or more basic processes as the primary cause of aging. Gonadal degeneration is now more modestly considered to be one of many changes that occur as a result of a more basic process of aging, but its importance as a possible immediate cause of senescent sterility in the female is the subject of current investigations, which will be discussed below.

Physiological Theories of Aging

Two other 19th century ideas have remained viable, in modified form, up to the present. E. Metchnikof (1908) proposed that the basic cause of aging was connective tissue degeneration; the current version of the theory, viz., that aging results from molecular changes in collagen and structural changes in connective tissue, is now widely held, and fits the evidence well. He also (1908) believed that toxins from putrefaction in the intestine caused aging, and cited correlations between short intestines and long life spans. It has recently been suggested that polyamines, e.g., putrescine, with histamine-like effects, may be absorbed from the intestine and provoke reactions of an allergic type. Korenchevsky (1961) has discussed more recent versions of the "auto-intoxication" theory. Toxins of various types are known to induce auto-immune reactions (Nikolayev, et al., 1970), which have been implicated in

certain degenerative diseases and are believed by some to be a cause of aging, that is, the primary event in aging is presumed to be the appearance of antibody producing cells incapable of distinguishing self from other. Virus and diet have been implicated in auto-immune disease in NZB mice (Fernandes, *et al.*, 1972). As a general cause of aging the auto-immune process would seem to be only a special case of somatic mutation, and A. Comfort (1969) has convincingly argued, in relation to Curtis's defense of the somatic mutation theory of aging, that somatic mutations are normally not numerous enough to account for aging, and that a cause prior to the mutations must be sought. What at first seemed to be a cause of aging turns out to be an effect of aging. D. G. Carpenter and J. A. Loynd (1968) similarly criticize the somatic mutation theory on the basis of the number of mutations that occur, but add that it fails to explain the effect of diet, and the changes that uniformly appear with age. P. Alexander and D. I. Connell (1963) have shown that the powerful mutagen ethyl methane sulphonate had no effect on the life-span of mice, in spite of causing more mutations than X-ray treatments which did shorten the life-span of mice, and conclude "that a non-genetic cause must be sought for the reduction of life expectancy which occurs in CBA mice after large doses of X-rays..." Although R. H. Mole (1963) has claimed that there is "no additive or synergistic effect of whole body irradiation by fast neutrons and natural aging," this does not apply to other forms of radiation, and some data will be mentioned below that show

a synergism between X-irradiation and dehydration in producing mutations and cell death. It is also interesting to note that Brachet (Bell, 1972) produced chromosomal abnormalities in amphibian eggs by merely heating the cortex. The evidence generally favors the idea that both aging and chromosome damage are physiologically caused, rather than the idea of Curtis and others that aging is produced by random somatic chromosomal mutations (Strehler, 1967).

The concept of accumulation of toxic material with age has the advantage of appearing to be a simple physical process that correlates very well with age (Dubina, 1970) and that could reasonably be expected to cause some of the functional changes associated with age, but again the process of metabolic detoxication, which can be assumed on the basis of various lines of evidence to decline with age, could account for the accumulation of toxic metals (Selye, 1967) and other materials with age. This criticism, however, is not as convincing as similar criticisms of other theories because of the peculiar vulnerability of the primary organ of detoxication, the liver, to toxins (Song and Kappas, 1968; Biskind, 1946). A very obvious positive feedback system must be involved, but the appropriateness of the feedback concept to a process of lifelong aging hasn't been demonstrated, and regenerative and higher control processes would add further complications. The capacity of heavy metals to act as catalytic centers, modifying the nature of the collagen system, suggests an overlap of the collagen and toxin theories of aging. The inactivation of enzymes by certain metals

(e.g., silver, mercury, arsenic) suggests that this theory would also have a degree of similarity to some of the somatic mutation and cybernetic theories of aging.

The theory of somatic mutations, resulting from background radiation or other random processes that would place the mutation as the first event in aging, is probably inadequate, as mentioned above, but there is evidence of some kind of physical change--possibly crosslinking--in at least some kinds of DNA, which increases with age (Hahn and Fritz, 1966). Increased melting temperature of DNA has been offered as evidence of a physical change assumed to involve inactivation of genes, but it is known that the melting temperature of another nucleic acid, transfer RNA, is very sensitive to ion concentration (Millar and Byrne, 1967). It might be that altered counter-ions are responsible for the observed change in DNA with age. It is known that general tissue concentrations and ratios of ions change with age (Kohn, 1971) and it seems that at least some of the change is intracellular (Friedman *et al.*, 1965). Repetition of these experiments with purified DNA might settle this question.

An idea that used to be generally believed and that is still frequently encountered is that the nucleus is the center of all control processes in the cell and that therefore a change of cell function requires a loss of some kind in the information stored in the nucleus; an extreme form of the idea was the doctrine that only the germ line possessed a full complement of genes, and that somatic

differentiation occurred by selective loss of information. Gurdon's (1962) transplanting of nuclei from differentiated gut epithelium into enucleated egg cytoplasm was a clear refutation of that doctrine, since the nuclei allowed development into a late embryo. Although discredited for most organisms, the idea has given moral support to at least two theories of aging, viz., somatic mutations and Hayflick's idea (descended from Weismann's) of germ immortality - somatic mortality. Vegetative reproduction in plants is an obvious exception to Hayflick's principle of somatic cells being limited to a small number of divisions. Hayflick attempted to discount changes of the culture medium and "time" as factors inhibiting mitosis after a certain number of divisions, but time was measured with the cultures in the frozen state, which suggests that he has a strange conception of how time affects chemical and biological reactions. His demonstration that the medium didn't become progressively inhibitory was simply to put new fetal cells into a culture of cells that had stopped dividing, and show that they were able to divide. Strehler (1967) has shown that the number of divisions depends on the dilution factor. What Hayflick argues for is some kind of internal clock, designed to stop after a certain number of divisions, independent to a high degree of its environment. This internal clock concept is still held by some theorists to account for control of growth and differentiation, in opposition to the concept that cells differentiate in response to a series of inductive and inhibitory influences, which varies with time and their position in the organism. It has been demonstrated in Strehler's laboratory (1967) that physical restraint (an agar film)

that prevented mitosis allowed some kind of aging process to occur, in either the cell or the medium. Strehler (1967) also demonstrated, by labeling stem cells in skin, a capacity for division that is apparently unlimited, or at least is on the order of 100 times greater than the number set by Hayflick as a limit. Skin (Krohn, 1966) and mammary gland (Daniel et al., 1968; Daniel, 1971; Daniel and Young, 1971) transplant experiments have been used to argue for or against the idea of somatic cell mortality resulting from a fixed number of mitoses, but the arguments have been inconclusive in both cases. (Apparently it is generally tacitly assumed that transformed malignant cells are no longer somatic cells.) Hayflick's inhibition could be the result of either the presence or the absence of a chemical substance, or a physical factor, such as might be involved in Auerbach's discovery of reprogramming of lymphocytes by contact with other cells. Failure to consider the dilution factor is an important weakness in Hayflick's theory, just as it was in the (clonal selection theory) experiments criticized by Auerbach (1970).

Daniel et al. used mammary transplants in mice to argue for Hayflick's theory, but their assumption that immune reactions are not involved is not beyond question, and the experiments really just show that a tissue can outlive an organism. The purpose of the experiment was to study aging as it relates to an organism, but the survival of the tissue for several generations shows that the process involved in its eventual death is not likely to be

relevant to aging in the normal life span. They found, in fact, that the age of the tissue donor did not affect the lifespan of the transplants. The real significance of the experiment is to weaken the theory of programmed cell death as a factor determining the lifespan of an organism. The absence of an effect of donor age tends to support Kohn's belief that intracellular changes are not the most important factor in aging. More recently, Daniel et al. (1971) reinterpreted the results as showing only that the tissue "gradually loses its capacity to respond by proliferating to a hormonal stimulus that is relatively constant and which continues over a long period of time."

The inhibitory property of serum from old animals (Kohn, 1971) possibly relates to Hayflick's results, but it is very interesting from several other points of view. For example, it has been proposed (Comfort, 1964) that the cell loss which is typical of aging is the result of inhibition of mitosis by chalones and adrenalin. According to Bullough (1971) both aging and cancer result when inhibition is lost, and stress, by maintaining high levels of adrenalin, can prolong life. Delayed regeneration in old animals might relate to a high level of chalones. Adrenalin concentration may be elevated in old age, and sensitivity to exogenous adrenalin increases with age (Strehler, 1967; Frolkis et al., 1970) which is consistent with a high basal level, and with the concept of increased mitotic inhibition with age.

D. G. Carpenter (Carpenter and Loynd, 1968), a nuclear weapons engineer engaged in systems analysis of space weapons, has proposed an integrated theory, which consists in saying that many partially true theories of aging when taken together are more completely true. Regardless of his conclusions, his interest in feedback and amplification as part of an organism's control system is probably valid.

Sacher (1959) has proposed that the brain is the control system most relevant to aging, and the idea of neurone loss with aging is so popular that one of the most frequently repeated remarks about one's body concerns the supposed daily loss of 100,000 brain cells. The suggestion has even been made that the loss is so selective that it could be the basis for memory (Dawkins, 1971). Regardless of the idea's popularity and its good foundation in some insects, it certainly is not unchallenged (Comfort, 1964). Even the concept of age-related decline of mental function (e.g., the limit of learned reaction time or nonsense memorization) has been challenged by experiments using populations with equivalent occupations or an opportunity for relearning (Murrell, 1969). Analogous to the supposed decline in mental function with age (as distinct from disease) is Terman's observation that bright females suffer a severe loss of mentality at the age of 23, with the shift of occupation from student to housewife. The critics of the concept of brain loss with age sometimes claim that the brain is one of the most durable populations of cells, and that the "horizontal" averages of brain quality include a majority of diseased individuals,

that obscures the lack of effect of mere aging (Pfeiffer et al., 1968). However, this controversy is not necessarily essential to Sacher's idea of the brain as the most important control system in aging. Sacher's observation is that mass of brain correlates very well with length of life (1959). His belief is that the brain maintains biological equilibrium, and that with accumulated damage to the brain as a control system (whatever its nature) the organism would become increasingly unstable and die.

Sacher's correlation of brain size with length of life ("it's an advantage to have a brain, and a disadvantage to have a body") recalls G. W. Crile's (1941) discovery of a close correlation between brain size and total metabolic activity (oxygen consumption per hour per animal) both between and within species. Crile's observation accounts in an odd way for the old approximate "two-thirds power" (actually 0.7 to 0.8 for most species) rule for increase of metabolic activity with increased body weight within a species (Florey, 1966), because this is approximately the proportion in which brain size increases with body weight (Lilly, 1963). The well-known actuarial data that show that short people live longer than tall ones might be relevant to this question, because of their proportionately larger brain size; the same would apply to the greater longevity of women. Energy production has been proposed as a crucial factor in life-span (Hershey, 1970; Calloway, 1971a).

Palladin (1964) has made a possibly related observation concerning "evolutionary level" (which in this case corresponded to brain size) and efficiency of oxidative phosphorylation. The efficiency of coupling was found to increase with higher evolutionary level, as well as with alertness. This observation suggests an interesting secondary factor to consider in relation to "rate of living" and Sacher's idea--the reason for Sacher's correlation might be metabolic efficiency, i.e., a less wasteful use of oxygen, possibly even a less destructive use of it.

The free-radical theory of aging has become very popular recently, probably partly as a result of radiation studies and partly because of the observation (Harman, 1968; Kohn, 1971) that antioxidants such as BHT added to the diet of mice increased their lifespan by several percent. It has been pointed out by other investigators that the mice ate less of the diet, probably because of the smell of the antioxidant, and that the restricted food intake alone could account for the increased life span (Comfort, 1972).

Restricted intake of calories has recently been found to slow the aging of collagen in rats (Everitt, 1971).

Relations of the Senescent Physiology to
Other Physiological Processes

Other studies (Lee et al., 1952; Visscher et al., 1952) have shown that limitation of caloric and protein intake lengthens

the life span of mice, delays development of cancer in a susceptible strain, suppresses estrus and delays onset of both reproductive maturity and reproductive senescence. These observations will be discussed further below.

Smith and Soderwall's (1962) finding that supplementing the diet with vitamin E delayed reproductive senescence in hamsters, and similar observations in rats tempt one in this context to suggest that excess free radicals from an overactive metabolism are the agent of alteration in the collagen, which in turn leads to accelerated functional senescence. However, several problems exist for this interpretation, for example the relation between suppression or acceleration of sexual maturity and the onset of senescence, and also the idea that excess calories will necessarily increase the production of free radicals, which has not been established. The literature regarding free radical concentrations in living tissue is still in an early stage, as a result of the lack until recently of equipment that could measure EPR in the presence of water. This interpretation also fails to explain the observation that the amount of vitamin E required to maintain fertility increases steadily and drastically with age, being at 59 weeks 67 times the amount required by the 10 week old rat. Verzar and Ermini's (1970) observation of poor creatine phosphate recovery in old animals is interesting in connection with the creatinuria and low tissue creatine levels in vitamin E deficiency (Houchin, 1942) and the restoration of creatine kinase activity by vitamin E

administration (Matusis, 1971) and the fact that X-irradiation also produces creatinuria (Kozalka and Andrew, 1972).

Besides the functional complementarity of aging and vitamin E deficiency, there is an interesting similarity in their ability to produce lipofuscin, "aging pigment", or ceroid pigment (Kaunitz, et al., 1948). This pigment has been widely discussed as an example of intracellular crosslinking, and is assumed to be a "clinker," or product of metabolism that the cell is incapable of removing (Carpenter and Loynd, 1968). Florey (1966) has referred to the existence of this material in carp which are accustomed to living under ice at low oxygen tension and suggests that it may serve as an alternate electron acceptor in the absence of oxygen. (It is supposed that unsaturated fatty acids can serve as electron acceptor in yeast cells in the absence of oxygen (Warburg, et al., 1967).) Regardless of the accuracy of this guess concerning its function, Florey's observation at least points out the feature that is common to the various situations in which it appears: fetal liver (Goldfisher and Bernstein, 1969) has relatively low oxygen tension, and the disappearance of the pigment from the liver following birth would correspond to increased oxygen tension; aged cells are increasingly embedded in collagen, and probably experience reduced oxygen tension (Casarett, 1963); in vitamin E deficiency, there is extremely high oxygen consumption, which would tend to lower oxygen tension, and the pigment appears as a result of vitamin E deficiency (Kaunitz, et al., 1948). That the intracellular oxygen tension is lowered by the high rate

of consumption associated with this deficiency is implied by the fact that muscular dystrophy can be induced in various experimental animals as a result of a vitamin E deficiency, and it has been demonstrated that raising dystrophic chicks in a high oxygen atmosphere suppresses development of the disease; similarly, restricted blood circulation also produces the disease, the most reasonable conclusion apparently being that low oxygen tension produces muscular distrophy, and a vitamin E deficiency "wastes" oxygen at a sufficient rate that oxygen tension is lowered in the tissue. Additional vitamin E allows animals to survive hypoxic conditions in a higher percentage than animals fed a normal or deficient diet (Telfer, 1954).

Bjorkerud (1963, 1964) has determined that there is NADH oxidase activity associated with the age pigment. NADH oxidase is, by consuming NADH, able to release one of the potential controls over fermentation (Racker, 1972) and, at the same time, can potentially reduce a large amount of the oxygen that enters the cell. Bjorkerud's observation would seem entirely consonant with the data concerning possibly impaired mitochondrial function in old age (Shikla and Kanungo, 1970) and the observations of Shock (1963) of undiminished oxygen consumption with age (in relation to active cell volume). This enzyme function should be elevated in vitamin E deficiency if the same system is really active in both cases. A microsomal NADPH oxidase is known to increase its oxygen consumption greatly in the absence of vitamin E, and in vitro pO_2 is rate limiting.

in the peroxidative conversion of polyunsaturated fatty acids to a dialdehyde by this enzyme. Estrogen is known to induce an NADH oxidase. It has frequently been observed that many cells in the senescent hamster uterus are filled with pigment granules (Orsini, 1962). These granules apparently require estrogen and the absence of supplemental vitamin E if they are to form (Kaunitz, *et al.*, 1948). Peroxidase, which is induced, or activated (Lucas, *et al.*, 1955), by estrogen, and which is known to have an NADH oxidase function (Lucas *et al.*, 1955; Beard and Hollander, 1962), may be involved. (Peroxidation is being widely studied in relation to both aging and cancer.) Catalase is apparently suppressed by estrogen, which suggests that peroxide, whatever its origin (it is universally associated with respiration, but it is not known how) is being spared for another function. In cancer, a condition similar to what is proposed here for aging exists, with low NADH combined with a "reducing environment" (Reid, 1965).

The proportionality, mentioned above, between onset of sexual maturity and age at which reproductive senescence, and even death, occur, has been recognized as a genetic, as well as a dietary, phenomenon by L. C. Strong (1969).

A well-known idea (Lansing, 1952) is that late maternal age tends to shorten the life-span of offspring; this seems to be in doubt even in the case of rotifers (Meadow and Barrows, 1971). (It is interesting that vitamin E happens to be necessary for sexual reproduction even in rotifers.) There is a general

belief that the effect exists in mammals, too (Lansing, 1947; Carpenter and Loynd, 1968); it is generally attributed to a higher rate of mutations in the older animal, which results in slightly defective offspring. Strong, Johnson, and Rimm (1963) found that late maternal age is associated with an earlier onset of sexual maturity in the offspring, which would be expected if the "Lansing effect" is true for mammals and if the reduction of life-span is associated with accelerated sexual maturity, as in the feeding experiments. However, Strong (1969) made the seemingly paradoxical observation that prolonged selection and inbreeding of the offspring of the 201-300 day maternal age group produces mice with fewer deviant traits, with fewer spontaneous cancers, and with longer life spans than those of strains produced by younger or older maternal age selection, and the earliest maternal age selection produced the poorest longevity.

Strong has rationalized this in terms of the concept of genetic homeostasis, which he associates with heterozygosity, as illustrated by the greater variability in age of first litter among homozygous than among heterozygous mice. He considers the 201-300 day maternal age descent to represent a genetically more stable or homeostatic group. Many observations have been made relating the occurrence of cancer in Strong's mice to an excess of estrogen, but the evidence for the existence of high levels of estrogen is mostly indirect. Abnormal levels of estrogen in the strain could explain many of the effects observed, through an effect in utero (or through milk), without recourse to more complex genetic theories.

Blaha (personal communication, 1972) has expressed the opinion that hamsters probably don't have elevated estrogen levels in old age as do old rats which often have continuous estrus, in spite of the continued existence of 17-HSD which he says suggests the presence of estrogen (1972), but no measurements have been made of their circulating estrogen level. He has found elevated progesterone levels in old hamsters (1971), and proposes that it is the cause of their "delayed parturition." High levels of estrogen are considered to be responsible for the frequent endometrial hyperplasia in rabbits and have been suggested as a cause of the similar hyperplasia which frequently occurs in menopausal women (Cowdry, 1952; Dove et al., 1970).

Radiation or free radicals or both are sometimes believed to be responsible for the increased cross-linking of collagen assumed to be involved in the progressive increase with age in the melting temperature and melting contraction force of collagen, but it has been found that rat tail tendons of irradiated animals do not have the characteristics of aged rats' tendons. Whatever life-shortening effect irradiation has, and it does seem to mimic aging in certain ways, it doesn't appear to act by way of accelerated cross-linking in collagen.

B. W. Casarett (1963) proposed that radiation and other "non-specific" injuries lead to an "increase in density and amount of collagenous substance interstitially and in sub-endothelial

regions of arterioles. These changes constitute a temporal advancement in the increase of the histohaematic barrier and in the development of arteriolocapillary fibrosis, which are progressive processes in 'normal' aging. Eventually these processes cause progressive reduction in number of dependent parenchymal cells due to relative hypoxia and malnutrition." These ideas conform to the known effects of radiation, and he traces in detail the idea that both radiation and aging involve increasing isolation of the cells, which in itself constitutes an injury.

Brooksbys, Sahinin, and A. L. Soderwall (1961) have reported that X-irradiation mimics certain features of reproductive senescence in hamsters, for example prolonged gestation equivalent to that of the senescent animal with 100 r whole body irradiation and, with all exposures used (100 to 700 r whole-body), increased connective tissue which infiltrates and thickens uterine muscles, glandular areas, and endothelial linings.

S. O. Brown, et al., have presented (1964) data that suggests that chronic low levels of radiation may accelerate the onset of reproductive maturity in rats. R. L. Brent (1964) observed some similarities between vascular clamping and maternal irradiation in effects on the fetus, suggesting the possibility that radiation may act partly through an effect on circulation or oxygen tension. Neonatal "heat" (Boling, et al., 1939) may result from hypoxia, since no hormonal basis is apparent. Estrogen and anoxia both improve resistance to irradiation (Katch and Edelman, 1964).

Radiation mimics estrogen stimulation in several other ways: vaginal cornification (Mandl and Zuckerman, 1956), uterine weight, estrus behavior, response to progesterone, etc., and appears to have its first physiological effect on respiration (Valentini and Hahn, 1971; Hahn and Ward, 1969). Ingram and Mandl (1958) claim that X-irradiated ovaries produce more estrogen than normal ones, without pituitary intervention.

Some of the other similarities between senescence and estrogen treatment observed in various animals are:

degree of corrugation and adhesiveness of luminal surface (Martin et al., 1970)

mitotic response in both lumen and stroma (Finn and Martin, 1969)

uterine weight

radiation resistance

vitamin E deficiency effect on uterine weight

vitamin E-estrogen antagonism on age pigments of uterus and other aging symptoms (Kaunitz et al., 1948)

catalase level (Baird and Samis, 1971; Adams, 1955)

Na/K ratio of cells (Friedman, et al., 1965)

basement membrane in reproductive organs (Fredricsson, 1969; Rowlatt, 1970)

increased tube-locking of ova (Connors, 1969; Greenwald, 1959)

chronic estrus (Bloch, 1961)

levels of uterine estrogen receptor protein (Kraay and Black, 1970; Trams, et al., 1971)

failure to implant, main embryo loss (Dreisbach, 1959; Connors, 1969)

tetrazolium reduction

oxygen consumption

oxygen partial pressure in lumen

Singhal and Valadares (1969) showed that very high levels of estrogen are required to restore ovariectomized old rats to their normal enzyme levels, which implies that high estrogen may be normal for old animals. In their mitotic response (Finn and Martin, 1969) to estrogen stimulation, however, old tissues showed greater sensitivity than did the young; residual bound estrogen might account for this, and the time allowed after ovariectomy might have differed in the two sets of experiments. Old animals have a similar high sensitivity to catecholamines (Frolkis, et al., 1970), which also can be interpreted as the effect of a high basal level. Catecholamines have also been associated with "oxygen wastage" (Raab, et al., 1962).

Metabolic rate (oxygen consumption) has long been known to decline steadily with age, although the steady state level of thyroxin in the blood (Gregerman, 1967) does not appear to change, but Shock et al. (1963) found that the loss of oxygen consumption per unit of tissue weight disappears when oxygen consumption is recalculated on a cell water basis. That is, there is a decline in the number or volume of active cells with age, as the mass of inert connective tissue increases, but there is no decrease in the rate of oxygen consumption of the remaining cells.

In spite of this evidence that oxygen consumption does not decrease generally with age, there is some evidence that mitochondria of various tissues (e.g., liver) lose efficiency, or decrease in number, or both, with increasing age (Shukla and Kanungo, 1970; Barrows, et al., 1960). Considering the oxidase activity of age pigment, it is possible that non-mitochondrial oxidation increases as mitochondrial oxidation declines.

Loeb (1939) has found remarkable similarities between the effect of excessive estrogen stimulation and senescence on the structure of the rat uterus. Both cause progressive increase in collagen deposits.

Arvay et al. (1971) have shown that stress accelerates the collagen changes of rat tail tendon and uterus, apparently acting through adrenal cortical hormones and estrogen.

These similarities in the effects of X-irradiation, estrogen, and senescence, suggest another experiment which found effects of X-irradiation and chemical carcinogens similar to those known for estrogenic stimulation. F. Devik (1963) has used the reduction of tetrazolium by skin cells to compare the effects of several carcinogenic and non-carcinogenic chemicals, and X-irradiation. Non-carcinogenic compounds caused a decreased deposition of the reduced form of the tetrazolium on the first day, while the carcinogenic compounds and the irradiation caused an increase, though the X-rays caused a more persistent increase. Damage to the mitochondria has been proposed as a common effect (Warburg, 1969; Devik, 1963),

but since it is generally believed that tetrazolium reduction is an indicator of dehydrogenase activity, all that is clearly indicated by these experiments is that carcinogens and radiation both activate or induce the dehydrogenases, or make more oxidizable substrate available. The increase is very rapid. Estrogen has very similar effects (several carcinogens are known to be estrogenic in the ovariectomized female (Turner, 1966; Needham, 1942)).

The fact that age, excess estrogen treatment, and irradiation all stimulate excessive collagen deposition, are sometimes associated with cancer development, can cause increased tetrazolium reduction, and produce similar effects on pregnancy, suggests that a new interpretation of the collagen theory of aging might be desirable. The enzyme system which synthesizes vitamin C, which is important in the development of collagen, loses activity with age, is inactivated by lipid peroxides and preserved by adding vitamin E, and also appears to be suppressed by estrogen (Patnaik, 1971; Chatterjee and McKee, 1965).

The failure of X-irradiation to increase cross-linking in collagen tends to weaken the free-radical theory of aging, and the mutation theory cannot explain satisfactorily either the generalized cross-linking or the generalized deposition of collagen. A common event, such as a metabolic pathway change, or a general interference with enzyme control systems, seems to be a more suitable alternative locus for the aging process, and it would seem especially suitable

if it were such as could be simply implicated in a self-stimulating (positive feedback) loop, tending to prevent reversal. For example, collagen accumulation could increase collagen synthesis or block its degradation. Casarett's scheme was of this kind, but he relied on the mere assumption that a non-specific injury would lead to increased density and amount of collagenous substance. What is lacking is information regarding the control of collagen synthesis, that could relate with each other the many specific stimuli that are known to accelerate collagen formation. Chvapil *et al.* (1970) found that in granuloma and in some organs in rats, prolonged hypoxia stimulates collagen synthesis and inhibits the synthesis of noncollagen protein, and enhances mitosis in fibrogenic cells. Hypoxia seems a likely common factor for the various non-specific injuries and provides a very simple positive feedback system.

A view of ionizing radiation's biological effects presented by Webb (1965) offers a radically different kind of action that would be at least potentially applicable to the intracellular control theory of aging proposed by Comfort and others (Comfort, 1969). Several Russian laboratories have proposed a similar mechanism to explain the biological activity of relatively low energy microwaves (Sharp and Paperiello, 1971). Webb found that, in bacteria, both the lethality and the mutagenicity of X-irradiation depended on the relative humidity of the atmosphere in which the experiments were performed, when the bacteria were suspended in an aerosol.

He showed that partial dehydration increased the bacteria's susceptibility to both effects. Various chemicals, such as inositol, that are known to protect proteins, cells, and organisms (Troshin, 1966) from destruction by various agents, were able to offset the effects of dehydration. Webb interpreted this effect in terms of the supposed ability of the molecules' hydroxyl groups to stabilize bound water which in turn would stabilize macromolecules, or larger structures such as membranes. A mechanism by which the pathway changes induced by estrogen, radiation, anoxia, and aging might result from a more or less direct action on intracellular water has been discussed elsewhere (Peat and Soderwall, 1971, 1972).

Alteration of the solvent properties of cell water could have very far reaching effects on the function of an organism (Atkinson, 1971).

One of the few factors, if not the only one, that shows a universal correlation with age, even before a many-celled stage is reached, is the concentration of water (Needham, 1964).

Calloway (1971a, 1971b; Calloway and Kujak, 1966) has, from an analysis of the literature, shown that the water content of all organisms fits the same exponential curve on an absolute time scale. Thus, the tissues of a 24 month old rat contain roughly the same percentage of water as the tissues of a 16 month old human baby (both being about the same age from conception).

Intracellular ratios of sodium to potassium tend to increase with old age (Friedman, et al., 1967). However, the opposite occurs

during early development, approximately parallel to the decline in water concentration (Hazelwood, 1971). If Hazelwood (1971) and Wiggins (1970) and Szent-Gyorgyi (Baird *et al.*, 1957) are correct in their theory of the control of the salt ratio, then one must assume that the intra-cellular water concentration, as opposed to the total organismic water concentration, would increase with age. This would be compatible with the known increase of mass and compactness of collagenous tissue with age, and with the data on the decrease of extracellular body water (thiocyanate diffusion space) with age (Kohn, 1971).

The conventional methods of measuring extracellular space have been criticized as over-estimating the true volume, at least in certain cases (Ling and Kromash, 1967). Whole organ concentrations of calcium, sodium, and chloride (Kohn, 1971) increase with age, and there are decreases in potassium, magnesium, and phosphate; Kohn interprets these changes as reflecting increasing extracellular space and decreasing cell volume, which accords with the microscopic picture, but there is also the likelihood (Friedman, 1967) that shifts of the same nature occur in the cell water itself.

If it is true that the intracellular water concentration is increased with age, it represents another feature of similarity among old cells, cancer cells, and estrogen stimulated tissue, since the others are already known to have a high water concentration (Hazelwood, 1971; Damadian, 1971).

The "Estrogenic" State

According to Pincus and Kirsch (1935) excess estrogen in vivo kills rabbit embryos at the blastocyst stage, but does not interfere with cleavage. Epinephrine prevents implantation in the rabbit, and hypoxia has been suggested as the cause (Auletta, 1971). The few measurements that have been made suggest that the increase of pO_2 in early pregnancy is delayed or inadequate in the senescent hamster, but since abnormally low pO_2 has been established in the cyclic senescent hamster, no special failure of a control system need be invoked to explain the difference at the time of implantation. These results suggest that it would be desirable to follow pO_2 levels throughout pregnancy, and especially at the time Thorncroft and Soderwall (1969) observed increased resorption of embryos. Schultz (1967) has observed metabolic changes in old rats at day 12 during the period when resorption of embryos by old mothers occurred, which he interpreted as a shift toward estrogen dominance. Reduced sensitivity to deciduoma induction in rats has been attributed to high endogenous estrogen levels (Horikoshi and Wiest, 1971), and Schultz has also proposed high estrogen levels as the cause of low fertility in rats selected for small body size (1968) or high uterine metabolism (1965, 1966).

The literature provides many kinds of evidence supporting this interpretation, in spite of the popularity of the idea of estrogen insufficiency and, in humans and sometimes in experimental animals, of "estrogen replacement therapy" for menopause. The

concept of such therapy has been challenged, but probably the strongest reasons for holding the belief in estrogen insufficiency are a) elevated gonadotropins in senescence, b) decreased urinary estrogen excretion, and c) reduction, in some species, of the number of active follicles. In hamsters, the last reason is not relevant, since the number of follicles is normal (though the number of corpora lutea may be reduced (Thorneycroft and Soderwall, 1969b), but Steinach's (1940) view might be relevant for some species, viz., that interstitial cells multiply and increase hormone production whenever the germ cells are destroyed, e.g., by heat or radiation (Kennedy, 1970; Grindelund and Folk, 1962). Brooksby, Sahinin, and Soderwall's (1964) study of the effects of X-irradiation on hamster ovaries is consistent with this idea. Thung and Hollander (1967) have expressed a similar view: "In the ovary, the declining number of follicles is matched by the gradual evolution of proliferative cell groups.... These hyperplastic changes in senile ovaries are found in various species, but have been studied extensively in mice...." Some of the cells "...look very similar to the oestrogen-producing interstitial cells of the ovary. Actually, the occurrence of these cells in old ovaries is found to correlate with signs of increased oestrogenic stimulation."

The other reasons given as support for the idea of estrogen insufficiency are subject to various interpretations. The evidence in support of the ovary-pituitary feedback system, existence of which is the assumption behind the argument that elevated gonadotrophins

imply ovarian and estrogenic insufficiency, has been reviewed (Borth, 1967) and it was concluded that the evidence is fragmentary and weak. However, accepting the existence of such a control system at certain times, there is evidence suggesting that it may be inoperative in senescence, that is, that adrenal estrogens (Vinson and Jones, 1964) or ovarian estrogens (Mestwerdt, et al., 1972) may be overproduced in senescence, or adrenal estrogens may be overproduced when ovaries are removed (Diczfalusy, et al., 1964). Pineal involvement has been suggested as a factor in prolonged estrus in old rats (Meyer, et al., 1961). Aschheim (1964) suggests that the hypothalamus loses sensitivity to circulating steroids in senescence, which would be consistent with the effect of a vitamin E deficiency on nerve tissue, and possibly with the observation of P'an, et al. (1949), that pituitary extracts of vitamin E deficient rats caused greater gonadal stimulation in rats on a normal diet, than did extracts from rats on a normal diet. Drummond, et al. (1939) made similar observations. Orsini and Schwartz (1966) found that senescence apparently involves failure to release LH from the pituitary. Vitamin E has been considered to be anti-estrogenic (Shute, 1940; Evans and Burr, 1927a, 1927b, 1927c, 1928), as have various B vitamins (Biskind, 1949) and vitamin A, possibly by an effect on the liver. Since the amount of vitamin E needed to prevent symptoms of a deficiency increases greatly with age (Fuhr, et al., 1944; Emerson and Evans, 1940; Ames and Ludwig, 1964), most animals on a normal diet eventually show some signs of a

vitamin E deficiency. Since kidney resorption of the glucuronide of estrogens appears to be about zero (based on ratios of serum urinary estrogen conjugates in pregnancy), a small decrease in liver (or kidney, or other conjugating tissue) activity would tend to produce great increases in the amounts of unconjugated estrogens retained in the body. These estrogens would be almost entirely bound to proteins and lipids, but the equilibria of protein-estrogen binding are such that almost all of the body's unconjugated estrogens would be bound in the uterus, with smaller amounts in the vagina and mammary glands. Therefore, decrease of urinary conjugated estrogens (Poortman, et al., 1971) may directly reflect an increase of tissue-bound estrogens. Estrogen induces the estrogen binding protein (Kraay and Black, 1970), and post-menopausal women probably have a higher concentration of the binding protein than younger women (McGuire, et al., 1972; Trams, et al., 1971). Post-menopausal bleeding in women, often assumed to result from estrogen insufficiency, has been found to be associated with increased estrogen activity (Frampton, 1966). However, irritation, such as an intrauterine object causes, is also known to increase estrogen binding and uterine weight (Labhsetwar and Perser, 1972). Other estrogen-like effects of intrauterine irritation include luteolysis (Chatterjee, et al., 1971), increased oxygen uptake (Kar, et al., 1965, 1966), increased alkaline and acid phosphatase (Karkun, et al., 1969), and uterine weight (Malajiya, et al., 1970).

It is now generally accepted that senile estrus in rats (Meyer, et al., 1961; Bloch, 1961) and cystic hyperplasia in old rabbits (Burrows, 1949) result from excessive estrogen in old age. Some old mice have a prolonged proestrus (Thung, Boot, and Muhlboch, 1956). Cowdry (1952) has discussed this as a possibility in human cystic hyperplasia of the endometrium and other genital diseases which become very frequent after menopause. Dove, et al., (1970) have shown that urinary estrogen levels in post-menopausal women do not provide an index of the degree of estrogenic stimulation on the cytological level. However, Ward and Thyssen (1970) found that an elevated ratio of estrogens/androgens correlates with persistent karyopyknotic smears in post-menopausal women. Progesterone insufficiency has been suggested as the cause of menopausal disturbances and senescent sterility (Poulson, 1970; Matthew, 1949; Jones, 1970; Thorneycroft and Soderwall, 1969). Insufficient progesterone causes both failure to implant and congenital abnormalities, depending on the amount of progesterone given (Poulson, et al., 1965). Progesterone has been used in attempts to offset the anti-fertility effects of estrogen in rats, with conflicting results (Martin, 1962; Edgren, et al., 1961), and in cows, with good results (Otel, et al., 1968). Progesterone can even improve fertility after irradiation, which mimics estrogen (Valentini and Hahn, 1971). Even though it is generally accepted that, within certain limits, it is the estrogen/progesterone ratio which is important, rather than the absolute level, one would expect progesterone to be

incable of balancing the effect of a certain amount of estrogen because of its solubility or binding properties. The effective progesterone/estrogen ratio is sometimes placed as low as 2000:1, but several studies have suggested that it may be as much as 300,000:1 (or even higher) (Challis, *et al.*, 1971; Nutting-Meyer, 1970), in which case it is obvious that the capacity of a tissue to absorb progesterone could easily be exceeded before reaching the concentration needed for the proper ratio. (Low solubility of progesterone has probably led to errors in protein binding assays: Clark and Gorpide, 1972).

In spite of Thorncroft and Soderwall's suggestion, based on the size, number, and growth of corpora lutea in senescent hamsters, Blaha (1971) found serum progesterone levels to be elevated in old hamsters. However, without data on estrogen levels, this can be considered as possibly only a compensatory response to elevated estrogen. Leavit and Blaha (1970) attribute prolonged gestation (observed by Soderwall, *et al.*, 1962, in senescent hamsters) to excess progesterone, through its supposed inhibitory effect on uterine contractility. Since the development of the embryo is retarded in older animals (Parkening and Soderwall, 1972; Connors, 1969) even before implantation, Blaha's theory seems inadequate to explain prolonged gestation. In vitro studies have shown permanent damage to the embryo from excess estrogen, but not from excess progesterone, yet both can interfere with cleavage (Daniel, 1964).

Glycogen deposits are diminished or at least delayed in their appearance in the decidual cells of senescent hamsters (Connors, 1969). This is usually attributed to estrogen insufficiency, but combined progesterone and estrogen appear to be responsible for increased glycogen deposition. Estrogen is known to activate glycogen phosphorylase (Takeuchi, *et al.*, 1962), which under known intracellular conditions (that is, a ratio of Pi/glucose-1-phosphate of more than 3:2; Reithel, 1967) accelerates the conversion of glycogen to glucose. Glucose or oxygen deficiency (Mitchell and Yochim, 1968) or rapid growth (Needham, 1942) leads to disappearance of glycogen. Since sufficient progesterone is necessary for decidual cell differentiation to occur (Orsini, 1962) and excessive estrogen will entirely suppress the decidual cell response (Finn and Emmens, 1961), it seems that the delayed and diminished deposition of glycogen in the senescent animals' decidual cells should be attributed to a relative progesterone insufficiency (Pous and Comas, 1959), or even to excessive estrogenic stimulation. The higher sensitivity of the senescent uterus to induction of mitoses in ovariectomized rats (Finn and Martin, 1969) would be consistent with an excess of bound estrogen. However, vitamin E deficiency may also prevent glycogen accumulation (Butturini, 1949).

While progesterone (and the chemically related androgens) seem to have no toxic effect, organismically or cytologically,

estrogen has many deleterious effects when its concentration is too high, for example on the adrenals (Kimeldorf and Soderwall, 1947), on liver mitochondria (Gonzales-Angulo, 1970), on deciduoma formation (Finn and Emmens, 1961), on ova, in vitro (Kirkpatrick, 1971), on liver (Song and Kappas, 1969; Biskind, 1949), on fertility (Smith, 1926), and as a carcinogen (Needham, 1942). Korenchevsky (1961) listed depression, salt retention, renal degenerative changes, hypercalcaemia, hyperlipaemia, and epithelial hyperplasia as other toxic effects.

Estrogen treatment is known to be able to cause liver damage, and prolonged treatment with estrogen lowers the activity of the "mixed function oxidases" (Song and Kappas, 1968; Biskind, 1946). Chronic estrogen treatment lowers UDP - glucuronyl-transferase activity (which is important for removal of estrogen from the body) and also lowers the activity of -glucuronidase, after first raising its level (Wakabayashi, 1971). Many liver toxins can lower the liver's capacity to inactivate estrogen (Biskind, 1946). Since urinary estrogens may be 1000 times more concentrated than those in the blood, the importance of conjugation in the liver for regulation of hormone concentration is obvious. Nutritional deficiencies are able to interrupt the liver's conjugation of steroids (and other substances), and vitamin E deficiency is known to be involved in various liver diseases (Schwarz, 1949). Since the requirements for vitamin E increase with age (Fuhr, et al., 1949), liver function

would tend to become increasingly precarious, to the extent that it depends on the vitamin. Most of the measurable symptoms of vitamin E deficiency have a precipitous onset (Fitch, 1968), appearing and becoming extreme within a few days or a few weeks, though serum tocopherol may not have been detectable for many months. Such a physiological process might be involved in the sudden "menopause" when it involves many large physiological changes, but a sudden onset of infertility without other sudden changes would suggest that a simple threshold of survival conditions of the embryo may have been reached.

Estrogen seems to lower the intracellular K/Na ratio, possibly causing the cell's "trans-membrane" potential to become lower (Spaziani and Suddick, 1967; Jones, 1968, 1972; Goodland, 1953). Such a change in salt ratio (Orr, et al., 1972) and in electrical potential (Cone, 1970) apparently is very closely involved with the regulation of DNA and RNA synthesis and with mitosis. Oxidative phosphorylation (Strickland, et al., 1955) is apparently necessary for maintaining high intracellular K/Na ratios though possibly not for maintaining the resting potential (Ling, 1962), so it is possible that estrogen might have its early action either on the cytoplasmic structure (Nemetchek-Gansler, 1967; Peat and Soderwall, 1972), i.e., producing a permeability or phase change, and thus affecting oxidation by modifying structural relationships and diffusion rates and solubilities, or on oxidative phosphorylation, with a phase change

(i.e., a salt ratio change) resulting from a reduction of the energy charge. Merely incubating uterus in Eagle's medium mimics estrogen action (Mueller, et al., 1958), and hypo-osmolarity duplicates some effects (Adams and Haynes, 1969). Progesterone has an opposite effect on sodium retention (Tomlinson, 1971; Jones, 1972). Sex hormone effects seem to be universal and specific; even yeast "sexual" types are selectively affected by the mammalian hormones (Takao, et al., 1971; Yanagishima, et al., 1970).

The best known scheme for estrogen action (Jensen, et al., 1968; Mueller, et al., 1958) suggests that estrogen binds to a cytoplasmic "transport" protein and then enters the nucleus where it modifies the chromosomes so that certain mRNAs are transcribed, leading to synthesis of new proteins which then produce all other effects by membrane and enzymic changes. This theory ignores the fact that lipids are the first substances (by several hours) to show net synthesis (Mueller, 1958), and the observations that salt ratio changes are sufficient to initiate, stop, or modify synthesis of DNA and RNA (Allfrey, et al., 1964; Lezzi, 1969). Engel (1970) suggests that certain enzymes have estrogen affinity sufficient to make them "receptors." Although the 9.5S and the 4-6S "receptors" are not known to have enzyme activity, this may simply reflect the fact that the leading investigators of these proteins are not interested in their potential enzyme activity.

The Problem of Senescent Sterility andOxygen Wastage as its Immediate Cause

Several of the best established events in the aging process -- changes in collagen, water, salts, pigment, and certain enzymes -- form a constellation, at least part of which reappears in several other physiological states, which may have useful similarities to aging: vitamin E deficiency, low oxygen tension, estrogen stimulation, and radiation damage. (Muscular dystrophy, and possibly even myocardial "oxygen wastage," may also be related conditions.) Two of these states--vitamin E deficiency and radiation damage, have been well studied in their relation to reproductive senescence. This thesis will pursue the concept that low oxygen tension is a probable immediate mechanism in reduced fertility, and that the above mentioned constellation of factors, possibly initiated by estrogen but also a self-stimulating process common to other physiological states mentioned, is a prior cause of reproductive senescence.

Senescent hamsters ovulate seemingly normal eggs in normal numbers. There is a much greater loss of embryos at the time of implantation among old hamsters than among the young (seven times greater, according to Thorncroft and Soderwall, 1969a). Some loss does occur by later resorption (two times greater than young: Thorncroft and Soderwall, 1969b), and some ova seem to be delayed in passage through the oviduct in the senescent animals (Connors, 1969), but their greatest difference from the young is at implantation

(Connors, Thorpe, and Soderwall, 1971). It has been fairly well established in several species (Adams, 1965; Finn and Martin, 1969; Talbert and Krohn, 1966; Biggers, 1969; Maurer and Foote, 1971; Finn, 1970) that the aged uterus or its hormonal environment is more likely to be the cause of sterility than the aged ova. Lack of adhesiveness and lack of nutritive support are two possible aspects of the uterus that have been considered as causes of aged sterility. Although differences of surface structure and content of glycogen and certain enzymes are known, it is difficult to argue that these are decisive. For example, even if glycogen stores are low, blood glucose should provide a substitute; if estrogen dominance leads to rapid consumption of glucose by the uterus, lactate should be available. Blaha (1964) found early embryos apparently adhering to the uterine epithelium in senescent hamsters, and only later did they disappear. The requirement of the embryo for molecules of three or four carbons at the two cell stage disappears by the blastocyst stage (Brinster, 1971; Biggers, 1971). However, there is a large increase in oxygen consumption by the embryo at the blastocyst stage, both in rate and in total quantity. (This seems to be true for all vertebrates.) Needham (1931, 1942) reviewed the work of Warburg, Loeb, and others on the relation of pO_2 to respiration and development, and for the organisms considered development was retarded and respiration inhibited by moderately low pO_2 . In the normal animal, according to several studies in

rats (Yochim and Mitchell, 1968; Yochim, 1971), the luminal pO_2 increases sharply at this time, just preceding implantation, and remains high as far into pregnancy as measurements have been made. This coincidence of increasing demand and increasing supply suggests that a failure of supply may be critical, since no substitute for adequate oxygen is known. Glenister's (1970) observation that embryos in tissue culture will develop farthest in an atmosphere of 30% to 50% oxygen suggests that oxygen may be the limiting factor in implantation. He found that implantation occurred in vitro irrespective of the physiological stage of the endometrium or the presence or absence of added hormones. Glenister found that hamster blastocysts would not attach to hamster endometrium which was arranged in the form of a "well", which suggests that the slightly greater diffusion distance in this configuration (which was unnecessary when he used larger rabbit embryos) might have been responsible for the failure to attach. It is known that the shape of the vessel can govern the rate of blastocyst development in vitro (Winterberger, Torres and Daniel, 1971). Some of the embryos in several species are retarded in their development in old animals. With even a moderate oxygen consumption rate, the pO_2 drops sharply with the distance the oxygen must diffuse (Longmuir, 1958). The apparent K_m for oxygen of cytochrome oxidase is high, but the actual K_m of the enzyme is almost infinitely small (Boag, 1970). The apparent K_m for oxygen of intact cells has been found to increase

with the diameter of the cell (Longmuir, 1958) and if this rule applies to ova and blastocysts, then the K_m of these must be very high: possibly close to the pO_2 of capillaries.

According to Yochim's calculations, the uterine blood flow does not increase as much as uterine respiration and uterine mass following estrogenic stimulation, with the result that the tissue becomes relatively hypoxic. The increased proportion of connective tissue (Brooks, Sahinin, and Soderwall, 1964) would probably have a similar effect. Parkening and Soderwall (1972) have presented evidence for impaired capillary permeability in old hamsters at the time of implantation, and suggest that this may be responsible for delayed development of the blastocyst and its failure to implant. Damage to uterine circulation prevents implantation in mice (Senger, Lose, and Ulberg, 1967; Wigglesworth, 1964; Bruce, 1971).

With these considerations, it would seem appropriate to look for the causes of senescent loss of fertility in the physiological differences that exist between the young and the old uterus. The cyclic uterus would reveal basic differences that exist as a function of age, independent of the effect of embryos (McLaren and Menke, 1971). (Embryos at different stages of development might make the meaning of pO_2 much more complex. Even with only the electrode consuming O_2 at the luminal surface or in luminal fluid, the problem of a local gradient and different diffusion rates is a possible source of error.) Besides oxygen tension, related factors will be

investigated, including rate of oxygen consumption, reductive activity, and some specific enzymes that are relevant.

METHODS AND MATERIALS

Animals

Hamsters (*Mesocricetus auratus*) which had been maintained under conditions of controlled light periods and partially controlled temperature and housed in individual cages were used in these experiments. They were fed lab chow pellets and lettuce; from August to December, 1971, they were given a vitamin supplement, including vitamin E. Two age groups were used: "young", three to six months old, and "senescent", thirteen to sixteen months old.

When the whole uterus was to be used, the animals were given 0.5 ml of sodium pentobarbital solution (50 mg/ml) by intraperitoneal injection, and when measurements were to be made in vivo 0.25 ml/100 gr. body weight was given. Animals were followed through at least 2 estrus cycles, using the method of Orsini (1961), and were used on day 4 of the cycle.

For enzyme studies, the uteri were removed, blotted and gently stripped to remove fluid, and weighed. Wet weight is used in all experiments, since large increases in collagen would make dry weight or protein basis misleading. The decreased water content would tend to offset increased collagen of old tissue. For the tetrazolium assay, 40 mg. pieces of whole uterus were blotted, weighed, and

immediately dropped into the tetrazolium solution at incubation temperature. When endometrium and myometrium were to be assayed separately, the uterus was opened by cutting one side longitudinally, and placed on a cool glass surface, where the endometrium was quickly removed by scraping and placed in cold saline until weighed. The tissue was homogenized in one ml. of saline for each gram of fresh tissue.

For respiration measurements, the tissue was prepared as above except that instead of being homogenized, it was cut with small scissors into pieces about 0.5 mm in diameter. Pieces of approximately 10 to 15 mg. of endometrium, myometrium, or whole uterus were blotted on dry glass and quickly weighed, then returned to cold Krebs-Ringers before being cut. The chopped tissue was immediately placed in 2 ml. of cold Krebs-Ringers in the respirometer flask, or in the electrode chamber.

Oxygen Consumption

GILSON MANOMETRIC METHOD: 10 to 15 mg. of uterine tissue prepared as described above was placed in 3 ml. of Krebs-Ringer solution, pH 7.1 in a manometer flask, with 0.2 ml. of concentrated KCl solution in the center well with a filter paper wick, and allowed to equilibrate for 5 minutes at 36°C before recording oxygen consumption. Readings were made at 5 or 10 minute intervals for one hour. In some cases, concentrated sulfuric acid was added to the vessel side-arms.

BECKMAN CLARK ELECTRODE METHOD: 0.2 ml. of Krebs-Ringers solution was injected into the recording chamber and allowed to reach 37°; 10 mg. of tissue, prepared as described above, was placed in the chamber and the Beckman Clark electrode was inserted, and recording was begun immediately, using the Beckman 160 Physiological Gas Analyzer and a Varicord recorder.

Luminal Oxygen Tension

Some measurements were made using a Beckman platinum micro-electrode inserted into the uterine lumen through an 18 gauge arterial needle. Most measurements were made by cutting an opening on the anti-mesometrial side of the uterus about 0.5 cm below the uterotubal junction and inserting the bare microelectrode directly into the lumen. One horn of the uterus was exposed at a time through a small incision in the back, so the same animal could be used for more than one measurement. Thirteen cycling animals were used, and two on day 4 1/2 of pregnancy. Insertion of the electrode through the vagina may allow air to enter.

Triphenyl Tetrazolium Chloride Reduction

Forty mg. of fresh tissue was dropped into 4 ml. of a 0.5% solution of TTC in 0.1 M phosphate buffer, pH 8.5, and incubated one hour at 37°C. The tissue was then removed and left

in 4 ml. of reagent grade acetone until all color was removed.

As a blank control, 40 mg. of fresh tissue was placed directly into 4 ml. of acetone. The color intensity was recorded photometrically (at 475 nm.) and the quantity of formazan present was determined by comparison with known quantities of formazan in acetone.

Enzymes

TOTAL LDH. 0.1 ml of enzyme solution (supernatant fluid of the homogenate) was diluted with 1.0 ml of saline and 0.1 ml of this solution was added to Sigma Standard Substrate, incubated for 30 minutes, developed with Sigma Color Reagent, and optical density was measured in a Beckman Model B spectrophotometer at 570 nm. wavelength.

LDH ISOZYMES. Enzyme solution was prepared as above, except that 0.1 ml of the enzyme was diluted with phosphate buffer, 0.1 M, pH 7.5, and heated to 57°C to inactivate the heat sensitive isozyme 1 (liver or muscle isozyme), and another 0.1 ml was heated to 65°C to inactivate all isozymes but the relatively heat stable isozyme 5 (heart isozyme). The heated enzymes were then treated as above, and their activities were compared to an unheated sample. Some samples were separated by electrophoresis and stained with Sigma color reagent and substrate.

Special care must be taken with the homogenate for LDH measurement, since the M₄ isozyme is cold-denatured (Zondag, 1963).

β -GLUCURONIDASE. Phenolphthalein glucuronide (0.01 M) was used as a substrate, and incubated at 37° in an acetate buffer at pH 4.5 for 1 hour, with 0.1 ml of the same enzyme solution used for LDH assays (Fishman and Green, 1956; Talalay, Fishman and Huggins, 1946; Fishman, Springer, and Brunetti, 1948). Released phenolphthalein was made visible by raising the pH to 10.5 with glycine buffer, and measured in the photometer at 540 nm. Fixed sections of uterus were also treated, for approximate localization of the enzyme.

PEROXIDASE. P-phenylenediamine (0.01 M) was used as substrate, and incubated at room temperature. The photometric measurement was made every minute during the reaction. 0.1 ml of the same enzyme solution as above was used. Fixed sections of uterus were also stained with p-phenylenediamine for general localization of peroxidase activity.

CATALASE. Using 0.01 M hydrogen peroxide as substrate, 0.1 ml of the enzyme solution described above was placed in a respirometer flask with 2 ml. of substrate solution (in phosphate buffered saline, pH 7.5, 0.15 M) and the rate of oxygen evolution was recorded. With the same substrate concentration, both enzymes should reach the same total amount of oxygen generated, though at different times, but the concentration of H_2O_2 used inactivates both enzyme solutions in about the same length of time, leaving some substrate unchanged when the reaction stops from enzyme damage.

Animals to be used in NMR studies of estrogen influence were injected subcutaneously with 5 μ g of estradiol in albumin solution three days before using, and ovariectomized animals were prepared at least a month before use. Whole uteri were kept in cool saline until they were placed in NMR vials.

Assays

BOUND-ESTROGEN ASSAY, BROWN-ITTRICH FLUORESCENCE METHOD.

(Lunaas, 1962). Whole uterine homogenate was extracted with 2 volumes of acetone, which was partially evaporated, and placed in fuming sulphuric acid, and then extracted with 4 ml of methylene chloride. This was placed in quartz cuvettes, and a Hitachi Perkin Elmer MPF-2A fluroescence spectrophotometer was used to scan at 565 nm while exciting with u.v. at 546 nm, using slits #5, and sensitivities 5 and 6.

DYE COUPLING METHOD. (4-amino-6-chloro-m-benzenedisulfonamide.) Urbanyi's method developed for pharmaceutical use was applied to a methanol extract of the uterine homogenate (Urbanyi and Reim, 1966).

BIO-ASSAY. Whole uteri were extracted in acetone (Jakubowska-Naziemblo, 1969), which was evaporated and the extract resuspended in 1% ethanol in saline solution. Half of this was injected as 2 subcutaneous doses (1 ml each) on consecutive days into each ovariectomized animal. Their uteri were inspected and weighed two days after the second injection.

UTERINE AGE PIGMENT EXTRACTION. Whole senescent uteri

which had been cleared in benzyl benzoate and which were heavily pigmented were soaked in reagent grade diethyl ether or pyridine until the solvent was slightly colored, about 24 hours. Ether or pyridine extracts of old uteri were scanned at visible frequencies in a Beckman Model B Spectrophotometer, and at ultraviolet frequencies in a Cary 14 ultraviolet photometer.

Uterine Weight, Water Content, and Diameter

After weighing the whole fresh uterus, pieces of 50 to 75 mg of whole uterus, or 10 to 20 mg of endometrium, or 50 to 100 mg of myometrium were occasionally taken for dehydration and determination of wet weight/dry weight ratio. Representative slides of fixed uterine cross sections were measured to determine overall diameter and thickness of myometrium and endometrium.

Vitamin E Treatment

Three animals were given 125 units of vitamin E once a week for six weeks, orally, and were then used for oxygen tension measurements.

NMR Spin Echo of Tissue

Recently removed uteri were examined in a Nuclear Magnetic Resonance Specialties Spin Echo Spectrometer, at 25 megahertz.

Inspection of Harderian Glands

The eyes of recently killed hamsters were pushed aside and the tissues of the eye sockets were examined under long-wave ultraviolet light for red fluorescence.

Uterine Age Pigment Extraction

Whole senescent uteri which had been cleared in benzyl benzoate and which were heavily pigmented were soaked in reagent grade di-ethyl ether or pyridine until the solvent was slightly colored, about 24 hours. Ether or pyridine extracts of old uteri were scanned at visible frequencies in a Beckman Model B spectrophotometer, and at ultraviolet frequencies in a Cary 15 ultraviolet photometer.

Statistics

Differences between means were evaluated by Student's t test, with Cochran's approximation to the Behrens-Fisher correction for samples of different variation when necessary.

RESULTS

It is really rather strange that human beings are normally deaf to the strongest arguments while they are always inclined to overestimate measuring accuracies.

--A. Einstein

OXYGEN CONSUMPTION. The uteri of forty-six animals, twenty-three young and twenty-three senescent, were studied by the Gilson manometric technique, and six (three young, three old) by the Beckman oxygen electrode method. The mean QO_2 (in $\mu l O_2/mg.$ wet weight/hour) were:

Senescent	Young	P
3.7	2.5	<.05

This is not a high level of significance, but the limitation is probably inherent in the method (Jusiak, 1970). Finer discrimination can be achieved by using an indirect method such as TTC reduction, assuming that these two events closely correspond to each other, as indicated by Cascarano and Zwifach (1955). The problem of ammonia production will be discussed below (under Oxygen Consumption, in Discussion).

LUMINAL OXYGEN TENSION. Cycling animals:

Senescent (5)	Young (5)	Vitamin E treated (3)
Range, mm Hg	0-2	4-8

The oxygen tensions measured in the senescent uteri are in the range known to limit in vitro respiration, and also to limit development of cultured embryos (Needham 1931), but the exact oxygen requirements of embryos are not accurately known, since oxygen tension is normally measured only in the gas phase. The effective K_m for oxygen increases with increased cell diameter (Longmuir, 1957), and the viscosity of the surrounding medium must also have an effect. The early period following ovulation is likely to be similar in both the regular cycle and following mating. This difference in pO_2 could easily account for the early retardation observed in senescent hamsters' embryos, such as lagging cleavage and delay in loss of the zona pellucida. The established lag in uterine changes (Connors, 1969; Stockton, 1972) suggests that this difference in pO_2 may become even more distinct around the time of implantation, when respiration is even higher, and the likelihood of damage even greater, but the fact that the lumen no longer exists at this time makes it necessary to depend on indirect evidence. If it can be generally accepted that TTC reduction does accurately reflect the QO_2 of a tissue, then a complete study of the differences in uterine reductive capacity between old and young animals should be done on each day of pregnancy, to build on the very suggestive work that has been done on rats of different ages by Schultze (1967).

One young and one senescent hamster on day 4 1/2 of pregnancy were used in an attempt to study the luminal oxygen tension directly, and the pO_2 's measured were 4 mm Hg in the senescent, and 15-40 mm Hg in the young. However, the procedure caused tremendous disruption

of the tissues. Since the closure of the young lumen is believed to be firmer than that of the old animal, this very large difference could probably be at least partly attributed to greater bleeding in the young. Nevertheless, differences such as these would be expected on the basis of decreased oxygen consumption of implantation sites, and the senescent lag in factors related to implantation.

TTC REDUCTION.

	Senescent	Young	P
% Transmittance at 475 nm	33	48	<.01

These values are equivalent to approximately .05 and .03 mg formazan per sample, respectively (22 animals). In a group of three young litter mates and three old litter mates, the variability was much less ($P <.001$).

β -GLUCURONIDASE. Mean % transmittance at 540 nm after one hour incubation:

Senescent (3)	Young (3)	P
34	20	<.01

Sections showed a generalized pale stain, except around blood vessels, where it was very intense.

PEROXIDASE. Mean % transmittance at 540 nm and at 90 seconds:

Senescent (4)	Young (4)	P
59	66	<.05

Sections showed intensely stained granules about 5 μ in diameter distributed throughout, but especially in the endometrium, and a

very intensely stained endometrial epithelium; a few larger areas, about 20 by 80 microns, were more lightly stained; these may have been blood vessels.

CATALASE. Mean $\mu\text{l } \text{O}_2$ at 5 minutes:

Senescent (4)	Young (4)	P
78	105	<.05

UTERINE WEIGHT.

3-6 months	14 months	P
Mean 385 mg	592 mg	<.001

Mean ratio dry weight/wet weight:

		Senescent	Young
25 animals	myometrium	1:4	1:6
12 animals	endometrium	1:6.5	1:6.6

The greatest increase of thickness was in the myometrium, but the endometrium also appeared to become thicker with age.

Estrogen Assays

BROWN-ITTRICH FLUORESCENCE METHOD. Five old and three young uteri showed a significant difference in fluorescence at the frequencies used for the oxidation product (or products) of estrogen. The shape of the spectrum was similar to that of estrogen products, and to that of the standard solutions used, within the resolution, which was very poor under these conditions of sensitivity and frequency.

Mean intensity of fluorescence (in arbitrary units):

Mean S (5)	Mean Y (3)	P
52	14	<.01

Estradiol Standard

1 ug	10 ug
19	27

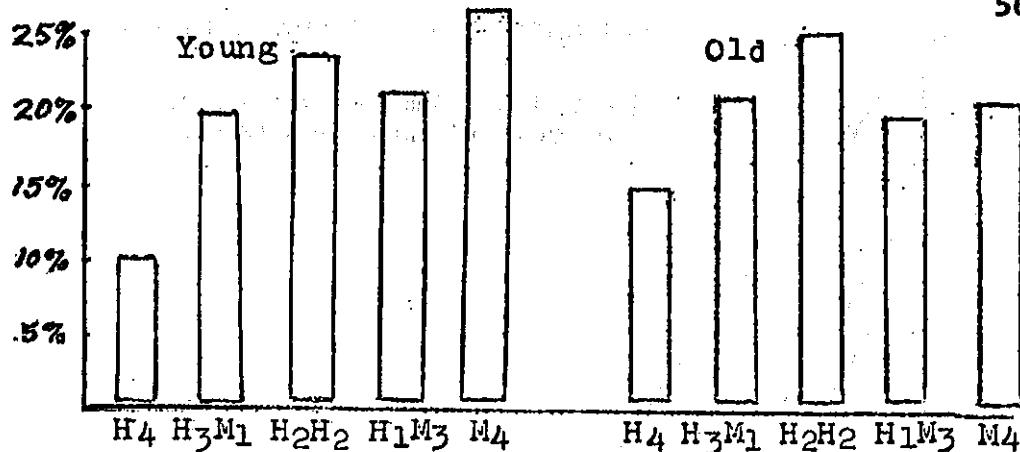
DYE COUPLING METHOD (4-amino-6-chloro-m-benzenedisulfonamide):

Rather than the red product resulting from condensation of estrogen with the dye both young and old gave a yellow product; the old gave a more intense yellow color. This would suggest either that there was no estrogen in the extract, or that there were other materials that condensed with the dye more easily. The technique was discarded as an estrogen assay, but the fact that there was an age-dependent difference, and that bile pigments similarly condense to form diazo products, suggest that the product's spectrum should be compared with known bile pigments similarly condensed.

BIOASSAY. Since this method was intended to assay for "estrogenicity" bound in the uterus, extraction was made in acetone, in which estrogen is highly soluble. An effect on the uterine weight was the intended object of investigation. However, it was noticed that after two injections (containing the extract of 1/2 an old uterus) the ovariectomized recipient had red fluorescence around one eye. This typical Harderian fluorescence has been reported not to occur in ovariectomized animals unless estrogen

has been administered, although injection of porphyrins into intact rodents can cause increased secretion, which has led to the concept that the gland may concentrate and secrete porphyrins produced elsewhere, rather than synthesizing them (Figge, 1947). No difference in uterine weight was observed.

AGE PIGMENT. The age pigment spectrum of certain tissues has been studied by Strehler (1967) and Karnaughov (1971). Strehler reported increasing but homogeneous absorption at higher frequency ultraviolet, but Karnaughov found several peaks of absorption around 260-280 nm (an acetone extract in petroleum ether) in nerve and salivary gland extracts. The uterine pigment, extracted in ether, shows increasing absorption at shorter wavelength (especially below 250 nm), except for a peak at 280 nm, similar to one observed in a blood extract and to one peak in Karnaughov's extract. This is consistent with the suggestion of Orsini and Meyer (1962) and Deno (1937) that the pigment is related to blood or hemosiderin, but the nearly smooth absorption curve could represent a complex mixture. The peak at 280 nm is lost in a methanol extract.



Typical electrophoretic distribution of isozymes

Mean % of H₄

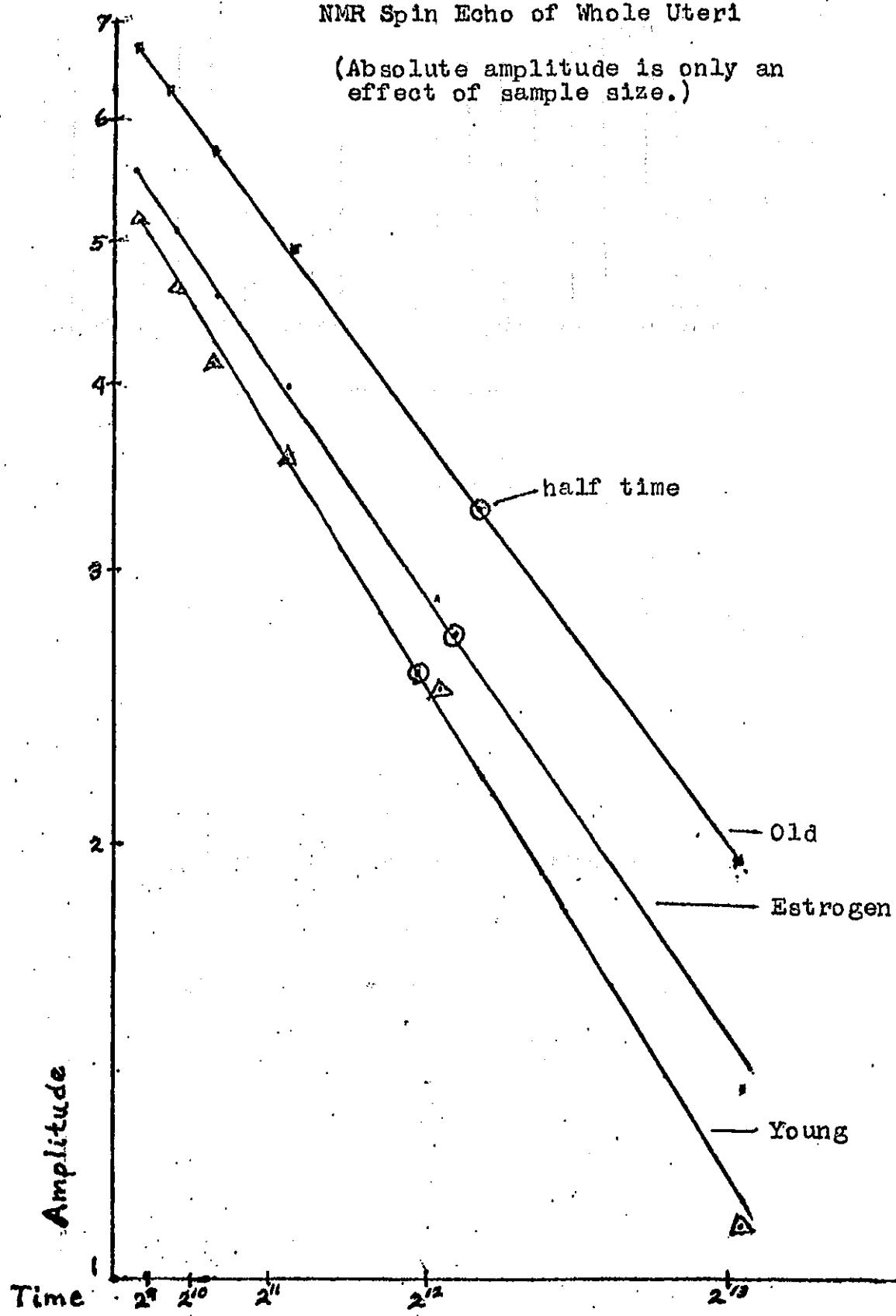
	S	Y	P
12 animals (6 old, 6 young)	14.3	10.7	.05

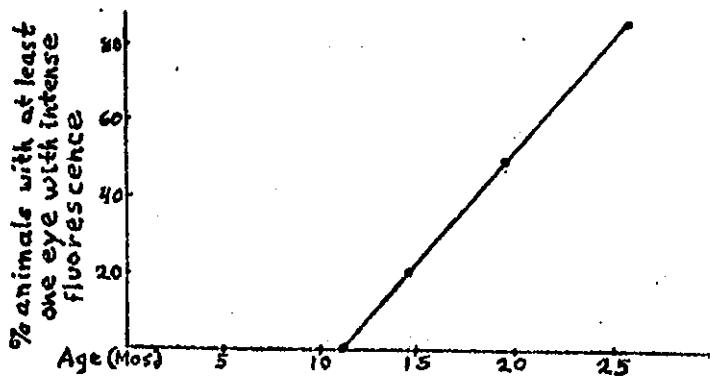
Total LDH Activity in B-B Units per ml:

	S	Y	P
	1700	1950	.05

NMR Spin Echo of Whole Uteri

(Absolute amplitude is only an effect of sample size.)



Harderian Fluorescence

Approximate incidence of red eye fluorescence with age in months.

25 animals.

NMR Spin Echo

The NMR spin echo results are similar to those of Damadian (1971) comparing tumor with normal tissue, and of Hazelwood (1971), comparing fetal with mature tissue. The results suggest that senescent uterine water is more bulk-like than that of young uterus, as tumor and fetal tissue water is more bulk-like than that of normal and mature tissues.

DISCUSSION

Every statement implies a metaphysic.
--A. N. Whitehead

The Paradigm Problem

Even seemingly simple and "straight-forward" problems and solutions are ultimately questions of probability, and interpretation is present in the most "objective" treatment of a question (Tart, 1972). A more complex problem requires a more complex context if a similar degree of "objectivity" is to be maintained. Whenever a paradigm is left, the question of interpretation becomes more apparent.

This thesis begins by questioning the paradigm of "aging as decline" of hormone concentration and metabolic rate. That paradigm was based on certain gross and organismic observations, and is valid with regard to androgenic, luteal, and certain adrenal hormones, at least in most mammals, and with regard to whole animal oxygen consumption per unit of tissue, and probably even with regard to mitochondrial respiration. However, it lacks generality. Highlights from a large literature regarding the physiology of aging are presented in the introduction, to show that a contrary, and much more general, paradigm exists, which can be stated

approximately as "in aging and other distress conditions, typical shifts of metabolic pathways occur." Measurements that only seem peculiar when considered in the former paradigm, may be definitive in the latter. The typical shifts of metabolism include:

- a) "damaged respiration" in the sense of a decreased efficiency of phosphorylation, or excessive hydrolysis of ATP, by high ATPase activity, which can result from mitochondrial damage;
- b) compensatory glycolytic phosphorylation;
- c) given mitochondrial damage and NADH production in glycolysis, a necessary step in compensation is the oxidation of NADH, for example by LDH, or peroxidase, or "age-pigment-related NADH oxidase." Either lactate formation, or oxygen consumption, or both, or another kind of oxidation, must increase if glycolysis is to proceed.

This paradigm forms an interpretive link between the various "distress conditions," and suggests that low oxygen tension will be a factor in these various conditions. The literature on mammalian loss of fertility under many conditions, e.g., X-irradiation, estrogen treatment, adrenaline treatment, senescence, vitamin E deficiency, progesterone deficiency, intrauterine devices, etc., is remarkable for the great similarities that exist in such diverse conditions. Estrogen action is the most common and easily manipulable of these conditions, and so each of the other conditions tends to be

described as "estrogenic." Knowledge regarding the states in which estrogen may be bound within the uterus is not adequate to provide a sound basis for its extraction and unambiguous assay. Whether these conditions that prevent gestation actually increase estrogen concentration in the uterus, or merely mimic it, is still debatable. The available metabolic information about the various "distress conditions" indicates that oxygen wastage is likely to be a common factor; data on embryos' requirement for oxygen in vitro show that oxygen deprivation in utero could be responsible for pregnancy wastage. The new paradigm suggests the hypothesis of loss of fertility through excessive reduction of oxygen, as a normal feature of aging, as well as in the various other "distress conditions." All aspects of the hamster uterus that have been measured are consistent with this. The old paradigm does not unambiguously predict any of the observed age changes. To make it do so requires a complex series of ad hoc assumptions, each one to explain away as "non-estrogenic" a change which to this point had been considered to be estrogenic. One clear prediction that it makes is that the senescent uterus will not have an elevated metabolic rate. This is refuted by the observations, especially the TTC reduction.

Another clear prediction made by the old paradigm is that senescent sterility and estrogen-induced sterility will not act by the same mechanism, since senescent sterility is conceived of as an effect of estrogen deficiency, exactly the opposite of an estrogen

excess. However, the data show that senescence lowers oxygen tension, just as estrogen treatment does.

It is remarkable that, in spite of in vitro studies that show sensitivity of mammalian embryos to changes in oxygen tension, the issue has been almost entirely ignored in studies of fertility, and has never previously been considered in relation to senescent sterility. Even percentages of oxygen higher than atmospheric can be limiting to embryos implanting in vitro, suggesting that implantation is especially sensitive to oxygen deficiency.

Implantation is also the main point of damage in the various "distress conditions," as far as the question has been investigated.

A paradigm by definition is open-ended enough to allow for further development, and so can never be overturned by data or reasoning, but it can become increasingly untenable. The smaller question, however, of whether oxidative changes are adequate to account for embryonic retardation, non-implantation, and resorption can be more decisively answered, because the less complex question calls on a less complex interpretive context.

The observations require rejection of the view that an oxygen deficiency cannot occur in the uterus from common physiological causes. Hypoxia has been proposed as the cause of abortion following vasoligation, but it has been generally believed that only such gross interference with circulation could produce sufficient hypoxia. That the cause can be physiological is indicated by the increased metabolic rate.

Beyond this, the observations characterize the senescent uterus as being in a very common physiological state, which can be called a "distress state." The causal relation between age and the other states is discussed on the basis of the literature. Senescence is physiological, and the physiology is such that sterility must result.

The correspondence of the changes in the senescent uterus to changes in the other distress states supports the view that the changes are patterned, paradigmatic, coherent and meaningful metabolic changes. Although the pathway details are not completely known, the existence of compensatory glycolysis and non-mitochondrial oxidation have been well established in various tissues.

The Senescent State

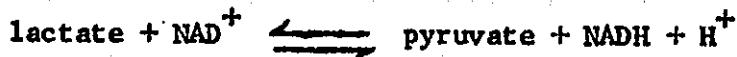
There is considerable evidence that the estrogen/progesterone ratio, or estrogen/17-keto steroid ratio, increases with age, at least in mice, rats, rabbits, and women, and that this change has metabolic effects similar to treatment with exogenous estrogen. However, irritation, low thyroid, excessive copper absorption, etc., probably could increase the estrogen effect without necessarily affecting the concentration of circulating estrogens. To find in senescence the metabolic state associated with estrogen treatment is interesting more for the possible effects of this state on functions such as implantation, than for its corroboration of a suspected excess of estrogen. The generality of this "distress state" of

metabolism is intrinsically interesting.

Enzymes

Many enzymes, including ATPase, spermidine synthetase, alkaline phosphatase, NADH-oxidase, β -glucuronidase, sulfatase, peroxidase, hexokinase (Gross, 1961), malate dehydrogenase (Wilson, 1969), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Schmidt, *et al.*, 1967), lactic dehydrogenase, and ornithine decarboxylase (Kaye *et al.*, 1971), change in activity under the influence of estrogen, but a few are especially important in relation to oxidative metabolism. An apparent increase of cytochrome oxidase following estrogen treatment turned out to be peroxidase (Lucas, *et al.*, 1955).

LDH was considered to be an especially suitable enzyme to study in relation to implantation because its total activity and isozyme composition are known to reflect oxygen concentration and degree of estrogenic stimulation, and to relate to the tissue's disposition of lactate and pyruvate (Johansson, 1966; Battelino, *et al.*, 1971; Goodfriend and Kaplan, 1964; Dawson, *et al.*, 1964). The enzyme can also function as a potential "transhydrogenase" system (Sanwal, *et al.*, 1970; Young, 1961), and thus is in a position to regulate the glucose-6-phosphate oxidation pathway of carbohydrate metabolism. Also, the equilibrium



implies that as the reaction proceeds toward lactate formation, the pH will tend to rise. In many tissues the ratio of lactate/pyruvate is much smaller than the chemical equilibrium, probably because of oxidative competition for the NADH (Olson, 1963). Restricting oxygen would probably allow the reaction to move farther toward equilibrium, with a consumption of protons. Elevated pH tends to weaken other controls (e.g., the inhibiting effect of ATP, involved in the Pasteur effect) over phosphofructokinase (Racker, 1972) which is an important control point in glycolysis. Waddell (1969) has pointed out that the addition of glucose to cancer cells tends to raise the intracellular pH, as the cells form large amounts of lactate. High pH has been observed to increase the rate of lactate formation without increasing respiration (Tobin and Mehlman, 1971). In aged rats, intracellular pH may be elevated (Comolli, 1971) as well as lactate production in certain tissues (Angelova-Gateva, 1968). The NAD^+/NADH ratio also controls the rate of glyceraldehyde-3-phosphate oxidation (Racker, 1972). LDH is thus in a position to exercise extensive control over glycolysis, possibly providing a mechanism for interfering with the Pasteur effect (which is decreased under the influence of estrogen: Greenstein, 1954). The LDH isozyme ratio may be of importance not only for determining the availability of pyruvate for oxidation, but for regulating other pathways. (Considering the low pO_2 of senescent or estrogen dominated hamster uterus, and the requirements of spermatozoa for oxygen - Nevo, 1965 - the X isozyme in male

producing spermatozoa - Stambaugh and Buckley, 1970 - may relate to the lower proportion of males in senescence or estrogen treatment: Hahn and Hays, 1963).

In the presence of very active NADH-oxidase, pyruvate and lactate might also have a "coenzyme" function in relation to this enzyme. Peroxidase, which is "induced" by estrogen, has an NADH-oxidase function (Lucas, *et al.*, 1955), and this activity is also found in association with age pigment (Strehler, 1967).

There is a slight decrease of total LDH activity in old animals, probably not significant considering their higher proportion of connective tissue and collagen.

Oxygen tension regulates the isozyme ratio, both in vitro and in vivo (Clark and Yochim, 1971; Yochim and Clark, 1970; Fujisawa, 1969; Johansson, 1966). Citric acid cycle intermediates can retard the formation of isozyme "M" by chick heart muscle cells in culture (Gahn, 1969), but oxygen tension has its isozyme inducing effect regardless of the presence of metabolic inhibitors, including fluoroacetate, potassium cyanide, 2, 4-dinitrophenol and sodium amyta (Johansson, 1966). Because of this, it has been speculated that oxygen might act directly on the genes regulating LDH isozyme synthesis, but other methods of control, such as preferential degradation (Pruitt, 1971; Greengard, 1967) might be more fruitful to investigate experimentally, especially considering the greater lability of the "liver" isozyme, both in heat and cold (Zondag, 1963;

Kreutzer and Fennis, 1964). Direct action of oxygen or peroxides on the labile isozyme, or an effect involving sequestering of the stabilizing coenzyme, NAD, might be mechanisms for regulating isozyme proportions. The H_4 isozyme seems to be the most lipophilic as well as the most stable, and M_4 the least lipophilic (Kabara and Konvich, 1972). This result is implicitly predicted by the theory which relates cytoplasmic phase changes to control of enzyme activity (Peat and Soderwall, 1972).

Finding the H_4 isozyme increased with age leads to at least two or three questions:

- 1) Does enzyme induction by oxygen relate to the rate of consumption of oxygen, rather than to oxygen tension itself?
- 2) If the function of this isozyme is to save some pyruvate for another use, rather than converting it to lactate (Lehninger, 1970), is carboxylation to oxaloacetate that use?
- 3) Would it lead to a situation in which (because of H-LDH's inhibition by high concentrations of pyruvate) pyruvate carboxylase tended to become rate limiting for glycolysis?

Although no mechanism is suggested, it is interesting that the age shift of isozymes is opposite to the shift which occurs in pregnancy, as early as day 5, when uterine oxygen consumption is known to decrease (Battelino, *et al.*, 1971).

β -Glucuronidase is increased in liver, uterus, kidney, and other tissues by injection of estrogen (Fishman, 1950) or various

poisons, and this effect has been likened to the induction of detoxifying enzymes in the liver by their substrates. Levvy (Karunairatnam and Levvy, 1949) has offered the contrary interpretation that β -glucuronidase is merely involved in tissue proliferation, regardless of what causes the proliferation. Prolonged estrogen treatment causes an eventual decline in β -glucuronidase, as it does of the mixed function oxidases (Song and Kappas, 1968). Its decline with age in the hamster uterus thus is consistent with the view that estrogen tends to dominate uterine metabolism in senescence, but of course doesn't imply that this is what occurs. However, if its normal function in the uterus and other tissues is to conjugate and remove estrogen to prevent estrogen toxicity, then its absence in the senescent uterus would suggest an explanation for other physiological changes that occur in the uterus with age. If estrogen no longer turned over in the uterus, then a much smaller supply could raise the uterine content. Estrogen treatment is known to "induce" larger amounts of the estrogen binding protein (Kraay and Black, 1970), as well as reducing the progesterone binding capacity of the uterus (Armstrong and King, 1971). Post-menopausal women have at least as high a concentration of uterine "estrogen receptor" protein as do younger women (McGuire, *et al.*, 1972; Trams, *et al.*, 1971).

β -Glucuronidase is known to have an *in vitro* transferase function (e.g., transfer of glucuronic acid from phenolphthalein to an alcohol) besides its common hydrolytic function (Fishman and

Green, 1957). It has been shown to have a slow synthetic function in vitro (Florkin, 1940). Fishman (personal communication) feels that an in vivo conjugating (transferase) function is plausible, considering the similar role of a "hydrolytic" enzyme such as glucose-6-phosphatase. β -Glucuronidase is so abundant in smooth endoplasmic reticulum that it is considered likely to be a structural protein (Wakabayashi, 1970).

CATALASE. Catalase activity of the uterus is interesting because it is believed to relate to the cytochrome system (Deisseroth and Dounce, 1970), is lower in the female, at least in some species (Adams, 1965), and since either its rate of synthesis or rate of degradation appears to change with age in mouse liver (Baird and Samis, 1971). The fact that its activity is lowered by riboflavin deficiency (Adams, 1965) and by serum or tissue extract from animals with tumors (Nakahara and Fukuoka, 1959), and is lower in females, suggests that its level will correspond to the amount of energy derived from mitochondrial oxidation. A similar extract is an abortifacient. The lower level in the aged uterus would not be particularly interesting if oxygen consumption were lower in that tissue. Since oxygen consumption tends to be higher, low catalase activity suggests that the oxidation may not be occurring in the usual way.

PEROXIDASE. The fact that estrogen activates the NADH-oxidase function of peroxidase, which itself is "induced" by estrogen (Lucas, et al., 1955; Yokota and Yamazaki, 1965; Jellink and Lyttle, 1971; Temple, et al., 1959; Hollander and Stephens, 1959; Beard

and Hollander, 1962) suggests that in the uterus peroxidase may be involved at an early stage in the process of cell activation by estrogen. Vitamin E, which is known to inhibit peroxidation, has anti-estrogenic effects, including inhibition of the formation of the estrogen-dependent uterine age pigment, which is associated with NADH-oxidase activity, and which is generally considered to result from peroxidation of lipids and proteins (Elftman, *et al.*, 1949).

VITAMIN E. Two classical symptoms of a vitamin E deficiency are increased tissue oxygen consumption (Houchin, 1942) and anemia (Fitch, 1968). The degree of stability of erythrocytes in the presence of oxidants, such as hydrogen peroxide, is an indication of the vitamin E status of the animal. Vitamin E has an anti-oxidant function, but other properties are also believed to be involved in its vitamin activity. It increases the viscosity of protein solutions (e.g., albumin), apparently by causing a more extended conformation of the protein. Such a physical or mechanical property, as well as a chemical anti-oxidant property, could be involved in its anti-hemolytic effect, and in its ability to stabilize other "membrane" systems such as lysosomes (McCay *et al.*, 1971). It lowers the extractability of muscle proteins (Matusis, 1970). The enzymes of vitamin C synthesis in microsomes are inactivated by lipid peroxides, and preserved by added α -tocopherol (Chatterjee and McKee, 1965). Estrogen's effects on vitamin C synthesis,

β -glucuronidase (of lysosomes), and pigment formation may be produced by a common structural event. Uterine age pigment requires estrogen for its development, but vitamin E treatment prevents its formation (Kaunitz, *et al.*, 1948; Atkinson, *et al.*, 1949).

If hemes, or other porphyrins, are related to the lipofuscins or age pigments, they may be responsible for the NADH-oxidase activity that Bjorkerud (Strehler, 1967) found associated with the pigments. The same hemolysis which leads to vitamin E deficiency anemia could therefore also be responsible for the increased oxidation of vitamin E deficiency. Since hypoxia stimulates porphyrin synthesis (Falk, *et al.*, 1959), oxygen wastage by porphyrins would tend to be a positive feedback process. The oxidase activity of age pigment granules is cold-inactivated (Bjorkerud and Cummins, 1963), which is consistent with the theory of pathway regulation by "structural temperature" (Peat and Soderwall, 1972). If estrogen's action is intrinsically related to a hypoxic state, this could also explain the fact that porphyrins are synergistic with estrogen, and similarly could explain the occurrence of porphyrins mainly in female and cancer-prone rodents (Strong, 1942; Figge, 1942).

Vitamin E is known to lower serum bilirubin related to liver damage (Schwarz, 1949).

The circulatory availability of oxygen, as well as the metabolic disposition of oxygen, may be directly related to the vitamin E status of the animal. Adaptive hyperemia in several glands

has been found to result from the action of peptide kinins, produced by proteolytic kallikreins (Schachter, 1969). Small molecule inhibitors (e.g., Trasylol, ϵ -amino caproic acid) are known, as well as the protein inhibitors, of the kallikreins and other proteolytic enzymes (cathepsins, trypsin). Age, X-irradiation, and estrogen have been implicated in various ways in proteinase inhibition. The "Shute test" (Shute, 1937; de Oliveira, 1949), a measure of the level of serum proteolytic inhibitory activity, clearly shows that a vitamin E deficiency increases the inhibition of proteolytic enzymes. The presence of inhibitors would tend to suppress formation of kinins, the local "inflammatory" vasodilators, and would also interfere with clot removal.

The hamsters that were given vitamin E in very large doses showed chronic leucocytic vaginal smears, as in rats given daily doses of progesterone (Labhsetwar, 1970).

A vitamin E deficiency causes muscles of rabbits (Morgulis and Osheroff, 1937) to increase their concentration of "extra-cellular" ions, especially Na^+ , Cl^- , and Ca^{++} , suggesting a possible loss of organic ions, as well as an energy loss: creatine phosphate may be largely responsible, since vitamin E deficiency causes creatinuria.

The observations of Thorneycroft and Soderwall (1969) regarding fewer and smaller corpora lutea in senescent hamsters may relate to the observations of Lecoq and Isidor (1949) that vitamin E deficient rats have no corpora lutea.

Progesterone's effect on Na^+ , Cl^- , Ca^{++} , and water is similar to that of vitamin E, and opposed to estrogen's effect. The most sensitive demonstration of vitamin E's anti-estrogen effect is probably Lecoq's (1949) on uterine chronaxy.

Vitamin E requirements increase with age (Fuhr, *et al.*, 1949; Ames and Ludwig, 1964; P'an, *et al.*, 1949; Emerson and Evans, 1940) and a deficiency acts mostly to block implantation (Blandau, *et al.*, 1949).

Oxygen Consumption

A likely source of error in measuring tissue oxygen consumption (besides the problem of balancing cell damage in thin slices or minces against limited diffusion in thicker slices: Jusiak, 1970) is in neglecting to account for other gaseous metabolites (Dudka, *et al.*, 1971; Maharajh and Walkley, 1972) than oxygen and carbon dioxide, of which ammonia is likely to be the most important by far. Tashiro (1922) observed ammonia production in nerve and muscle, which increased when the tissue was stimulated. Needham (1942) wrote that ammonia is not likely to be produced by embryonic tissue unless sufficient carbohydrate is not available, implying that it results from protein catabolism. A popular contemporary argument (Lowenstein, 1972) is that ammonia emission results from the activity of adenylate deaminase, but in many cases glutamic dehydrogenase seems a more likely enzyme. In estrogen stimulation protein synthesis and turnover are accelerated (Mueller, 1968); glutamic acid is an important link between carbohydrate metabolism and

proteins, and is also closely related to synthesis of pyrroles.

Aspartate, however, seems to be the first amino acid synthesized after estrogenic stimulation (Jervell, et al., 1958), probably by amination of oxaloacetate (Barker and Warren, 1966). Glutamate dehydrogenase may have alanine dehydrogenase activity when dissociated into subunits. Estrogen can regulate this dissociation and change of activity, probably by increasing the supply of NADH (Tomkins, et al., 1961). Estrogen-increased L-alanine pyruvate conversion by this form of glutamate dehydrogenase would account for some of the diversion of pyruvate into protein synthesis. Lacking carbohydrate, a reversal of the reaction could form pyruvate, with a high rate of protein catabolism and production of ammonia. (A shift of the enzyme activity away from glutamate dehydrogenase may spare glutamate for synthesis of **δ**-levulinic acid, and porphyrins.)

Senescent uterus has reduced glycogen levels in pregnancy (Connors, 1969), and if this lack of glycogen exists in the cycling senescent uterus, or if the rate of metabolism is high in relation to available carbohydrate, aged uterus may release more ammonia than young uterus. In vitro, where small glycogen deposits may be quickly consumed and no external glucose is provided, the tissue with the highest metabolic rate will probably release the most ammonia, and if this is not taken into account, the manometric method will read this as diminished oxygen consumption. In the first measurements, samples often showed negative "oxygen consumption," and this occurred more often with senescent tissue, especially

myometrium, and probably was intensified by delay in preparing the tissue. Although the literature on uterine oxygen consumption does not mention this problem, it may be a general factor in measurements of oxygen consumption. When sulfuric acid was added to the vessel side arms, oxygen consumption measurements became consistently positive and rates became more consistent, especially in the case of senescent tissue.

Dissolved oxygen measurements would tend to show alteration in the opposite direction when ammonia is released, so additional measurements were made in the Beckman apparatus. No removal of ammonia is possible in this case, so the data should be considered only in relation to the manometric data, unless it can be assumed that the much shorter time needed to get measurements eliminates the factor of ammonia production.

Increased oxygen consumption by estrogen-activated tissue does not imply increased mitochondrial oxidation, coupled to phosphorylation or not. Lucas et al. (1955) determined that what had been assumed to be elevated cytochrome oxidase activity was actually the NADH-oxidase activity of peroxidase. Wilson (1969) reported the puzzling observation that estrogen increased the activity of only malate dehydrogenase of all the citric acid cycle enzymes, and that others may have decreased. It is interesting that MDH is one of the few enzymes known to increase in some tissues in senescence (Finch, 1972). The carboxylation of pyruvate to oxalo-

acetate, followed by amination to form aspartic acid (Barker and Warren, 1966; Jervell, et al., 1958) may have its equivalent in this situation, viz., production of oxaloacetate for amino acid formation.

Barker, Nielson, and Warren (1966) claim that lipid synthesis, mediated by NADP^+ , regulates the hexose monophosphate shunt.

(Elsewhere it has been suggested how a cytoplasmic phase change could accelerate lipid synthesis: Peat and Soderwall, 1972).

They found that the specific activity of glucose-6-phosphate dehydrogenase decreased significantly during an early period in which G6P and 6PG were being oxidized at an increased rate. Activation of either lipid synthesis or oxidation of NADPH would be able to stimulate this pathway.

Oxygen Tension

The measurements of intrauterine pO_2 that have been made with the Beckman microelectrode inserted through an 18 gauge arterial needle (Yochim and Clark, 1971; Mitchell and Yochim, 1968, 1969b; Salderini and Yochim, 1967) are apparently inaccurately high.

The sharp end, and additional thickness, possibly cause still-oxygenated blood to be freed into the cavity surrounding the electrode tip, where it would not be subject to oxidation by the tissue.

The cyclic variation of about 20 mm Hg pO_2 could be a further artifact, since the electrode itself reduces oxygen and will quickly

lower the oxygen tension of a small volume of fluid that is not stirred. The end of the needle immobilises a few microliters of liquid. Periodic contractions of the uterus would replace the fluid in the tip of the needle with fresh blood (or other oxygenated fluid produced by introducing the electrode) from elsewhere in the lumen. This procedure gave measurements in the hamster's uterus that were about the same as those obtained by Yochim and others in the rat, except that instead of one minute cycles, in the hamster the cycles were about three to four minutes long. Different means or maxima determined by this method would therefore reflect indirectly the rate of oxygen consumption by measuring the level of oxygen present in the blood which is released into the lumen. The rate of oxygen consumption would not be entirely unrelated to tissue pO_2 (Yochim, 1971), but it would present an entirely false picture of the conditions experienced by the pre-implantation embryo.

Using the "bare" electrode (with stainless steel jacket, but no needle around it) with a diameter of 1.0 mm and a blunt tip, a much lower pO_2 was measured, and there was no cyclic change that could be recorded. The oxygen tension is so low that an accurate absolute measurement is extremely difficult, but relative measurements of differences between animals can be easily and clearly made. The oxygen tensions observed are lower than any known mammalian tissue except cancer. Senescent animals' uteri had a pO_2 in the range of 0-2 mm Hg, young animals in the range of 4-8 mm Hg, and

vitamin E treated senescent, 6-12 mm Hg. The color of the uterus varied from maroon in the old animals to pale pink in the vitamin E treated animals; young animals' uteri were bright red.

At such low oxygen tension it might be advantageous to have hemoglobin with high oxygen affinity; it has been reported that sheep with hemoglobin A (with higher than normal oxygen affinity) are better able to withstand the abortifacient effects of estrogenic pasture than are other sheep (Obst and Seaman, 1971). A lower "unloading tension" would correspond to a lower tissue oxygen tension, with possibly adaptive effects, such as a more controlled or gradual release of oxygen. Oxygen consumption decreases at implantation sites in the rat uterus at the time of implantation (Surani and Heald, 1971), and this probably causes a rise in pO_2 to levels the embryo requires. Progesterone by counteracting estrogen, may be the cause (Goodland, et al., 1953). As early as the fourth day of pregnancy there are greatly increased obstructions in the uterus, probably from increased adhesiveness of the epithelium which at this time is closing over the blastocysts. Even the "bare" electrode has to be forced if it travels more than a centimeter from the opening, and obviously is causing luminal bleeding. The force required was such that at one obstruction the electrode passed entirely through the wall of the uterus. Before hitting an obstruction the pO_2 reading was 40 mm Hg, which might have been high because of being near the opening, though the soft and abundant endometrium formed

a seal. Beyond the obstructions the pO_2 measured 5-10 mm Hg, or about that of the vitamin E treated cycling hamsters. The average blood would be expected to have more oxygen than the respiring tissue but if the deeper stromal or myometrial tissue were respiring faster than the implanting epithelium, bleeding might cause a lowering of pO_2 .

Yochim's system of measuring uterine luminal oxygen tension with an arterial needle may be measuring something equivalent to the "bulk" pO_2 in organ culture, i.e., possibly uterine fluid that has been contaminated with oxygenated blood or gas introduced with the needle, so they are possibly correct about the effect of pO_2 on LDH isozyme proportions in the uterus, which they compare to the effects in cultured uteri.

However, the pO_2 at the surface of the cell in culture, where the effect of pO_2 on LDH isozymes is to be studied, will be much lower, because of an O_2 gradient in the unstirred layer. A bare electrode, able to contact the epithelial tissue in the uterus, and less likely to cause bleeding, would probably more closely approach the pO_2 actually experienced by tissue either in vivo or in culture.

The pO_2 Yochim's group measured would not, according to Needham's review (1931), be low enough to approach limitation of embryonic development. However, when the lumen "surface" pO_2 is measured, by the bare electrode, the tension is seen to be close to that which limits respiration and development of embryos; the actual pO_2

the embryo experienced in the experiments reviewed by Needham is not known, nor is the actual pO_2 in the old hamster's uterus, since it is at the extreme lower limit of the measuring system's capacity. A better technique is needed, to determine the real minimum.

TTC Reduction

The reduction of triphenyl tetrazolium chloride is considered to reflect dehydrogenase activity (Racker, 1955; Novikoff, 1959) or the cell's reductive capacity, including substrate availability, and is believed to be one of the most sensitive indicators of estrogenic stimulation. It is known to accurately correspond to QO_2 in many tissues (Cascarano and Zweifach, 1955). Martin (1959) has reported that the TTC reducing response of the ovariectomized mouse vagina to exogenous estrogen is linear in relation to dosage from about two picograms of estradiol per mouse up to about one microgram. Estrogen increases phosphorylase activity (Takeuchi, et al., 1962) and ATPase activity, so would tend to release inhibition of glycolytic enzymes while providing increased amounts of glucose. Ammonia is a very effective activator of phosphofructokinase, and may be produced by cell activation (Lowenstein, 1972). The production of NADH would therefore be increased. The earliest effect of estrogen may be on glycolysis, rather than on respiration (Roberts and Szego, 1953). Since estrogen causes "induction" of peroxidase, which has NADH oxidase activity (for which estrogen serves as a coenzyme: Beard and Hollander, 1962), at least part of the increased

NADH would tend to reduce oxygen by this short-circuit route, increasing oxygen consumption without necessarily increasing usable oxidative energy production. That is, an extramitochondrial uncoupling of oxidative phosphorylation could occur through these known enzymic effects of estrogen. Whatever the mechanism, increased oxygen consumption must lower the pO_2 of a tissue if circulation doesn't increase proportionately. According to Yochim, *et al.* (1971) circulation doesn't increase as fast as the mass of tissue increases, under estrogenic stimulation. With the rapid "uptake" of water (or possibly metabolic production of water) immediately after estrogen treatment, the average distance of cytoplasm from capillaries must increase, which would possibly decrease the efficiency of tissue oxygenation.

The fact that the senescent hamster uterus shows distinctly increased TTC reduction is probably the best evidence that senescent sterility is the result of an active physiological process (or malfunction). Schultz (1964, 1965, 1966, 1967, 1968) has found that strain differences in rats suggestive of estrogen differences correlate with fertility and TTC reduction. Selection for large bodies produced low uterine TTC reduction and large litters, while selection for small bodies produced high uterine TTC reduction, and small litters. He also found increased TTC reduction at the time of senescent litter loss by resorption in rats.

Racker's (1955) "nothing dehydrogenase" of well-dialyzed protein fractions, which give a strong nitroprusside reaction, reduces NAD

or NADP without the addition of substrate, and the reduced nucleotide can then reduce tetrazolium. Racker and others have guessed that protein SH groups are the source of this reducing capacity. The present tendency is probably to assume that starches or fats contaminate the protein fraction and provide the reductive capacity. However, G. Ungar (1957, 1959) has presented some evidence to support the view that cell excitation involves an increase of protein SH groups, resulting from conformation changes or "partial denaturation" after excitation or hormone treatment. The technique of determining uterine protein SH concentration is very hard to use reliably, but might eventually be useful to study the more exact nature of the uterine reducing capacity which changes with age and estrogen treatment.

Age Pigment

Uterine "age pigment," which develops in the presence of estrogen and vitamin E deficiency (Kaunitz, *et al.*, 1949), is not noticeably soluble in ethanol, benzene, benzyl benzoate, acetone, or chloroform, but is highly soluble in alkaline pyridine solution which is an excellent bile pigment solvent, and is partly soluble in ether, which is also a solvent for certain bile pigments (With, 1968), and the spectrum is somewhat similar to that of a blood extract. The presence of an ether soluble fraction of bile pigment of a

certain magnitude in human serum is considered to be indicative of cancer (With, 1968). Bjorkerud (1964) has observed that age pigment has NADH oxidase activity, and contains the heme group. Aged uteri (Zinkat, 1931) contain remarkably large amounts of iron, which might be bound by the hemes. The fact that the pigments appear under conditions in which red blood cells are hemolyzing rapidly, further supports the possibility that they are porphyrin-related (Gyorgy and Rose, 1949). Bjorkerud (1964) also observed that this NADH oxidase activity of the pigment is cold-inactivated, which tends to support the theory of entropic activation of oxygen consumption (Peat and Soderwall, 1972).

NMR

The evidence is very good that water near interfaces is structured differently from bulk water (Drost-Hansen, 1971, 1972). Probably the most dramatic demonstration was Shereshevsky's (1928) experiment showing very low vapor pressure of the water in capillary tubes. What is still in dispute is whether the particular structure induced by a surface represents an intrinsic "phase" or crystal-like state of water (Drost-Hansen, 1971, 1972), or whether the particular repeating pattern of the surface (such as a macromolecule) imposes its structure onto the water (Ling, 1972). It has been pointed out that enzyme biochemists (the "Pauling School") emphasize the role of ionic side chains of proteins in ordering (or binding) water, while the fiber chemists emphasize the role

of the protein backbone (Ling, 1972). Since estrogen stimulation involves a decrease in protein, DNA, and RNA concentration, because of the great increase in water concentration, the average distance of intra-cellular water from macromolecular surfaces must increase, and this should mean that the water molecules experience fewer restrictions of orientation. High resolution NMR spectra show a distinct broadening of the water line in tissue, relative to bulk water, and less broadening (with a shift upfield) in estrogen-treated uterus than in atrophic uterus from ovariectomized hamsters. Old uteri were not significantly different from young uteri, and both were intermediate between tissue from ovariectomized and estrogen-treated animals. The method, of course, can't determine the quantity of extracellular and intracellular water, unless their movement or shielding is very different, and this apparently is not the case. Diffusion, proton exchange, paramagnetic ions, and lipids are all probable sources of error in this method.

Spin echo NMR can eliminate the possibility that the line broadening results from proton exchange, and shows a slight difference between old and young uteri, as well as between estrogen-treated and untreated young animals, with young uteri showing the greatest difference from bulk water. The old uteri, by being intermediate between younger uteri and bulk water, are somewhat analogous to the malignant tumors in Damadian's study (1971). Even very dense connective tissue (rat knee joint) responds to irritation by a

similar change in water relaxation time, without a large uptake of water (Rokityanskiy and Yakimov, 1971).

Harderian Fluorescence

This strain of hamsters apparently has less active porphyrin secretion from the Harderian glands than do many other rodents, unless their diet is simply richer in B vitamins. A B vitamin (especially pantothenic acid) deficiency is known to cause increased porphyrin secretion by the Harderian glands. Since World War II, animal feeds have become more standardized and are generally supplemented with adequate vitamins, and this may account for the loss of interest in the Harderian glands and their porphyrin secretions. Most (about 80%) barely senescent (14 months) hamsters that have been examined in this lab showed no fluorescence of the eye socket, or only a weak green fluorescence. Beyond this age, however, the typical bright red fluorescence becomes more common. By the time their body weight begins to decrease, which is usually after 15 months, most of the females showed some red fluorescence, but no male did. This is consistent with the observations of Strong (1942) and Figge (1942) regarding the relation of the secretion to estrogen (no males showed fluorescence) and possibly to the likelihood of an animal to develop cancer. Its appearance around the time of reproductive senescence suggests that the liver function may be decreasing at this time, and that the pigment may be

related to the altered uterine metabolism.

Porphyrins are synergistic with estrogen, as well as with carcinogens (Strong, 1942; Figge, *et al.*, 1942; Bittner and Watson, 1946; Kennedy, 1970), and the pyrroles and porphyrins are also conjugated by the liver for excretion via the kidneys. With liver damage, they accumulate, causing porphyria (With, 1968). At least in rodents, concentration of porphyrins in the Harderian glands corresponds to estrogen levels and to the tendency to develop cancer, as well as to deficiencies of the B vitamins essential for liver conjugation (Figge, 1944; Hoffman, 1972).

Uterine Weight, Water Content, and Thickness

Organ weight has been said to be a more sensitive index to metabolic change than any other single condition (Criscuolo, *et al.*, 1955; Rusin, 1971). All dimensions of the hamster uterus that were observed, diameter, thickness of endometrium and myometrium, and total weight, increased with age. Water content of the myometrium decreased with age, but there was no significant difference between young and old endometria's water content.

Though all parts of the old uterus are enlarged, the biggest change with age appears to be the thickness of the myometrium. A large part of the collagen that accumulates with age is in the myometrium (Loeb, 1939).

Estrogen inhibits collagenase (Jeffrey, *et al.*, 1971; Woessner, 1968) and increases collagen synthesis (Henneman, 1968; Kao, *et al.*,

1964). Loeb (1939) found that estrogen and age both cause collagen accumulation in the uterus. Schaub (1965) found a three-fold increase of uterine collagen in the aged rat, and observed the same age changes (crosslinking) that occur in other tissues.

Finn (1963) found that collagen accumulates in mouse uterus with age, both in a barren uterine horn and in a repeatedly pregnant horn, when the mouse was unilaterally ovariectomized. This weakens the arguments that maintain that collagen formation is largely the result of mechanical stress.

Alkaline phosphatase activity increases with estrogenic stimulation. Therefore, if excessive estrogenic stimulation is responsible for the failure of implantation in senescent animals because of an inadequate level of progesterone, alkaline phosphatase activity should be demonstrable.

In slides prepared by T. J. Connors for a study of enzyme changes in implantation (1969), at all times examined--days 3 1/2, 4, 4 1/2--the epithelium of the senescent uterus was more intensely stained. The senescent stroma was more intensely stained on day 4 1/2, but on day four it was in several cases more lightly stained than in the young.

Since the decidual cell reaction (to an artificial stimulus, viz., the injection of air: Stockton, 1972) is delayed by about twelve hours in the old hamster (and also fails to produce a weight

increase as great as in the young animal), this difference in stromal alkaline phosphatase probably corresponds to that delay.

Horikoshi and Wiest (1971), among others, have attributed "uterine refractoriness" to a small progesterone/estrogen ratio.

The epithelial reaction, however, seems to reflect a chronic difference. The senescent myometrium stains nearly as much as the endometrial stroma, but the young myometrium is almost unstained, on all days examined. Again, this suggests a possible chronic difference in degree of "estrogenic" stimulation.

An Organismic Change

These diverse but interlocked observations suggest that a profound reorganization, probably consisting of many compensatory tissue responses to the altered internal environment, occurs during aging. It seems that these changes may be most easily observable in the female mammal, but analogous processes may be practically universal. The mutual adjustment of many parts of a system makes the evaluation of a single component--such as estrogen--very difficult and ambiguous, yet the "meaning" of the configuration to the organism is reasonably consistent and determinate. If the many relevant components of the system can be identified, then it should be possible to evaluate each part without ambiguity. A partial example is the estrogen/progesterone ratio, but it will probably eventually be possible to interpret quantitatively the concentration of other

hormones (especially the salt and sugar regulators), the density and composition of connective tissue, composition of bile pigments, enzymes, etc. For an organismic process, such as aging, it will be appropriate to have precise and quantifiable organismic concepts.

CONCLUSION

Several methods were used to characterize the physiological state of the hamster uterus, with regard to oxygen metabolism, in two age groups, young and senescent. Most of the factors studied were consistent with the concept that the aged uteri are in a relatively "estrogenic" state, but this does not necessarily imply an elevated concentration of steroid estrogens. Factors such as vitamin E deficiency, age pigment deposits, porphyria, ammonia, collagen accumulation, and progesterone deficiency could promote this state, as well as possibly elevated levels of adrenal or ovarian estrogens or other hormones such as catecholamines. In senescent animals oxygen consumption was increased, TTC reduction was high (TTC reduction may simply reflect the oxidative capacity--Cascarano and Zweifach, 1955), catalase was low, peroxidase and uterine pigment were high, and uterine weight was increased considerably. Oxygen tension of the uterine lumen was extremely low in cycling animals of all ages, but was distinctly lower in senescent animals. These changes might be ascribed to a mere vitamin E deficiency, except that the requirement for vitamin E is a function of age. It is possible that vitamin E's "oxygen sparing" effect may be responsible for its effect on reproduction, as it seems to be for its effect in cardiac angina. Considering the anti-fertility effects of small

amounts of exogenous estrogen, it is suggested that this state is responsible for senescent infertility, and it is proposed that luminal oxygen tension may be the crucial factor in both the artificially induced estrogenic sterility and in senescent sterility. Since any irritation promotes estrogen binding by the uterus, the oxygen effect may be even more general. The extraordinarily low oxygen tension recorded in the lumens of both young and senescent hamsters--apparently lower (especially in the older animals) than any mammalian tissue that has been studied except cancer--suggests that a drastic increase of oxygen tension must be achieved around the time of implantation if the embryos are to survive. A few measurements suggested that this is the case, and a similar change has been reported in other animals, but the closure of the lumen at the time of implantation makes measurements at that time very questionable. If the equivalence of TTC reduction to QO_2 can be accepted, then a study of age differences in TTC reduction by uterine tissues at implantation sites throughout pregnancy would be a very desirable step in confirming this mechanism.

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