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ROLE OF MITOCHONDRIA AND HISTONES IN DEVELOPING AND AGING *DROSOPHILA* HYBRIDS

by

Andrew Orlando Martinez

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Andrew Orlando Martinez

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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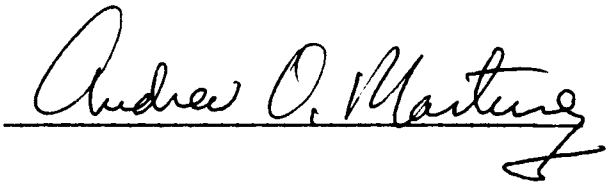
SIGNED: 

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ABSTRACT

Differences and temporal changes of mitochondrial activity and histone composition have been investigated in *Drosophila melanogaster* F_1 reciprocal hybrids and respective inbred parents.

Mitochondria were isolated by differential centrifugation, and mitochondrial activity as judged by oxidative phosphorylation (ADP:O), respiratory control, and State 3 oxidation was measured by oxygen polarography.

Mitochondria isolated from adult flies readily oxidized pyruvate and alpha-glycerophosphate, but not acetate. The addition of exogenous magnesium to the reaction medium resulted in lower efficiency of phosphorylation (ADP:O) with pyruvate and alpha-glycerophosphate. Respiratory control (RC) was also decreased with pyruvate.

Heterosis in terms of longer life spans and heavier body weights was exhibited by one reciprocal cross. One of the crosses also exhibited enhanced mitochondrial activity over the theoretical midparent value (mitochondrial heterosis). This enhanced activity was maintained over several age intervals. *In vitro* mixtures of mitochondria from parents of the heterotic hybrids also exhibited mitochondrial efficiency higher than the midparent (mitochondrial complementation). Nonheterotic hybrids and mitochondrial mixtures of their inbred parents did not show enhanced mitochondrial activity. Aging effects were observed in all three mitochondrial parameters measured. However, hybrids and parents showed similar trends with respect to any particular parameter.

Histones were acid extracted from isolated nuclei of two different growth stages and of adult flies at various age intervals. Five major histone fractions were identified from adult flies. Distinct quantitative differences and a possible qualitative change were found between early third instar larvae and one-day-old adults.

Age-related quantitative changes were detected in both F_1 hybrids and inbred parents. No qualitative differences in histone composition were observed with advanced aging. Differences in proportional changes of specific histone fractions were noted between heterotic hybrids and inbred parents which correlated with heterosis.

The histone composition of a paralytic temperature-sensitive mutant was also investigated. No qualitative changes in histone pattern correlated with the mutation were observed.

INTRODUCTION

Heterosis is a genetic phenomenon commonly observed when two inbred strains are crossed. The concept of heterosis, although formulated a half century ago, is still not well understood either at the genetic, physiological, or biochemical level. Two main hypotheses have been invoked to explain heterosis at the genetic level: (1) deleterious genes are masked in their action by dominant alleles (dominance, Jones, 1952), or (2) the heterozygous condition results in an interaction between alleles to produce an effect greater than the homozygous condition (overdominance, East, 1936).

In *Drosophila melanogaster*, crosses among inbred populations may result in F_1 progeny that are superior to the inbred parental strains in a variety of attributes: viability (Langridge, 1962), fecundity (Gowen, 1952a), growth rate (Bonnier, 1961), body weight (Steele, Young, and Childs, 1969), longevity (Tantawy, 1961), and mitochondrial activity (McDaniel and Grimwood, 1971). Heterosis in terms of prolonged duration of life is of special interest in studies involving the "aging process." Life span is thought to be to a great extent genetically determined (Clark, 1964). The synthesis of hybrid strains differing in life span is important in studying not only mechanisms of aging but also mechanisms of heterosis.

The superiority of hybrids over their inbred parents may be a reflection of differences in genetic activity. Furthermore, the differences observed in life span between hybrids and parents may prove to be

a consequence of such differences in genetic activity. For example, inbred organisms may lose the capacity to synthesize essential proteins sooner than hybrid organisms because of progressive losses in the capacity to synthesize messenger-RNA.

Mitochondria are considered important structures in maintaining intracellular homeostasis (Caspari, 1956). A correlation between superior mitochondrial activity and heterosis has been suggested by McDaniel (1973). This phenomenon has been termed mitochondrial heterosis. In addition, *in vitro* mixtures of parental mitochondria that produce heterotic hybrids have been reported to demonstrate mitochondrial complementation (McDaniel, 1973). If heterosis in *Drosophila* is also a reflection of superior oxidative and phosphorylative activity, differences in mitochondrial activity between F_1 hybrids and their inbred parents should occur. Furthermore, age-related changes in mitochondrial activity may progress at different rates in hybrids and inbreds.

Histones are basic proteins found associated with DNA. These proteins are generally considered to be involved in genetic regulation through general repression of the genome. If heterosis is a reflection of differences in genetic activity between hybrids and inbred parents as suggested, detectable qualitative or quantitative differences between their histone compositions should be expected. Furthermore, differential changes in histone composition during aging could also occur. Theories of aging based on changes in genetic regulation have been proposed (Hahn, 1970). Age-related losses in the ability to synthesize m-RNA and proteins may result from irreversible repression of essential genes. Another way of looking at heterosis is to examine physiological

characteristics of an organism not only at a single arbitrary period of time but throughout the life span of the organism.

Drosophila is a particularly attractive organism for physiological, biochemical, and genetic research because of the extensive genetic information available and the ease of obtaining large numbers of individuals in a relatively short period of time. In spite of these facts, histone composition and mitochondrial activity during their life cycle have not been investigated extensively. Thus, *Drosophila* hybrids and parental inbreds would seem to be ideal systems in attempting to correlate differences and temporal changes in histone composition and mitochondrial activity with the "heterosis concept" and the "aging process."

MITOCHONDRIA

Literature Review

Heterosis

Heterosis, a genetic phenomenon associated with heterozygosity, has been demonstrated in many organisms. Plant and animal hybrids that express positive heterosis are of special interest to man because of the beneficial qualities associated with them.

Heterosis was described as early as 1766 by Koelreuter while working with tobacco hybrids (Shull, 1952). However, the scientific basis for the concept of heterosis was not clearly established until the first half of the 20th century. This concept resulted from the extensive investigations of plant breeders on hybrid maize. A comprehensive history of the development of the heterosis concept prior to 1952 is presented in the book edited by Gowen (1952b). The current status of the concept is discussed by Brewbaker (1964) and Milkman (1967). Discussions of heterosis at the genetic and molecular levels are presented in the books by Fincham (1966) and Manwell and Baker (1970).

Various theories have been proposed to explain the genetic nature of heterosis. The dominance theory proposed by Jones (1917, 1952) attributes heterosis to the action and interaction of dominant alleles. According to this theory, deleterious genes are masked in their action by dominant alleles. The hybrid organism is thought to receive dominant alleles in a greater number of loci than either of the inbred parental

lines and to express superiority because of additive effects. Overdominance (East, 1936; Hull, 1945) explains heterosis in terms of interallelic interactions. This theory has been associated with single gene heterosis and interallelic complementation (Fincham, 1966). The heterozygous condition is thought to result in an interaction between alleles to produce an effect greater than either the recessive or dominant homozygous state. Nonallelic interaction or epistasis, where one genetic locus influences the genetic expressions of other loci, has also been considered as a possible mechanism of heterosis (Brewbaker, 1964).

Several other theories attempt to explain heterosis at the physiological/biochemical and molecular levels. A molecular explanation as a function of temperature for heterosis in *Arabidopsis* and *Drosophila* has been proposed by Langridge (1962). He suggested that heterosis could result from a greater stability of hybrid proteins over a wider range of temperatures.

Manwell and Baker (1970) proposed a hypothesis for heterosis at the physiological and molecular levels on the basis of protein complementation. The classic case cited by these authors was heterocaryon formation in fungi in which two strains, each deficient in a different enzyme needed in a metabolic pathway, combined to restore the pathway (Pittenger, 1956). They suggested that heterosis could be an expression of greater activity of hybrid proteins resulting from complementation. Evidence for hybrid proteins resulting from gene interaction have been reported by Irwin (1971) in birds, Schwartz (1965) in maize, and Johnson and Denniston (1964) in *Drosophila*.

Caspari (1956) suggested that mitochondria might be important structures in maintaining intracellular homeostasis and that increased homeostasis of mitochondria could provide a physiological basis for heterosis. Hybrid vigor in plants has been correlated to mitochondrial respiration and energy production (Hanson, Hageman, and Fisher, 1960; Duvick, 1965; McDaniel and Sarkissian, 1966).

McDaniel and Sarkissian (1966) reported that mitochondria from hybrid maize exhibited increased oxidative phosphorylation, respiratory control, and oxidation rates, and enhanced enzymatic activity. This was termed mitochondrial heterosis and was directly related to polymorphic properties of mitochondria (Sarkissian and McDaniel, 1967). Furthermore, McDaniel and Sarkissian (1966, 1968) and McDaniel (1967) reported that 1:1 *in vitro* mixtures of mitochondria isolated from two inbred lines of maize also exhibited enhanced efficiency of energy conservation, as determined by ADP:O ratios, over the theoretical midparent as well as the highest parent. However, the enhanced efficiency did not surpass that of the F_1 hybrids. This phenomenon has been termed mitochondrial complementation and assumes that mitochondria are metabolically different and that some type of interaction, perhaps particle-to-particle contact, between mitochondria is necessary (McDaniel and Sarkissian, 1970). More recently, mitochondrial heterosis and complementation have been reported in barley (McDaniel, 1971, 1972a) and wheat (Sarkissian and Srivastava, 1969; Hobson, 1971).

Mitochondrial heterosis and complementation has also been reported in animal systems (McDaniel and Grimwood, 1971; Grimwood; 1972). These investigators demonstrated mitochondrial heterosis and

complementation in *Drosophila* in terms of enhanced efficiency of oxidative phosphorylation and higher respiratory rates.

The Aging Process in *Eucaryotes*

Insects, like all other multicellular organisms, undergo deteriorative biological changes with the passage of time. These changes can be observed from the level of the whole organism to tissues, cells, and subcellular organelles and, finally, to molecules themselves. Such changes are progressive and irreversible, and result in decreased homeostasis and eventual death of the organism (Strehler, 1962). Time correlated variations that limit life span are characteristic of the "aging process." In recent years a vast amount of literature on aging has accumulated (Andrew, 1964; Blumenthal and Berns, 1964; Walford, 1967; Bender, Kormendy, and Powell, 1970; Adelman, 1972; Gusseck, 1972; and the symposium edited by Woolhouse, 1967). Discussions specific to aging in insects include those by Maynard Smith (1966), Rockstein (1966), Lints (1971), and the recent book edited by Rockstein and Baker (1972). A historical background of early research in aging may be found in books by Comfort (1956) and Strehler (1962).

Major theories that attempt to explain the aging process in insects, as well as higher organisms, have been classified into either of two categories: (1) those theories that view aging as being genetically predetermined or (2) those that consider aging to result from accidental cellular events (Lints, 1971; Strehler, 1972).

Supporters of the first theories attribute aging to a predetermined genetic program that results in a gradual but progressive

deterioration of the organism. Bakerman (1969) has suggested that life might be genetically programmed for development, differentiation, maturation, and ultimately deterioration. Evidence of a genetic basis for the aging process comes from studies that indicate that each species has its own characteristic life span (Clark, 1964).

Origins and evolutionary manifestations of aging are discussed by Comfort (1956), Williams (1957), and Strehler (1962). Strehler (1972) has suggested that life span resulted from selection pressures acting during the evolution of the organism. Medvedev (1964, 1967) postulated that the loss or reduction of genetic information and synthetic capacity which decrease homeostasis and lead to aging is a process of differentiation. He suggested that life-limitation might itself be an evolved adaptation, which has provided the individual organism with a self-destruction mechanism.

The theories in the latter category are collectively classified as random or stochastic processes that act at the cellular or molecular levels and result in a gradual and progressive accumulation of irreparable damage (Sacher, 1956). Random changes associated with age have been reported to occur in many cellular systems (Kormendy and Bender, 1971). Comfort (1956) suggested that loss of information caused by random accumulation of mutations in the genetic material could result in a progressive impairment of cellular function. The somatic mutation theory (reviewed by Welch, 1967; Curtis, 1971) has been proposed as a primary source of errors and, therefore, a primary cause of aging. This theory suggests that a gradual accumulation of random mutations in DNA impairs an increasing number of somatic cells through faulty RNA and protein

synthesis and leads to eventual loss of function and, ultimately, death.

More recently, Harman (1972) has attributed the rate of cellular aging to free radical damage in mitochondria. He suggested that the life span of cells might be largely an expression of genetic control over the rate of respiration. Cells that have a higher rate of oxygen consumption accumulate more mitochondrial damage and have a shorter life span than cells with a lower rate of respiration. Damage may occur in the cytochrome system or respiratory chain, interfering with electron transport and adenosine triphosphate (ATP) synthesis.

It is very unlikely that the "aging process" can be explained by a single theory. One unifying concept is that aging is a very complex syndrome involving many different processes at all levels of organization and proceeding simultaneously. Which one will be central to the detriment of the organism depends finally upon its genetic constitution (Curtis, 1971).

Mitochondria

Mitochondria are organelles found in the cytoplasm of aerobic eucaryotic cells. Energy for chemical, osmotic, and mechanical work performed by cells is provided through the process of respiration, the enzymatic oxidation of nutrient molecules by molecular oxygen. The enzyme systems concerned with respiration are located in mitochondria.

These organelles received the attention of Köliker and other cytologists in the latter half of the 19th century. They were particularly prominent in muscle tissue and were named "sarcosomes." Benda, in 1897,

was the first investigator to apply the term mitochondria to these cellular structures, but he thought they were part of the hereditary mechanism of the cell (see Lehninger, 1965).

The first study to give some insight as to the probable function of mitochondria was reported by Michaelis in 1900. He demonstrated that these particles could be stained specifically *in vivo* by the oxidation/reduction indicator Janus green B. Several years later Kingsbury suggested that mitochondria were sites of cellular oxidations. Warburg in 1913 supported this suggestion by reporting that oxygen uptake of liver extracts was associated with large cytoplasmic structures (see Lehninger, 1965).

The isolation of mitochondria by differential centrifugation allowed investigators to carry out physiological and biochemical experiments that eventually led to the elucidation of the functional role of mitochondria. Keilin and Hartree (1938) and Green, Loomis, and Auerbach (1948) isolated the reactions involved in substrate oxidation. Hogeboom, Schneider, and Palade (1948) reported the association of respiratory activity specifically with mitochondria. Lardy and Wellman (1952) reported that oxygen utilization in mitochondria was dependent on adenosine diphosphate (ADP) and inorganic phosphate. Chance and Williams (1955) made use of this observation to study respiration of isolated mitochondria using the technique of oxygen polarography. The term "respiratory control" and the various metabolic states of mitochondrial respiration were defined. This technique has been fully developed and is widely used in studies with isolated mitochondria. Hagihara (1961) reported that ADP:O ratios derived from polarographic methods were numerically

equivalent to P:O ratios determined by manometric techniques. Reviews of recent developments in the field of mitochondrial research may be found in books by Racker (1965), Lehninger (1970, 1971), Wainio (1970), and Green and Baum (1970) and in recent symposia edited by Slater et al. (1968), Ernster and Drahota (1969), Boardman, Linnane, and Smillie (1971), and Mehlman and Hanson (1972).

Energy Transformation and Conservation

The mechanism by which the mitochondrion transforms and conserves the energy released by electron transport is not completely understood. Three main hypotheses have been postulated to explain these processes.

The chemical hypothesis proposed by Slater in 1953 (reviewed by Pullman and Schatz, 1967) is based on the concept of energy transfers involving chemical intermediates possessing high energy bonds. These "high-energy" bonds become precursors of the "high-energy" phosphate bond of adenosine triphosphate (ATP). Two weaknesses in this hypothesis have been pointed out by Lehninger (1970): (1) the "high-energy" intermediate has not yet been isolated or detected, and (2) oxidative phosphorylation does not occur in preparations with dissociated membranes as would be expected.

The chemiosmotic hypothesis initially introduced by Mitchell in 1961 (reviewed by Pullman and Schatz, 1967; Greville, 1969) assumes that the mitochondrial membrane is essential in oxidative phosphorylation. The membrane is thought to be permeable to H^+ ions generated by electron transport. The decrease in free energy that takes place is postulated to cause the formation of ATP from ADP and inorganic phosphate.

The conformational hypothesis proposed by Boyer in 1965 (reviewed by Green and Harris, 1969) has arisen from investigations on isolated mitochondria which indicate that they undergo structural and volumetric changes in the presence of certain factors and contraction in the presence of ATP. These changes are thought to be dependent on electron transport. This view has been supported by studies that show that inhibitors of electron transport also inhibit volume changes (Lehninger, 1960). Electron transport energy is thought to be directly converted into an energy-rich conformational state of the inner membrane, a state postulated to be the immediate driving force for ATP formation (Lehninger, 1970).

Other hypotheses are discussed in the reviews by Lardy and Ferguson (1969), Slater (1969, 1971), and Van Dam and Meyer (1971). In spite of all the experimentation in this field of bioenergetics, a mechanism to adequately account for oxidative phosphorylation has not been unambiguously described. Results that seem to be in agreement with one hypothesis may nevertheless also be compatible with the others.

Morphological, Biochemical, and Bioenergetic Aspects of Insect Mitochondria During Development, Maturation, and Aging

Insects derive most of the energy needed for biological work through the process of respiration and are appropriate organisms for studying structural, physiological/biochemical, and energetic properties of mitochondria at various stages in their life cycle. Flight muscles, which contain numerous mitochondria that supply the immense energy needed for sustained flight in adult insects, have been of special interest.

Morphological development of mitochondria has been studied in many different species (Herold, 1965; Gregory, Lennie, and Birt, 1967; Larsen, 1970). Tanguay and Chaudhary (1970) reported few small mitochondria lacking internal organization, in the African desert locust at an early stage of flight muscle development. At eclosion an increase in number, size, volume, and internal complexity was noted. Lennie, Gregory, and Birt (1967) and Lennie and Birt (1967) conducted similar studies with blowfly flight muscle mitochondria and reported gradual changes in mitochondrial morphology at different levels of development. The most obvious change was the number of cristae per mitochondrion. Pupa flight muscle tissue contained mitochondria with areas devoid of cristae. However, at 24 hours after adult emergence, the mitochondria were uniformly packed with cristae.

Herold and Borei (1963) studied biochemical changes in the cytochromes of honeybee flight muscle mitochondria during development and observed both qualitative and quantitative changes. Cytochromes b_5 and $a+a_3$ were the only ones detected during most of the pupal stage. In late pupa and early adult flight muscle, a progressive increase in cytochromes $a+a_3$ and the appearance of cytochromes b+c and c were noted. They suggested that the adult cytochrome system might be associated with increased energy demands in the flight muscle after emergence. Variations in amounts and distributions of respiratory enzymes have also been reported for blowfly flight muscle (Lennie and Birt, 1967). Cytochrome c oxidase and the dehydrogenases were observed to initially appear in small particles in pupae. During adult development these enzymes were redistributed and the activity was present in larger particles. These

investigators also studied the distribution and content of nucleic acids in mitochondrial preparations during early adult development. Mitochondrial deoxyribonucleic acid (DNA) increased sharply one day before adult emergence and then decreased after emergence. Mitochondrial ribonucleic acid (RNA) showed a similar pattern but the increase was not as great and its decline was more rapid. Walker and Birt (1969) suggested that development of flight muscle mitochondria might involve the asynchronous incorporation of enzymic and structural proteins, with the latter being incorporated most rapidly over the period of eclosion. This development might be preceded by an increase in nucleic acid required for the synthesis of "structural" protein. Furthermore, a considerable development of intramitochondrial membranes was expected (Lennie, Gregory, and Birt, 1967). This was supported by electron microscopy studies that showed an increase in internal organization of mitochondria during the same period (Lennie and Birt, 1967).

Very little is known about the energetics of flight muscle mitochondria during development. The question arises whether the small mitochondria that lack internal organization are functional in oxidative phosphorylation.

Sacktor (1965) reported that, in adult Diptera, carbohydrate served as the major substrate during flight. Crompton and Birt (1967) investigated variations in carbohydrate levels during metamorphosis of the blowfly and observed a decrease in glucose during the larval-pupal transition. The level of glucose increased again after adult emergence. They suggested that the major source of energy during pupation might be oxidation of total amino acids and fatty acids. D'Costa and Birt (1966)

reported that, during the time of greatest biosynthetic activity in the thoracic muscles of developing blowflies, the only appreciable loss of nutrient reserves was in the fatty acids. Gregory, Lennie, and Birt (1967) discussed the possibility that the energy required for development might be provided by the oxidation of fatty acids and that the activity of flight in mature insects could be energized only by the oxidation of carbohydrates, which accumulated after emergence.

Walker and Birt (1969) studied the redox and phosphorylation capabilities of mitochondrial fractions at several stages of development in the blowfly. They demonstrated that the phosphorylation capacity of the mitochondria developed with the oxidase activity and that the respiratory chain was extremely sensitive to ADP before emergence. However, a marked decrease in respiratory control was observed after emergence although the mitochondria were still coupled. Both pyruvate and alpha-glycerophosphate were oxidized, but the oxidases for the two substrates did not develop synchronously.

Balboni (1967) studied the respiratory metabolism of honeybee flight muscle during adult maturation and reported distinct patterns in the development of energetic capabilities of the mitochondria. The alpha-glycerophosphate oxidase system exhibited considerable activity after emergence, but the pyruvate system was not fully functional until late in adult maturation. He suggested that the alpha-glycerophosphate system might be responsible for energy production during early adult life, which is characterized by limited flight activity, whereas sustained flight, exhibited by mature adults, could be possible only when the pyruvate metabolizing system was fully functional. Childress and

Sacktor (1966) utilized the technique of oxygen polarography to determine rates of oxidation of various Krebs cycle substrates and reported that mitochondria are not readily permeable to all substrates. Selective permeability and rapid oxidation of pyruvate + proline and alpha-glycerophosphate was exhibited by isolated blowfly flight muscle mitochondria. It was suggested that these substrates might readily penetrate the membrane and be the normal substrates for flight muscle mitochondria in adult insects.

They also reported a correlation between the decrease in rate of pyruvate oxidation and increase in mitochondrial swelling and suggested a functional relationship between the two. A rapid rate of pyruvate oxidation has also been reported for *Drosophila* mitochondria (Tupper and Tedeschi, 1969; McDaniel and Grimwood, 1971).

The theory that a decrease in respiration and associated production of ATP may account for decreased homeostasis exhibited by old organisms has been tested by several investigators (Weinbach and Garbus, 1959; Gold, Gee, and Strehler, 1968; Angelova-Gatava, 1969). These studies have yielded conflicting and inconsistent data. Chen, Warshaw, and Sanadi (1972) investigated the respiratory activity of heart and skeletal muscle mitochondria from aging rats and reported that neither the efficiency of energy conservation as determined by ADP:O ratios nor controlled respiratory rate with limiting ADP (State 4) changed with age. However, a significant change in active respiration when ADP phosphorylation is not limiting (State 3) was observed.

The ability of insects to fly declines markedly with age (Rockstein, 1966). The mechanism for this decline is not known. A decreased

capacity to meet energy demands of bioenergetic processes may be one factor. A change in the bioenergetic properties of mitochondria with age may be reflected in the rates of oxidation or respiratory control or in the efficiency with which substrates are oxidized.

Rockstein and Bhatnagar (1965) studied changes in mitochondrial morphology in relation to age in the common housefly. Age-dependent and sex-dependent changes in number and size of flight muscle mitochondria were noted. These changes were preceded by wing loss and decline of alpha-glycerophosphate dehydrogenase activity in male flies. In female flies, such changes occurred with decreased physical and biochemical functions of flight.

Tribe (1967) conducted a study of mitochondrial respiratory efficiency in relation to age in the blowfly and reported significant declines in P:O ratios, respiratory control, and ATP-ase activity. He suggested that these reductions might indicate changes in the degree of coupled phosphorylation with advanced age. Similar age-correlated changes in ATP-ase activity have been reported for honeybee brain tissue (Cheng and Cutkomp, 1972) and housefly flight muscle (Rockstein and Gutfreund, 1961; Clark and Rockstein, 1964).

Recently, Bulos, Shukla, and Sacktor (1972) investigated structural and bioenergetic properties of flight muscle mitochondria from aging blowflies. They reported ultrastructural changes in intramitochondrial membranes with aging. Significant decrease in respiratory control and maximal respiratory capacity (State 3) with pyruvate+proline and alpha-glycerophosphate were also observed. However, State 4 rates of oxidation and phosphorylation efficiency (ADP:O ratios) did not change

significantly. They suggested that the decrease in maximal respiratory capacity might be associated with alterations in electron transport.

Materials and Methods

Experimental Material

Four inbred wild-type strains and one temperature-sensitive mutant strain of *Drosophila melanogaster* [Oregon (Ore-RC), Swedish (Swe-C), Lausanne (Lau), Urbana (Urb), and paralytic temperature-sensitive mutant ($Para^{ts}$)[1]] were maintained in one-pint Boston round culture bottles on Difco Bacto *Drosophila* medium M and incubated under normal laboratory conditions between 22° and 24°C. Adults were removed from the culture bottles when third instar larvae were observed. Newly emerged imagoes from the wild strains were collected at 8-hour intervals, and virgin females were separated from males. The females were aged for 48 hours before being used to make appropriate reciprocal crosses. A total of 15 flies in a ratio of two females to one male was placed in each culture bottle. Males used in the crosses were of the same age as the females.

In order to obtain adult flies of relatively uniform age, egg deposition was allowed for 4 hours; then the parents were transferred to fresh culture bottles and the procedure was repeated. The parents were discarded after four such transfers. Parental stocks used as controls and the mutant strain were prepared in a similar manner.

The F_1 progeny, controls, and mutants were collected at 24-hour intervals and either utilized in experiments that required one-day-old

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1. The author is very grateful to Dr. D. T. Suzuki of the University of British Columbia for providing the $Para^{ts}$ mutant stock.

imagoes or were transferred to aging cages and aged for the desired period. Samples for experimentation were taken from the aging cages at 7-day intervals and utilized immediately.

Isolation of Mitochondria

Mitochondria were isolated from adult flies of appropriate age intervals by modification of the methodology described by McDaniel and Grimwood (1971) and Childress and Sacktor (1966). The procedure is outlined in Fig. 1.

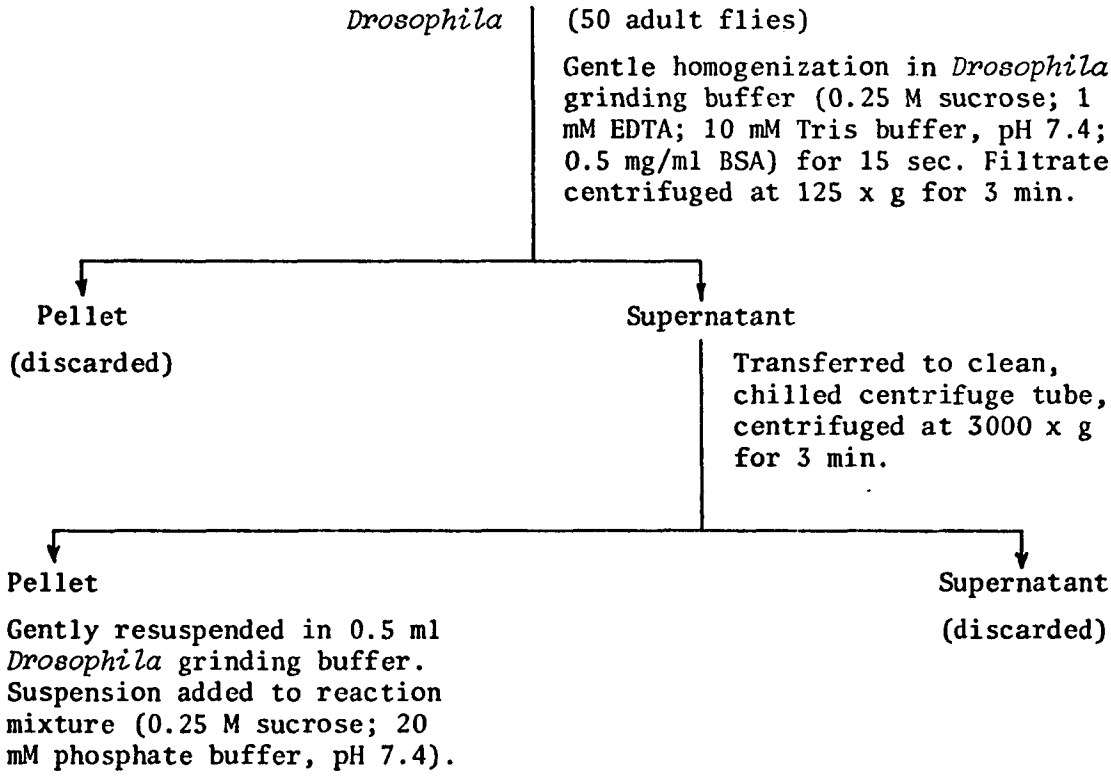

Fig. 1. Flow Sheet for the Extraction and Isolation of Whole-Fly Mitochondria.

Samples of flies were taken from aging cages or culture bottles, immobilized by chilling on ice, and subsequently placed in a cold petri plate for counting. Fifty flies (mixed sex) were transferred to a chilled mortar containing 2 ml of *Drosophila* grinding buffer (0.25 M sucrose; 1.0 mM ethylenediamine-tetracetate, EDTA; 10.0 mM hydroxymethyl-amino methane, Tris buffer, pH 7.4; and 0.5 mg/ml BSA, Bovine Serum Albumin) for immediate isolation of mitochondria. All isolation procedures were conducted on ice between 0^{o} and $5^{o}C$. The flies were ground very gently with the pestle for 15 sec. The pestle was then rinsed with 5 ml grinding buffer and the homogenate was filtered through one layer of monofilament nylon cloth (approximately 4,600 apertures/cm^2) into 50-ml nylon centrifuge tubes. The mortar and nylon cloth were then rinsed with an additional 3 ml of grinding buffer, and the filtrate was centrifuged in a Sorvall refrigerated RC-2B centrifuge at 125 x g for 3 min. The supernatant was transferred into a clean chilled centrifuge tube and recentrifuged for an additional 3 min at 3000 x g. The final pellet containing the mitochondrial fraction was gently resuspended in 0.5 ml grinding buffer and poured into the reaction vial containing 1.0 ml of *Drosophila* reaction buffer (0.25 M sucrose and 20 mM phosphate buffer, pH 7.4). The suspension was subsequently stirred and allowed to equilibrate to the temperature of the water bath (Haake, adjusted to $27^{o}C$) for 3 min. The five metabolic states of mitochondrial respiration were defined as follows: (1) State 1, mitochondria suspended in reaction medium; (2) State 2, substrate added; (3) State 3, adenosine diphosphate added; (4) State 4, adenosine diphosphate concentration limiting; (5) State 5, oxygen concentration limiting. Each mitochondrial preparation contained

0.8 to 1.5 mg protein as determined by Chaykin's (1966) modification of Lowry's protein assay.

Measurement of Oxidative Phosphorylation

Oxygen uptake of the mitochondrial preparation was monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Company) and recorded on a Sargent-Welch model DSRLG 100 mV linear recorder. When State 1 had been established, 5 µl pyruvate (5 mM, pH 7.4) was added to the reaction vial with a 25 µl Hamilton syringe through an access slot on the probe. Subsequent additions of adenosine-5'-diphosphate (ADP, Grade I, Sigma, 150 µM, pH 7.4) were added to the reaction vial with a 10 µl Hamilton syringe after establishing State 2 and State 4 respiration rates until all oxygen had been utilized in State 5.

ADP:O and respiratory control ratios were determined as described by Chance and Williams (1955). ADP:O ratios were calculated as the ratio of µM ADP added to µM O_2 uptake per mitochondrial reaction from the point of ADP addition to initiation of the subsequent State 4. Respiratory control ratios were calculated as the ratio of State 3 oxidation rates to State 4 oxidation rates. Oxygen uptake was calculated from State 3 oxidation rates and adjusted to a per-milligram mitochondrial protein basis.

One-to-one mixtures of mitochondria from inbred parental lines were used to study mitochondrial complementation. Complementation was calculated as percentage of a theoretical midparent.

Determination of pH Optimum

The pH optimum for isolated *Drosophila* mitochondria was determined by adjusting all solutions used in isolation and oxygen polarographic techniques at five different pH's in the range 6.8 to 8.0. The effect of pH on ADP:O and respiratory control ratios (RC) is presented in Figs. 2 and 3. The pH corresponding to the highest ratios (pH 7.4) was used in all subsequent experiments.

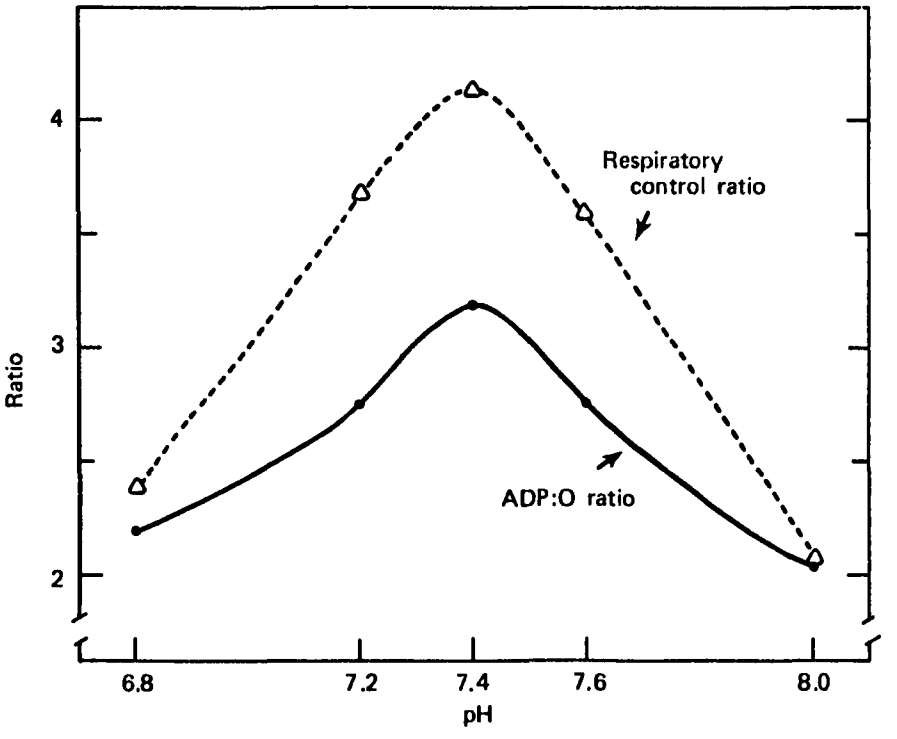

Fig. 2. Effect of pH on Oxidative Phosphorylation (ADP:O) and Respiratory Control of *D. melanogaster* Mitochondria.

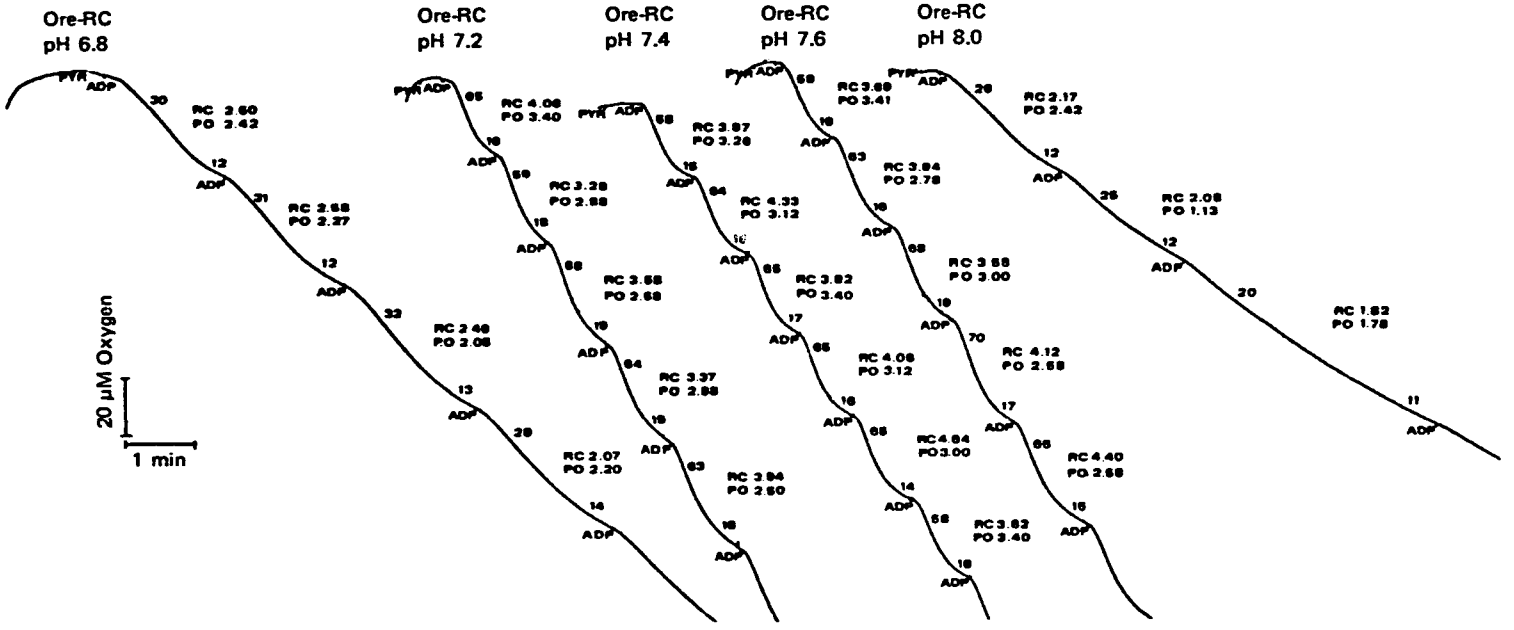

Fig. 3. Polarographic Traces of Oxygen Uptake by Isolated *Drosophila* (Ore-RC) Mitochondria Showing the Effect of pH on Oxidative Phosphorylation (ADP:O) and Respiratory Control (RC).

pH: 6.8, 7.2, 7.4, 7.6, 8.0, respectively. Concentrations: substrate, pyruvate, 5 mM; oxygen, 240 µM; ADP, 150 µM addition.

Results and Discussion

Measurements comparing two quantitative traits in *D. melanogaster* inbred parental strains and F_1 reciprocal hybrids are presented in Table 1. The reciprocal crosses SxO and OxS demonstrate heterosis in terms of both longer life spans and heavier body weights. However, the reciprocal hybrids LxO and OxL demonstrate heterosis in neither trait. Hybrids show less statistical variability than the inbred controls. There was no substantial difference in mortality rates among heterotic hybrids, inbred lines, and nonheterotic hybrids, but the onset of death was delayed in heterotic hybrids.

Table 1. Life Spans and Body Weights for Inbred Parental Strains and Reciprocal Hybrids.

Genotype	Mean life span (days) ± s.e.[a]	Mean body weight, 7-day-old adults (mg/100) ± s.e.[b]
Ore	49.27 ± 1.84	79.25 ± 3.43
Swe	50.76 ± 1.73	77.67 ± 3.89
Lau	49.12 ± 1.68	82.48 ± 3.64
SxO	56.69 ± 1.46	85.76 ± 3.20
OxS	55.38 ± 1.35	84.98 ± 3.14
LxO	50.09 ± 1.19	80.54 ± 2.82
OxL	50.44 ± 1.08	81.13 ± 3.26

[a]Each sample contains 200 individuals.
[b]Each sample contains 500 individuals.

It is generally accepted that life span is, to a great extent, genetically determined. The existence of species and strains differing in life span lends support to this hypothesis. How genes are acting to cause these differences is not known. Life span in itself should be

looked upon only as a standard of reference and becomes meaningful only when such strains are compared with respect to physiological differences and progressive changes.

Mitochondria are considered important structures in maintaining intracellular homeostasis and may play an important role in determining heterosis. Furthermore, age-related changes in respiration and efficiency of energy conservation may contribute to decreased cellular homeostasis associated with aging. Isolated mitochondria provide a convenient tool for investigating possible differences and changes in metabolic activities. For these reasons, the studies reported here are designed to evaluate the importance of mitochondrial metabolism in the heterotic expression and in the "aging process" of hybrid and inbred lines. Inquiries into the efficiency of oxidative phosphorylation of mitochondria among these lines were conducted.

Substrate Oxidation by Whole-Fly Mitochondria

Oxidation of several substrates by *Drosophila* mitochondria was examined. These mitochondria oxidize pyruvate and alpha-glycerophosphate quite readily, as shown in Fig. 4. Acetate is oxidized more slowly with little or no coupling. Addition of pyruvate to these mitochondria increases the respiration rates, indicating that they are capable of oxidative phosphorylation. ADP:O and respiratory control ratios were considerably higher with pyruvate than with alpha-glycerophosphate.

Rapid oxida ıon of pyruvate has also been reported for blowfly mitochondria (Childress and Sacktor, 1966; Van den Bergh and Slater, 1962). These findings suggest that pyruvate may be a main energy source

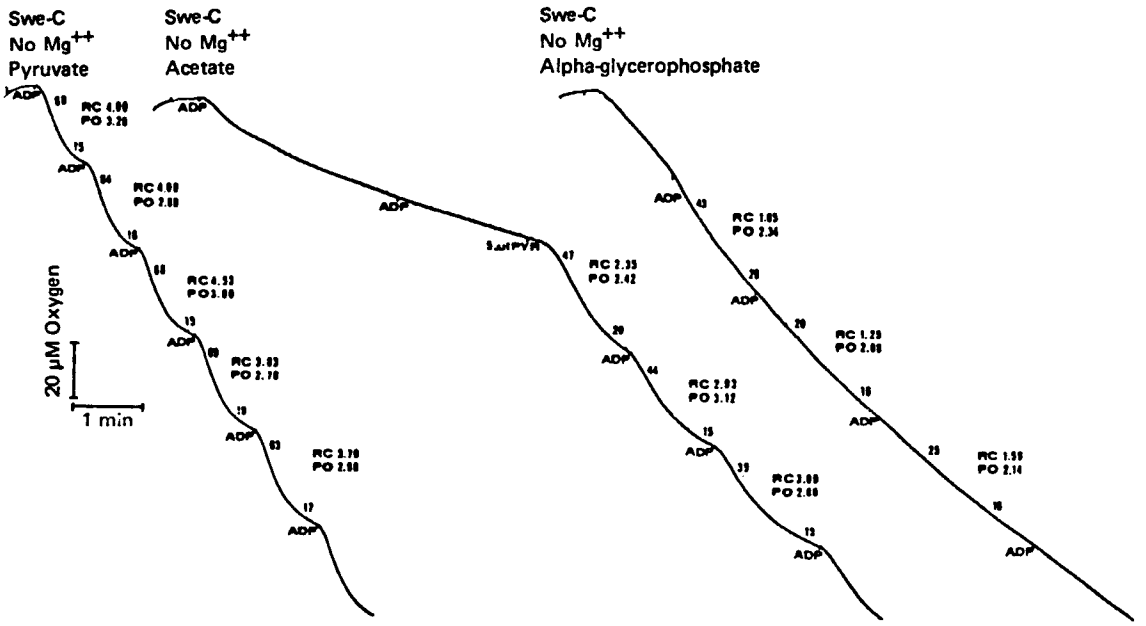

Fig. 4. Polarographic Traces of Oxygen Uptake by Isolated *Drosophila* (Swe-C) Mitochondria with Three Different Substrates.

Substrate concentrations: pyruvate, 5 mM; acetate, 5 mM; alpha-glycerophosphate, 5 mM. Pyruvate added to second trace to restore coupling was 5 mM. Concentrations: oxygen, 240 µM; ADP, 150 µM/addition.

in adult flies and that it penetrates the mitochondrial membrane quite readily.

The utilization of pyruvate is thought to involve three phosphorylations by means of the electron transport system, since electrons enter the chain at the DPN level. The theoretical ADP:O ratio with pyruvate is 3.00. As is evidenced from the data in Table A-1 in the Appendix, ADP:O ratios substantially greater than 3.00 were not uncommon. Data from oxygen polarograph traces (Fig. 4) show that the first respiratory cycle generally gives higher ADP:O values than subsequent cycles. Analysis of variance (Table A-2 in Appendix) showed that cycles were significantly different from each other with respect to all three parameters measured (ADP:O, respiratory control (RC), and State 3 respiration rate). There is a gradual decrease in ADP:O ratios with subsequent cycles.

A number of investigators have reported the oxidation of endogenous substrates by isolated mitochondria (Chance, 1961). Metabolic studies with isolated mitochondria are probably influenced by the presence of these endogenous substrates. This could possibly account for the over-all higher ADP:O ratios with pyruvate. Furthermore, the decline during subsequent cycles could be attributable to the depletion of endogenous substrates, in which case only pyruvate is being oxidized and ADP:O ratios are closer to the theoretical ratio. Von Korff (1965) has reported that, when pyruvate is used as the substrate in oxygen polarographic experiments, endogenous substrates supply a source of dicarboxylic acid to maintain a complete Krebs cycle at a maximum rate. Furthermore, he suggests that endogenous substrates contribute to oxygen

consumption. Another possibility may be that, in *Drosophila*, substrate level phosphorylation associated with glycolysis is active and may also be utilizing ADP. The precise nature of electron transport and coupled phosphorylation is not completely understood. The high ADP:O ratios could also be explained at this level. It is possible that multiple electron transport chains are present or that there are more than three phosphorylating sites. Still another possibility is that one electron is transported at some or all sites rather than two as is generally thought.

Magnesium ions notably increased State 3 respiratory rates and decreased ADP:O ratios with both pyruvate and alpha-glycerophosphate, as is indicated by oxygen polarograph traces shown in Figs. 5a and b. A decrease in respiratory control with pyruvate is also evident but not with alpha-glycerophosphate, which actually shows an increase. Packer (1957) found that addition of Mg^{2+} to rat heart mitochondria increased oxidation rates and ATP-ase activity and decreased respiratory control. The changes observed for *Drosophila* mitochondria could be attributable to enhanced steady-state levels of ADP resulting from increased Mg^{2+}-ATP-ase activity, as suggested by Tarjan and Von Korff (1967) for rabbit heart mitochondria. Because of these adverse effects on ADP:O and respiratory control ratios with pyruvate, Mg^{2+} was not utilized in subsequent studies.

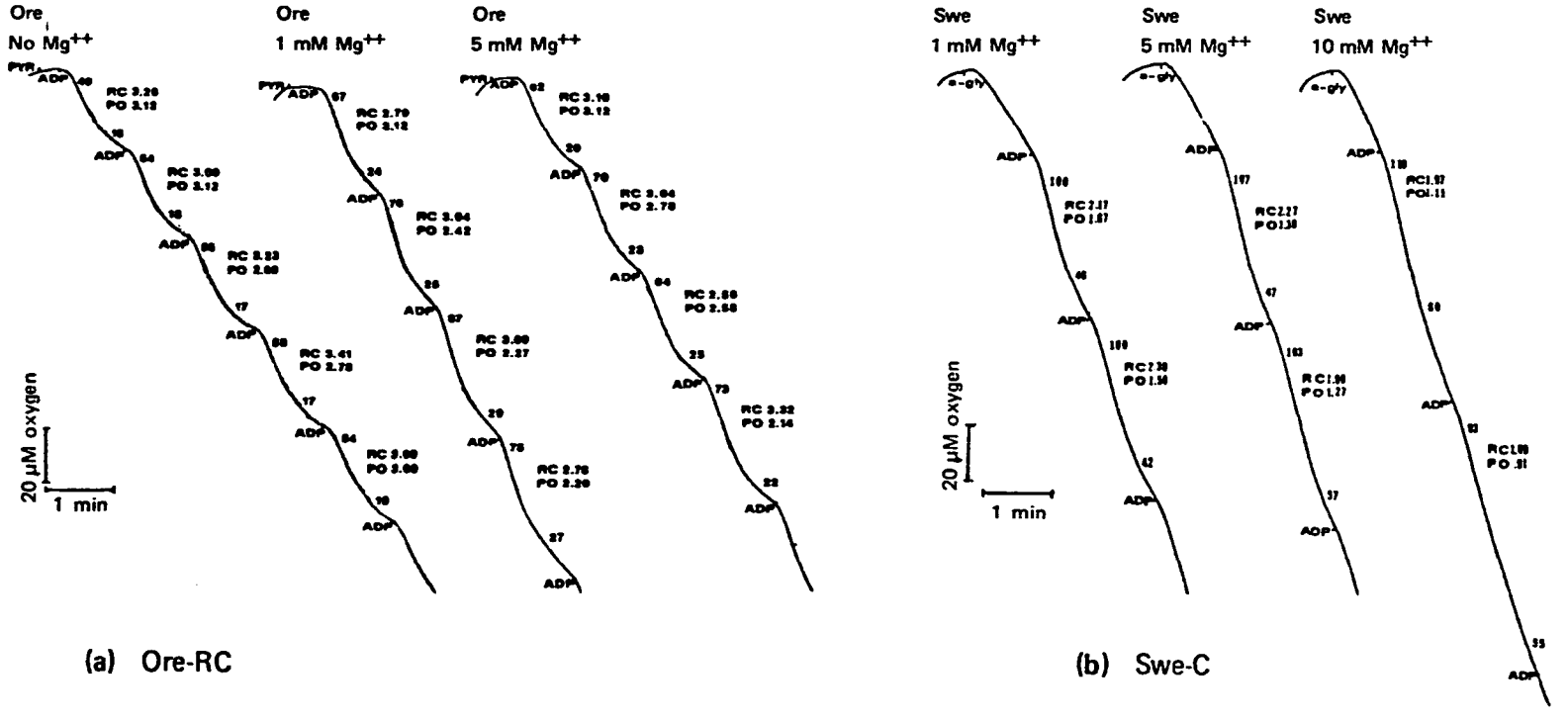

Fig. 5. Polarographic Traces of Oxygen Uptake by Isolated *Drosophila* Mitochondria Showing the Effect of Exogenous Mg^{2+} ($MgCl_2$) on Oxidative Phosphorylation (ADP:O) and Respiratory Control (RC).

a. Ore-RC. Mg^{2+} concentration added to traces 2 and 3: 1 mM, 5 mM, respectively. Concentrations: substrate, pyruvate, 5 mM; oxygen, 240 µM; ADP, 150 µM/addition.

b. Swe-C. Mg^{2+} concentration added: 1 mM, 5 mM, 10 mM, respectively. Concentrations: substrate, alpha-glycerophosphate, 5 mM; oxygen, 240 µM; ADP, 150 µM/addition.

Comparative Studies of Respiratory Activity of Isolated Mitochondria from Inbred Parental Controls, F_1 Reciprocal Hybrids, and Parental Mitochondrial Mixtures at Various Stages of Their Life Cycle

Oxidative phosphorylation was studied using pyruvate as the substrate. The efficiency of phosphorylation was estimated and compared between F_1 hybrids, inbred controls, and parental mixtures.

An initial screening experiment of the four inbred lines and all possible reciprocal hybrids was conducted in order to select appropriate F_1 hybrids and controls for subsequent investigations. These data are presented in Table A-1 in the Appendix. Enhanced efficiency of oxidative phosphorylation of mitochondria isolated from hybrid flies, as reflected by higher ADP:O ratios, over the inbred parental lines was used as the criterion for making selections. The heterotic hybrid, SxO (Swe-C x Ore-RC) exhibited a consistent advantage over its two inbred parents, and furthermore this advantage was maintained through several age intervals as is indicated by these data. This functional superiority of mitochondria from heterotic hybrids, reflected as higher efficiency of oxidative phosphorylation, over mitochondria from inbred parents has been termed mitochondrial heterosis (McDaniel, 1971). The heterotic reciprocal crosses, SxO and OxS, and the nonheterotic reciprocal crosses, LxO and OxL, were selected for further study.

Table 2 lists the mean values for three mitochondrial respiratory parameters (ADP:O, respiratory control, and State 3 respiration) at different age intervals. The respective inbred parents of the hybrids do not differ significantly from each other with respect to these parameters; thus comparisons of hybrids and 1:1 mitochondrial mixtures with

Table 2. Mean Values for ADP:O Ratios, Respiratory Control (RC) Ratios, and State 3 Respiration Rates from Polarograph Traces of *D. melanogaster* Mitochondria from Inbred Parental Controls, Reciprocal F_1 Hybrids, and *In Vitro* Parental Mitochondrial Mixtures at Various Age Intervals.

Each sample contains three replications of five observations. Respiration rates in O_2 uptake/min/mg mitochondrial protein.

	Age in days					
Genotype	1	7	14	21	28	35
			ADP:O ratios			
Ore	3.16	2.84	2.78	2.94	3.10	3.24
Swe	3.20	2.82	2.80	2.93	3.32	3.21
Lau	3.40	2.87	2.87	3.04	3.34	3.14
SxO	3.56	3.08	3.18	3.17	3.22	3.13
OxS	3.30	2.83	2.92	3.06	3.29	3.19
O+S	3.27	3.03	2.98	3.23	3.21	3.29
LxO	3.36	2.70	2.85	3.00	3.01	2.86
OxL	3.19	2.87	2.82	2.94	2.87	2.75
O+L	2.96	2.88	2.80	3.02	3.04	3.10
			RC ratios			
Ore	3.43	3.96	3.85	4.12	3.59	2.48
Swe	2.97	4.17	4.13	3.76	3.68	2.65
Lau	2.70	4.26	3.99	3.22	3.77	2.57
SxO	3.09	3.79	3.96	3.55	3.67	2.44
OxS	3.16	3.82	3.49	3.41	3.53	2.47
O+S	3.32	3.70	3.81	3.92	3.49	2.89
LxO	3.31	4.54	3.80	3.92	3.06	2.19
OxL	3.57	4.04	4.07	3.77	3.68	2.31
O+L	3.69	3.31	3.23	3.50	3.18	2.65
			State 3 respiration rates			
Ore	50.00	76.93	87.45	86.53	71.60	58.98
Swe	48.00	77.78	88.18	84.02	71.70	67.34
Lau	49.43	75.80	86.88	85.29	76.62	66.04
SxO	52.31	78.22	89.93	89.92	69.31	52.65
OxS	50.52	75.35	90.56	86.80	70.80	56.55
O+S	46.77	75.07	88.56	86.27	70.25	55.45
LxO	49.39	72.60	90.07	88.65	72.53	65.53
OxL	50.56	72.17	91.26	85.26	74.41	66.34
O+L	48.57	73.50	87.99	82.20	72.18	51.71

theoretical midparent averages gives an accurate measure of mitochondrial heterosis and complementation. The data obtained from oxygen polarograph traces were evaluated by analysis of variance (Table A-2 in Appendix). Energy conservation, respiratory control, and State 3 respiration rates of theoretical midparents, appropriate reciprocal hybrids, and 1:1 mitochondrial mixtures were compared by using an F-test on orthogonal contrasts of means as shown in Tables 3, 4, and 5.

Efficiency of oxidative phosphorylation (ADP:O) of hybrid SxO is greater than the midparent average up to day 21 (Table 3). Figures 6a and b show polarographic traces of mitochondrial respiratory activity of inbred parents, reciprocal hybrids, and *in vitro* parental mixtures at two different age intervals. These traces illustrate the enhanced oxidative phosphorylation efficiency typical of the heterotic hybrid, SxO. An apparent loss of mitochondrial heterosis occurs after 21 days. This loss does not appear to be due to a decrease in the mitochondrial efficiency of the hybrid, which remains quite stable throughout all age intervals, but rather to be due to a substantial increase in the efficiency of parental mitochondria. This is reflected by the increase in ADP:O values (Table 2) of the respective parents and midparents (Table 3). The reciprocal, OxS, was not significantly different from the midparent, with one exception (14 days), although mean ADP:O values are higher than the midparent average at most age intervals. The nonheterotic reciprocal hybrids, LxO and OxL, were either not significantly different from the midparent with respect to ADP:O values, or were significantly lower, as is indicated by the data in Table 3. This can be termed negative heterosis.

Table 3. Statistical Analysis of Oxidative Phosphorylation (ADP:O Ratios) of Heterotic F_1 Reciprocal Hybrids and Nonheterotic Hybrids, Parental Mitochondrial Mixtures, and Theoretical Midparent Averages.

Age (days)	Cross or mixture	Means: Midparent	Means: Hybrid or mixture	F-test
1	SxO	3.18	3.56	24.90[a]
	OxS	3.18	3.30	n.s.
	O+S	3.18	3.27	n.s.
7	SxO	2.83	3.08	10.63[a]
	OxS	2.83	2.83	n.s.
	O+S	2.83	3.03	15.58[a]
14	SxO	2.79	3.18	23.42[a]
	OxS	2.79	2.92	6.74[b]
	O+S	2.79	2.97	14.46[a]
21	SxO	2.94	3.17	17.23[a]
	OxS	2.94	3.06	n.s.
	O+S	2.94	3.23	31.20[a]
28	SxO	3.23	3.22	n.s.
	OxS	3.23	3.29	n.s.
	O+S	3.23	3.20	n.s.
35	SxO	3.23	3.13	n.s.
	OxS	3.23	3.19	n.s.
	O+S	3.23	3.29	n.s.
1	LxO	3.29	3.36	n.s.
	OxL	3.29	3.19	n.s.
	O+L	3.29	2.96	32.59[c]
7	LxO	2.85	2.70	8.03[c]
	OxL	2.85	2.87	n.s.
	O+L	2.85	2.88	n.s.
14	LxO	2.81	2.85	n.s.
	OxL	2.81	2.82	n.s.
	O+L	2.81	2.80	n.s.
21	LxO	2.99	3.00	n.s.
	OxL	2.99	2.94	n.s.
	O+L	2.99	3.02	n.s.
28	LxO	3.22	3.01	18.02[c]
	OxL	3.22	2.87	49.81[c]
	O+L	3.22	3.04	13.25[c]
35	LxO	3.19	2.86	25.02[c]
	OxL	3.19	2.74	44.16[c]
	O+L	3.19	3.09	n.s.

[a] F value significantly higher at the 1% level.
[b] F value significantly higher at the 5% level.
[c] F value significantly lower at the 1% level.

Table 4. Statistical Analysis of Respiratory Control of Heterotic F_1 Reciprocal Hybrids and Nonheterotic Hybrids, Parental Mitochondrial Mixtures, and Theoretical Midparent Averages.

Age (days)	Cross or mixture	Means: Midparent	vs Hybrid or mixture	F-test
1	SxO	3.21	3.09	n.s.
	OxS	3.21	3.16	n.s.
	O+S	3.21	3.31	n.s.
7	SxO	4.07	3.80	4.72[a]
	OxS	4.07	3.83	n.s.
	O+S	4.07	3.70	8.56[b]
14	SxO	4.00	3.97	n.s.
	OxS	4.00	3.50	27.81[b]
	O+S	4.00	3.81	n.s.
21	SxO	3.95	3.55	13.48[b]
	OxS	3.95	3.42	24.98[b]
	O+S	3.95	3.92	n.s.
28	SxO	3.64	3.67	n.s.
	OxS	3.64	3.53	n.s.
	O+S	3.64	3.49	n.s.
35	SxO	2.58	2.45	n.s.
	OxS	2.58	2.48	n.s.
	O+S	2.58	2.89	14.57[c]
1	LxO	3.07	3.31	5.09[d]
	OxL	3.07	3.57	21.86[c]
	O+L	3.07	3.68	33.61[c]
7	LxO	4.11	4.55	11.99[c]
	OxL	4.11	4.04	n.s.
	O+L	4.11	3.31	40.82[b]
14	LxO	3.93	3.80	n.s.
	OxL	3.93	4.08	n.s.
	O+L	3.93	3.23	52.26[b]
21	LxO	3.68	3.93	5.85[d]
	OxL	3.68	3.77	n.s.
	O+L	3.68	3.50	n.s.
28	LxO	3.68	3.06	28.33[b]
	OxL	3.68	3.68	n.s.
	O+L	3.68	3.18	18.66[b]
35	LxO	2.54	2.20	16.20[b]
	OxL	2.54	2.31	7.14[b]
	O+L	2.54	2.65	n.s.

[a] F value significantly lower at the 5% level.
[b] F value significantly lower at the 1% level.
[c] F value significantly higher at the 1% level.
[d] F value significantly higher at the 5% level.

Table 5. Statistical Analysis of State 3 Respiration Rates of Heterotic F_1 Reciprocal Hybrids and Nonheterotic Hybrids, Parental Mitochondrial Mixtures, and Theoretical Midparent Averages.

Age (days)	Cross or mixture	Means: Midparent	vs Hybrid or mixture	F-test
1	SxO	49.00	52.31	25.37[a]
	OxS	49.00	50.52	5.36[b]
	O+S	49.00	46.77	11.57[c]
7	SxO	76.51	78.22	n.s.
	OxS	76.51	75.35	n.s.
	O+S	76.51	75.00	n.s.
14	SxO	87.87	89.93	n.s.
	OxS	87.87	90.56	n.s.
	O+S	87.87	88.55	n.s.
21	SxO	85.28	89.92	12.29[a]
	OxS	85.28	86.80	n.s.
	O+S	85.28	86.26	n.s.
28	SxO	71.65	72.31	n.s.
	OxS	71.65	73.80	n.s.
	O+S	71.65	70.24	n.s.
35	SxO	63.16	52.65	57.79[c]
	OxS	63.16	56.55	20.43[c]
	O+S	63.16	55.45	27.88[c]
1	LxO	49.72	49.39	n.s.
	OxL	49.72	50.56	n.s.
	O+L	49.72	48.57	n.s.
7	LxO	76.37	77.60	8.16[a]
	OxL	76.37	72.17	10.72[c]
	O+L	76.37	73.49	4.99[d]
14	LxO	87.17	90.97	4.96[b]
	OxL	87.17	91.26	5.74[b]
	O+L	87.17	87.98	n.s.
21	LxO	85.91	85.65	4.29[d]
	OxL	85.91	85.26	n.s.
	O+L	85.91	82.20	7.79[c]
28	LxO	71.61	70.53	8.83[c]
	OxL	71.61	71.41	5.04[d]
	O+L	71.61	72.18	n.s.
35	LxO	57.51	54.53	4.17[d]
	OxL	57.51	52.34	12.55[c]
	O+L	57.51	51.71	15.77[c]

[a] F value significantly higher at the 1% level.
[b] F value significantly higher at the 5% level.
[c] F value significantly lower at the 1% level.
[d] F value significantly lower at the 5% level.

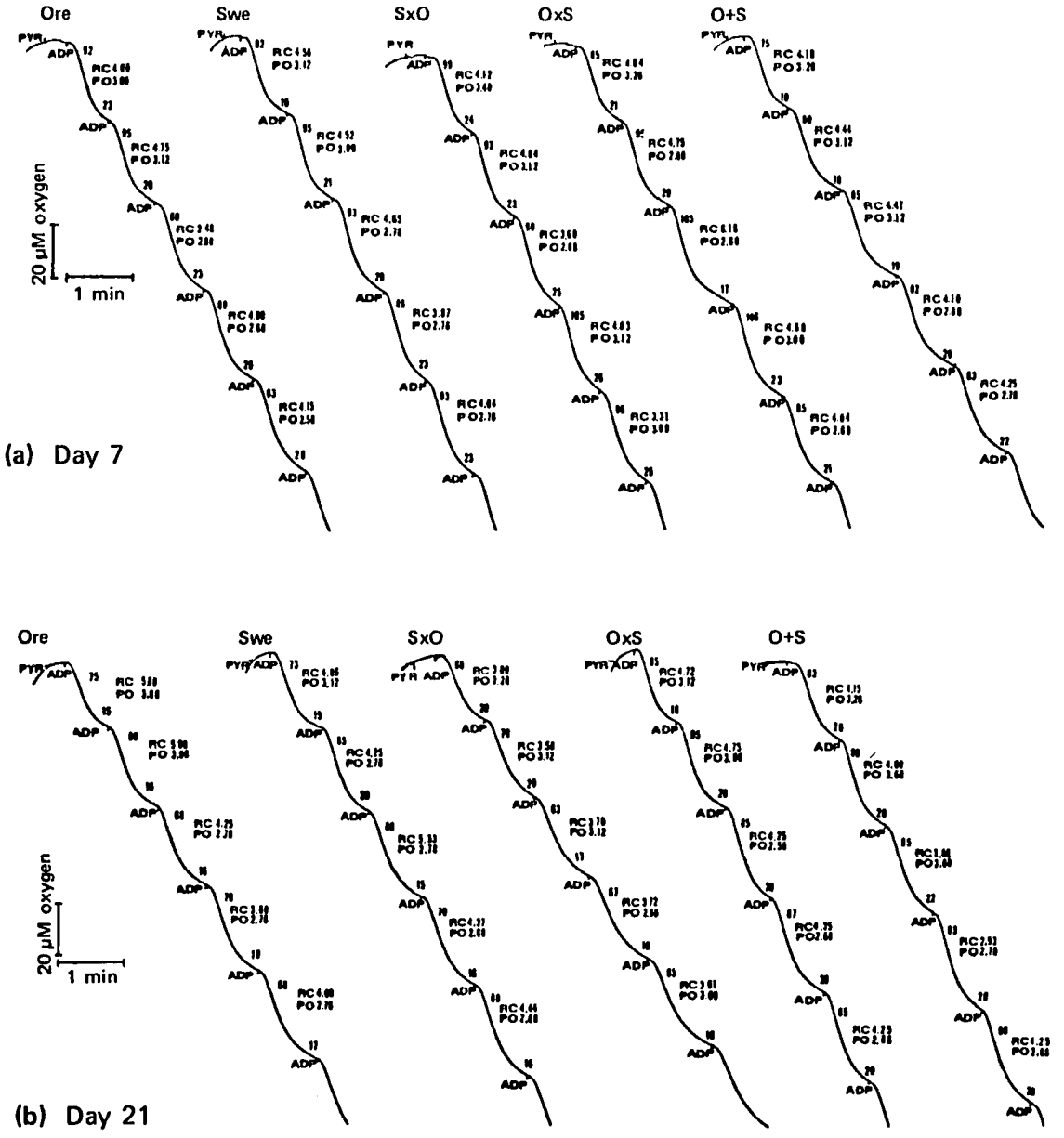

Fig. 6. Polarographic Traces of Oxygen Uptake by Isolated *Drosophila* Mitochondria of F_1 Reciprocal Hybrids, Parental Mitochondrial Mixture, and Inbred Parents, Showing the Enhanced Efficiency of Phosphorylation of the Heterotic Hybrid SxO and the Parental Mixture O+S.

Concentrations: substrate, pyruvate, 5 mM; oxygen, 240 µM; ADP, 150 µM/addition. (a) Day 7. (b) Day 21.

Respiratory control, which is a measure of the biochemical integrity of the isolated mitochondria, was found to be the most variable of the three parameters. This was probably because individual respiratory cycles were significantly different in most preparations. Respiratory control ratios of hybrids are either not significantly different or are significantly lower (negative heterosis) than the midparent values with the exceptions of LxO at days 1 and 14 and OxL at day 1 (Table 4).

State 3 respiration rates are on a per-milligram mitochondrial protein per minute basis. Again, with few exceptions the hybrids were either not significantly different from respective midparents or demonstrated negative heterosis (Table 5).

In vitro 1:1 mitochondrial mixtures (fresh weight) were made from inbred parents that produced heterotic hybrids as well as from parents that produced nonheterotic hybrids. Mixtures of mitochondria of the parents of the heterotic hybrid SxO exhibit greater efficiency of oxidative phosphorylation than the midparent but do not surpass that of the hybrid (Table 6). Complementation is shown as a percent of the theoretical midparent. Oxygen polarograph traces of *in vitro* mitochondrial mixtures showing enhanced efficiency of oxidative phosphorylation over the parents are depicted in Figs. 6a and b. This functional superiority of 1:1 mitochondrial mixtures over the parents has been termed mitochondrial complementation (McDaniel, 1971). The advantage of the mixture O+S over the midparent is not present at day 1 and is also observed only up to day 21 (Table 3). Mitochondrial mixtures of parents of nonheterotic hybrids do not exhibit complementation, as indicated by the data in Table 3.

Table 6. Statistical Analysis of ADP:O Ratios from *D. melanogaster* Mitochondria of F_1 Reciprocal Hybrids, Inbred Parents, Mitochondrial Mixtures, and Theoretical Midparents at Several Age Intervals.

Age (days)	Means						% complementation
	Ore	Swe	SxO	OxS	Mp	O+S	
7	2.84^{b}	2.82^{b}	3.08^{a}	2.83^{b}	2.83^{b}	3.03^{a}	107.07
14	2.78^{c}	2.80^{c}	3.18^{a}	2.92^{bc}	2.79^{c}	2.97^{b}	106.46
21	2.94^{b}	2.93^{b}	3.17^{a}	3.06^{ab}	2.94^{b}	3.23^{a}	109.87

Note: Horizontal means followed by the same letter are not significantly different at the 5% level according to Duncan's new multiple range test.

Genotype means calculated over all age intervals for the three respiratory parameters are shown in Table 7. Efficiency of energy conservation (ADP:O) of the heterotic hybrid SxO was significantly higher than the respective parents but was not significantly different from the reciprocal OxS. The nonheterotic reciprocal hybrids LxO and OxL exhibited ADP:O ratios lower than their inbred parents. Respiratory control ratios and State 3 rates of oxidation were not significantly different between hybrids and parents, however.

Comparisons of parental mitochondrial mixtures and respective inbred controls over all age intervals with respect to the three parameters are presented in Table 8. The mitochondrial mixture of the parents that produced heterotic hybrids is significantly higher than the respective inbred parents in phosphorylation efficiency (ADP:O). However, the mitochondrial mixture of the parents that produced the nonheterotic

hybrids is not significantly different from their inbred parents. No changes are found in respiratory control (RC) and State 3 respiration between mixtures and parents.

Table 7. Statistical Analysis of Polarographic Data from *D. melanogaster* Mitochondria of Reciprocal Hybrids and Respective Parents over All Age Intervals.

Parameter	Parents			F_1 hybrids			
	Ore	Swe	Lau	SxO	OxS	LxO	OxL
ADP:O	3.00^{bc}	3.04^{bc}	3.11^{ab}	3.22^{a}	3.10^{ab}	2.98^{bc}	2.90^{c}
RC	3.57^{a}	3.56^{a}	3.42^{a}	3.42^{a}	3.31^{a}	3.47^{a}	3.57^{a}
State 3 respir.	71.91^{a}	72.83^{a}	71.67^{a}	72.55^{a}	72.26^{a}	71.11^{a}	69.35^{a}

Note: Horizontal means followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls' mean comparison test.

Table 8. Statistical Analysis of Polarographic Data from *D. melanogaster* Mitochondria of Inbred Parents and Parental Mixtures over All Age Intervals.

Parameter	Parents			Mixture	
	Ore	Swe	Lau	O+S	O+L
ADP:O	3.00^{b}	3.04^{b}	3.11^{ab}	3.17^{a}	3.01^{b}
RC	3.57^{a}	3.56^{a}	3.42^{a}	3.52^{a}	3.26^{a}
State 3 respir.	71.91^{a}	72.83^{a}	71.67^{a}	70.39^{a}	69.35^{a}

Note: Horizontal means followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls' mean comparison test.

Mitochondrial heterosis and complementation have also been reported in several plant systems (McDaniel and Sarkissian, 1966; Sarkissian and Srivastava, 1969; McDaniel, 1973). The precise nature of mitochondrial heterosis and complementation either at the biochemical, physiological, or genetic level is not well understood, however. McDaniel and Sarkissian (1966) suggested that these phenomena could be a reflection of mitochondrial interaction and that hybrids may be polymorphic with respect to their mitochondria. Supporting evidence for this hypothesis has been reported by Sarkissian and McDaniel (1967) from qualitative studies on maize mitochondria that demonstrated polymorphism. The heterotic hybrids contained the two parental types plus an intermediate type. Furthermore, these investigators compared the cytochrome c oxidase activities of the different mitochondrial types and found high enzymatic activity associated with the "hybrid" type of mitochondria. It has not been possible to determine whether the mitochondrial heterosis and complementation exhibited by *Drosophila* in these studies are also the result of interaction between different mitochondrial types. The fact that mitochondrial mixtures exhibited complementation suggests this possibility, but more conclusive evidence is needed. Further studies to determine whether *Drosophila* heterotic hybrids are also polymorphic with respect to their mitochondria are needed.

As it is clearly shown by the data obtained from these experiments, hybrids that are not heterotic in other attributes, as measured by longer life span and heavier body weights, do not exhibit mitochondrial heterosis. Mitochondrial mixtures of the parents do not exhibit mitochondrial complementation either. This suggests the possibility

that a correlation between these two phenomena and heterosis with respect to other attributes may exist and also that the mitochondrial mixture may be used to select heterotic hybrids. Similar results have been reported with several plant hybrids (McDaniel, 1972a, 1973). Positive correlations of mitochondrial heterosis, complementation, and heterosis with respect to yield, growth rates, and other beneficial attributes have been obtained. Mitochondrial mixtures are used in selecting possible beneficial hybrids.

The possibility of a polymorphic nature of mitochondria raises the question of their origin and mode of inheritance. One possibility that appears to be compatible with the idea of mitochondrial polymorphism is that mitochondria arise from existing mitochondria and that they are transmitted gametically to the hybrid by both parents (McDaniel and Sarkissian, 1966). This is contrary to the hypothesis that mitochondria are maternally inherited. The fact that the reciprocal hybrids SxO and OxS are both heterotic with respect to life span and body weight but not with respect to oxidative phosphorylation appears to support the latter hypothesis, although the differences in mitochondrial activities may also indicate an unequal parental contribution of mitochondria. Further work is needed in order to determine why the two heterotic reciprocal hybrids demonstrate different mitochondrial activity and the mode of inheritance of mitochondria in *Drosophila*.

Effect of Age on Mitochondrial Activity

The effect of age on ADP:O and respiratory control ratios as well as State 3 oxidation rates is presented in Tables 3, 4, and 5.

Analysis of variances showed that there was no age x genotype interaction with respect to any of the parameters; thus F_1 hybrids, parental controls, and mitochondrial mixtures all behave similarly with respect to each parameter measured although significant genotypic differences may occur.

Efficiency of oxidative phosphorylation (ADP:O), respiratory control (RC), and State 3 respiration values calculated over all genotypes for all age intervals are presented in Table 9. Significant differences in ADP:O ratios occur during maturation and aging. The ADP:O ratio is high at day 1, decreases significantly at day 7, and increases significantly at day 21 but remains stable with advanced aging.

Table 9. Statistical Analysis of Polarographic Data from *D. melanogaster* Mitochondria of F_1 Reciprocal Hybrids, Parental Mitochondrial Mixtures, and Inbred Parents over All Genotypes.

Parameter	Age in days					
	1	7	14	21	28	35
ADP:O	3.26^a	2.88^c	2.88^c	3.06^b	3.15^b	3.10^b
RC	3.24^b	3.96^a	3.82^a	3.69^a	3.51^{ab}	3.52^{ab}
State 3 respir.	49.50^c	75.26^b	89.08^a	86.10^a	72.26^b	56.17^c

Note: Horizontal means followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls' mean comparison test.

This cyclic change in ADP:O for reciprocal heterotic and nonheterotic hybrids and respective inbred parents is depicted in Figs. 7 and 8. It is possible that the cyclic behavior reflects turnover in mitochondrial populations, but it could also be attributable to changes in metabolic states of mitochondria. Respiratory control of mitochondria increases at day 7 and then decreases with advanced age. This decrease in respiratory control may reflect lower ADP-stimulated rates of oxidation (State 3 respiration) although the decrease could also reflect a greater sensitivity of mitochondria to damage during extraction procedures with aging. State 3 respiration increases significantly at days 7 and 14 and then decreases with advanced aging (Figs. 9 and 10). Typical oxygen polarograph traces of an inbred parent and a heterotic hybrid showing these aging effects are presented in Figs. 11 and 12.

The data obtained in these experiments demonstrate that there are associated with aging significant changes in the three parameters measured. The decrease in respiratory control and State 3 rate of oxidation are of special interest because these changes may reflect a decline in mitochondrial homeostasis that accompanies advanced aging and senescence. State 3 respiration rates represent the maximal respiratory capacity of the mitochondria. A decline in respiration could also decrease ATP production. The decrease in State 3 rate of oxidation may reflect a decrease in the ability to oxidize pyruvate and may be correlated with ultrastructural alterations of mitochondria as has been reported for other insects (Bulos, Shukla, and Sacktor, 1972). This decrease in the capacity of mitochondria from aging flies to oxidize pyruvate with a concomitant loss in respiratory control may be accounted

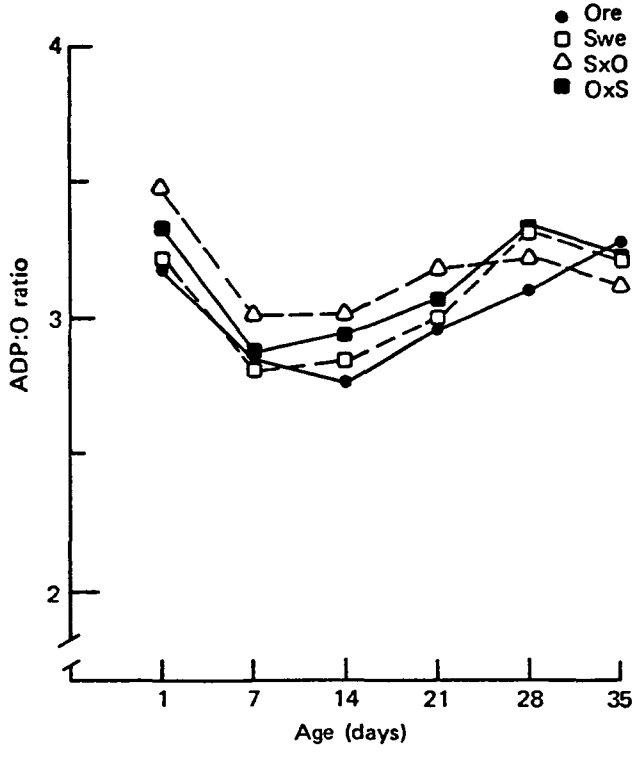

Fig. 7. ADP:O Values for Heterotic F_1 Hybrids and Respective Inbred Parents over All Age Intervals.

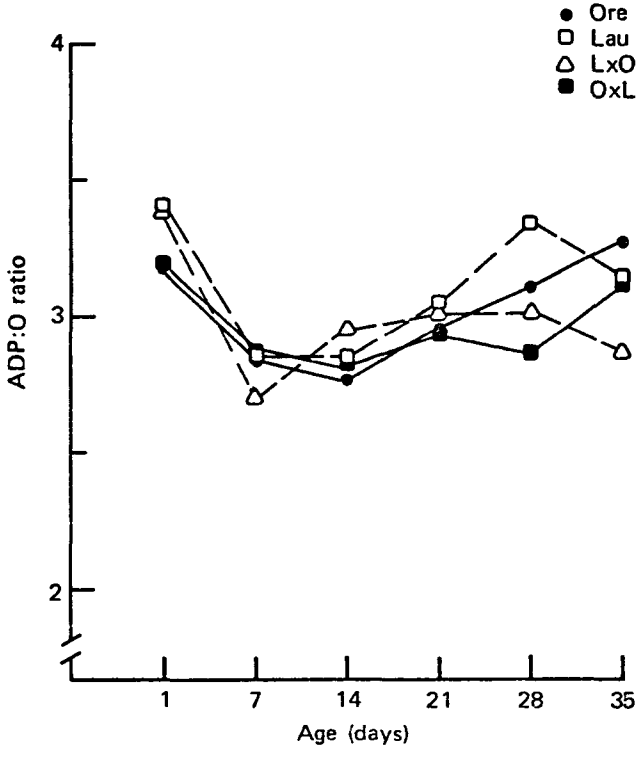

Fig. 8. ADP:O Values for Nonheterotic F_1 Hybrids and Respective Inbred Parents over All Age Intervals.

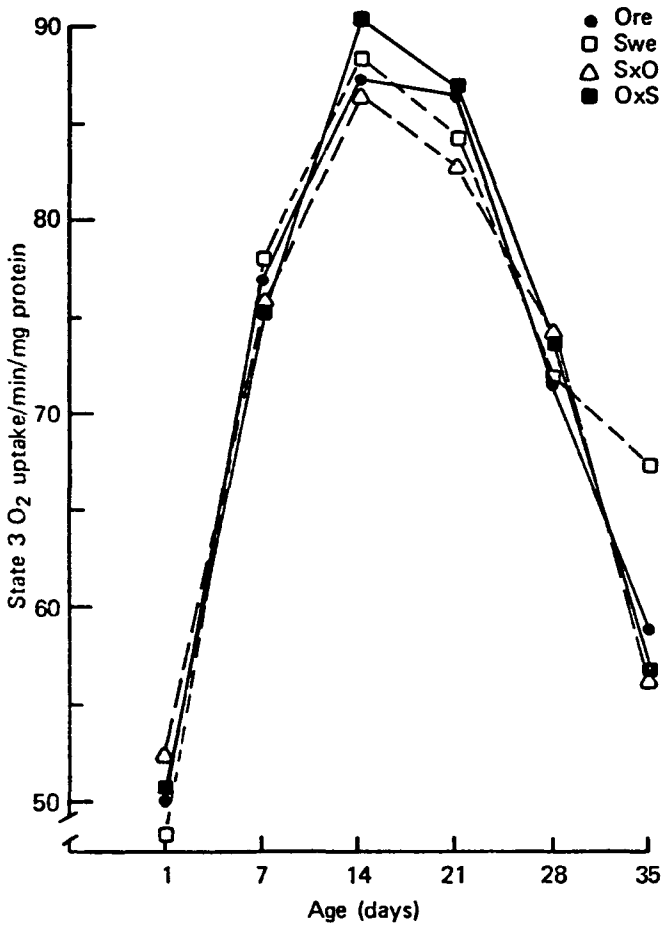

Fig. 9. State 3 Respiration Rates for Heterotic F_1 Hybrids and Respective Inbred Parents over All Age Intervals.

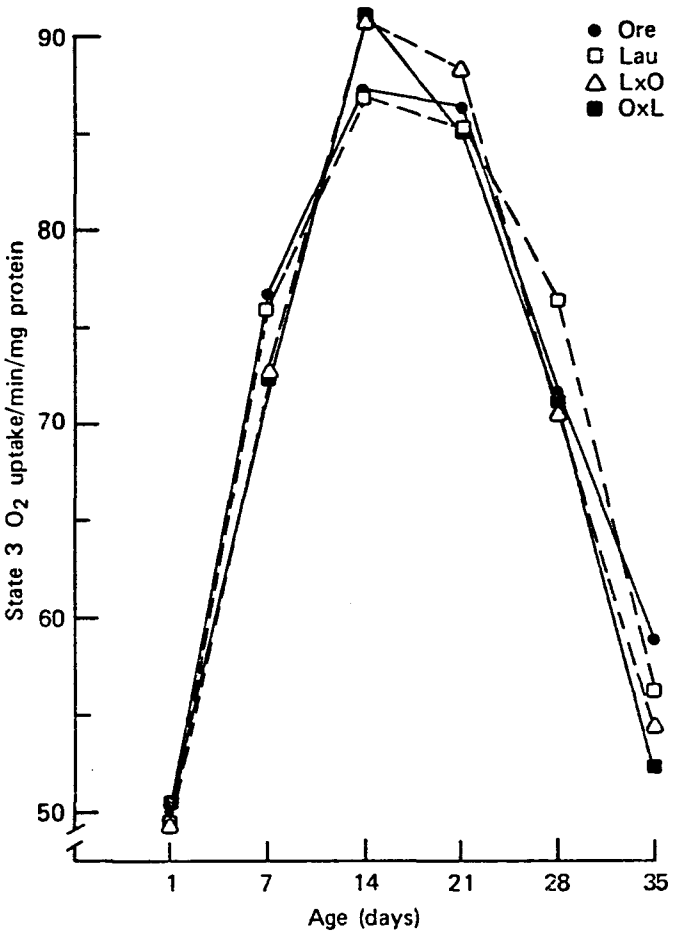

Fig. 10. State 3 Respiration Rates for Nonheterotic F_1 Hybrids and Respective Inbred Parents over All Age Intervals.

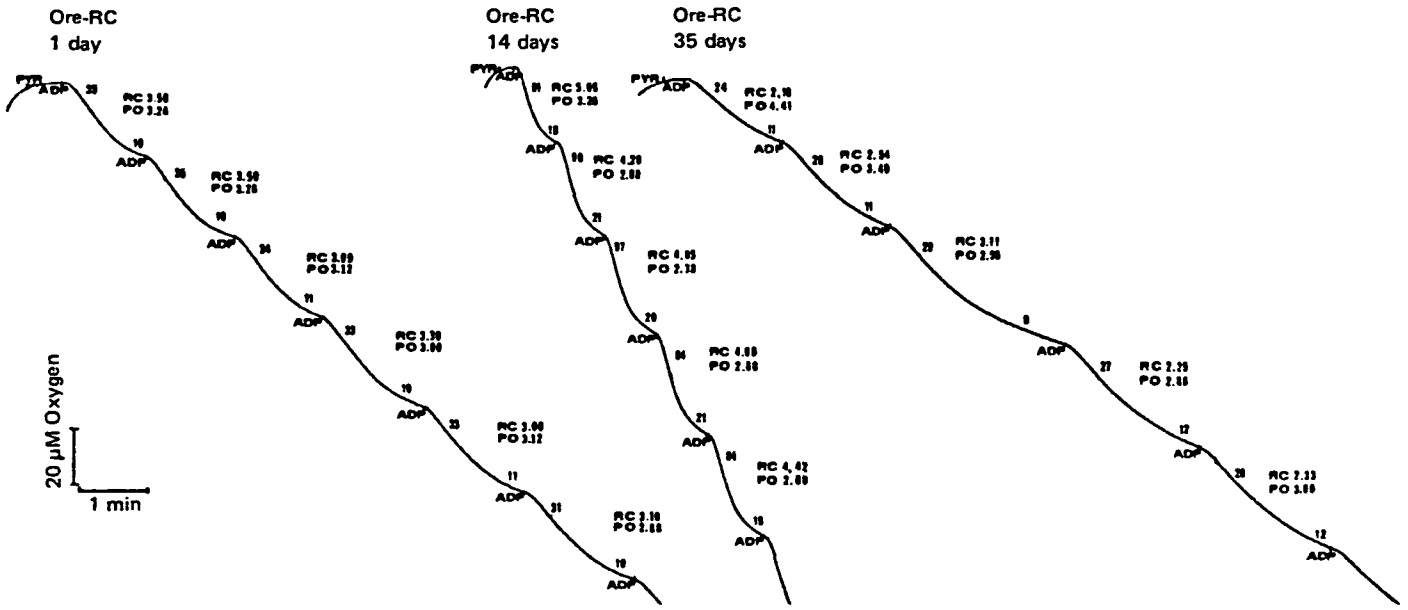

Fig. 11. Polarographic Traces of Oxygen Uptake by *Drosophila* (Ore-RC) Mitochondria, Showing the Effect of Aging on Oxidative Phosphorylation (ADP:O) and Respiratory Control (RC).

Concentrations: substrate, pyruvate, 5 mM; oxygen, 240 µM; ADP, 150 µM/addition.

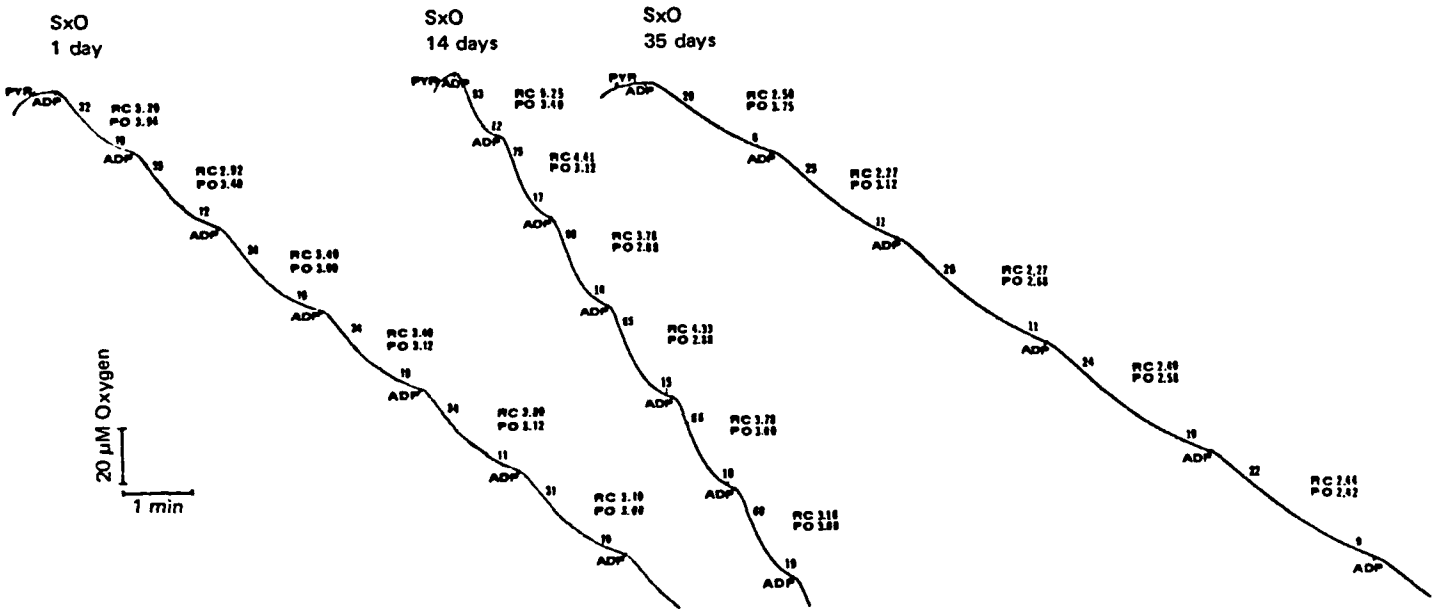

Fig. 12. Polarographic Traces of Oxygen Uptake by *Drosophila* Mitochondria of the Heterotic Hybrid SxO, Showing the Effect of Aging on Oxidative Phosphorylation (ADP:O) and Respiratory Control (RC).

Concentrations: substrate, pyruvate, 5 mM; oxygen, 240 µM; ADP, 150 µM/addition.

for by a decrease in the total number of functional cristae as indicated by the studies of Sacktor and Shimada (1972). However, the alteration in mitochondrial structure does not appear to affect oxidative phosphorylation since ADP:O ratios do not change significantly during the last three age intervals. The decline with age in State 3 respiration and respiratory control of *Drosophila* mitochondria is in agreement with the findings of Weinbach and Garbus (1959) and Bulos, Shukla, and Sacktor (1972).

Even though both heterotic hybrids and their inbred parents behaved similarly with respect to State 3 respiration rates, substantial differences in the rate of decline between hybrids and parents occurred at days 28 and 35, as shown by the data in Table 2. These differences may in turn reflect differences in genetic control of respiratory metabolism between hybrids and parents that could account for the longer life spans exhibited by heterotic hybrids. Harman (1972) has suggested that life span is to a great extent an expression of genetic control over the rate of oxygen uptake. Furthermore, this investigator believes that the rate of oxygen uptake determines the rate of accumulation of mitochondrial damage produced by free radical reactions, the rate of damage being proportional to the rate of oxygen consumption, which ultimately causes death. The data obtained in these studies are compatible with this hypothesis. With advanced aging, the inbred parents and nonheterotic hybrids showed a higher rate of State 3 respiration than the heterotic hybrids. Further studies attempting to correlate change in State 3 oxidation rates with rate of mitochondrial damage would be useful in trying to determine why the heterotic hybrids have longer life spans.

Comparison of Respiratory Efficiency of
Paralytic Temperature-Sensitive Mutants and
Wild-Type Flies at Four Different Temperatures

Measurements of oxidative phosphorylation (ADP:O) and respiratory control (RC) made on mitochondria extracted from the temperature-sensitive mutant, $Para^{ts}$, and the wild-type strain, Ore-RC, are recorded in Table 10. Changes in mitochondrial activity over the experimental temperature range, as measured by the above parameters, followed similar trends in both genotypes. ADP:O and RC ratios are comparatively higher for $Para^{ts}$ at 23^{o} and 27^{o} but not at 29^{o} and 32^{o}C. However, these differences were not statistically significant. ADP:O and RC means calculated over all temperatures are also not significant. However, temperature means calculated over all genotypes do show significant differences for respiratory control although no definite trend with increased temperature is noted.

Paralysis of mutants occurs at 29^{o}C in adults; these flies survive in a state of paralysis for at least 2 hours. Furthermore, they recover mobility immediately upon being shifted from 29^{o} to 23^{o}C. The potential of temperature-sensitive mutations for studying development through biochemical means has been discussed by Suzuki (1970). A comparison of the effect of temperature on mitochondrial activity may suggest that mitochondria of mutant flies are more sensitive to exposure to moderately high temperatures than mitochondria from wild-type flies. However, more work is needed to determine whether the lesion that causes paralysis of mutant flies is correlated with changes in mitochondrial activity.

Table 10. ADP:O Values and Respiratory Control Means of Mitochondria Isolated from Para^{ts} and Ore-RC Adults over a Range of Temperatures.

Each mean represents three replications of five observations.

Genotype	Temperature (°C) 23°	27°	29°	32°	Genotype means over all temperatures
			ADP:O*		
Para^{ts}	3.22	3.23	3.11	3.08	3.15^{a}
Ore-RC	3.14	3.06	3.22	3.14	3.15^{a}
Temperature means over all genotypes	3.18^{a}	3.15^{a}	3.16^{a}	3.11^{a}	3.15
			Respiratory Control		
Para^{ts}	3.32	3.72	2.93	3.15	3.26^{a}
Ore-RC	3.27	3.55	3.03	3.17	3.28^{a}
Temperature means over all genotypes	3.29^{b}	3.64^{a}	2.98^{c}	3.16^{b}	3.27

*ADP:O means have been corrected for differential solubility of O_2 in the reaction mixture at the temperature used.

Note: Means followed by the same letter are not significantly different at the 5% level according to Student-Newman-Keuls' mean comparison test.

HISTONES

Literature Review

In multicellular eucaryotes nearly all cells contain the same genetic information, and yet different patterns of genetic activity may be elicited in different tissues. This suggests that specific genes are activated while others are selectively repressed, and that such differential genetic activity necessitates some type of regulatory mechanism(s). It is currently thought that a mechanism of regulation similar to that proposed by Jacob and Monod for procaryotes is present in eucaryotic cells (Comings, 1972). In the Jacob-Monod model, gene activation and repression are enacted by regulatory molecules that interact with specific regions of the genome (reviewed by Reznikoff, 1972).

The genetic material in eucaryotes is found in close association with chromosomal proteins and a small amount of ribonucleic acid (RNA). Chromosomal proteins are divided into two classes, histones and nonhistones, both of which are considered to assume the role of regulatory molecules. Because of this close association with deoxyribonucleic acid (DNA) and their regulatory function, histones have been the subject of a vast amount of research in recent years (Georgiev, 1969; Stellwagen and Cole, 1969; DeLange and Smith, 1971; Elgin et al., 1971; Hnilica, McClure, and Spelsberg, 1971; Wilhelm, Spelsberg, and Hnilica, 1971; McClure and Hnilica, 1972).

Histones are basic proteins that contain a high proportion of the basic amino acids lysine and arginine. All higher plants and animals

have been found to contain essentially the same types of histones and approximately the same number of distinguishable fractions (DeLange et al., 1968). The histones can be electrophoretically separated into at least five major types. Separation into subfractions has been reported (Busch, 1965; Oliver and Chalkley, 1972a). The five major fractions have been designated as f1, f3, f2b, f2a2, and f2a1 (nomenclature of Johns, 1964) on the basis of their chemical properties and were grouped into three classes on the basis of lysine and arginine content: (1) lysine-rich (f1), (2) slightly lysine-rich (f2a2 and f2b), and (3) arginine-rich (f3 and f2a1).

Histones have been reported to be electrostatically bonded to DNA. The exact nature of the histone:DNA interactions is not precisely known although several models have been proposed (Leng and Felsenfeld, 1966; Shih and Bonner, 1970). It has been reported that histones have a greater affinity for double-stranded DNA than for single-stranded DNA. This is one indication that double-stranded structure contributes to more favorable binding sites for histones (Akinrimisi, Bonner, and T'so, 1965). One conclusion is that the histones bind in one of the grooves of the double-stranded configuration. Evidence favors the major groove (Shih and Bonner, 1970), but others disagree (Kleinman and Huang, 1971).

Interaction of individual histone fractions with DNA has also been reported (Bartley and Chalkley, 1970; Edwards and Shooter, 1969). These studies suggest different types of binding of the various fractions with DNA. Smart and Bonner (1971) have reported that histone f1 is more exposed to the aqueous environment than the other fractions and have proposed a model whereby the histones form a sheathlike structure

around the DNA with fractions f2a2, f2a1, f3, and f2b on the inside and fraction f1 on the outside.

The hypothesis that histones inhibit genetic activity was originally developed by Stedman and Stedman (1950, 1951). They believed the evolutionary appearance of the histones coincided with the appearance of differentiation and, therefore, could be involved in prolonged gene repression. Huang and Bonner (1962) reported evidence that supported the Stedman and Stedman hypothesis. They demonstrated that histones inhibited DNA-dependent RNA synthesis. Furthermore, *in vitro* denaturation of the DNA-histone complex was found to enhance template activity and renaturation of the complex to decrease it. This phenomenon has been substantiated in subsequent studies (Marushige and Bonner, 1966; Bekhor, Kung, and Bonner, 1969). Shih and Bonner (1970) suggested that the inhibition of DNA template activity might result from interference with unwinding of the DNA double-helix during the process of transcription. Littau et al. (1965) have reported that histone f1 was required for condensation of DNA and was thus involved in formation of crosslinks between DNA strands. Jensen and Chalkley (1968) obtained results that supported this concept and indicated that crosslinking was inversely proportional to template activity. Johns and Forrester (1970) suggested that supercoiling of DNA prevents large RNA molecules from interacting with chromatin because of unfavorable steric relationships.

Even though much evidence seems to support the proposed regulatory function of the histones, the precise mechanism is not known. Electrophoretic studies have shown limited heterogeneity of histones. Specificity in genetic regulation cannot be a function of histones alone

(Shih and Bonner, 1970; Elgin et al., 1971). Conversely, histones do influence DNA-dependent RNA synthesis; therefore, histones should function in general repression of the genome.

Allfrey, Faulkner, and Mirsky (1964) suggested that the inhibitory activity of histone is not constant but may vary with modification of certain chemical groups. Repression could be mediated by the histones, and gene activation could be effected by chemical modification of histone side chains by degree of acetylation, methylation, or phosphorylation (DeLange and Smith, 1971).

Histone Variations During Development

The early stages of development in most organisms are characterized by rapid changes in patterns of genetic activity. Many investigations have been conducted in an attempt to correlate such patterns with variations in histone composition.

Major differences in proportions of histone fractions have been observed during embryogenesis (Vorobyev, 1969; Bekhor, Kung, and Bonner, 1969; Thaler, Malcolm, and Villee, 1970; Benttinen and Comb, 1971; Auer, 1972). Qualitative and quantitative changes in histones have been reported between blastula and gastrula stages in sea urchins (Vorobyev, Gineitis, and Vinofradova, 1969; Ord and Stocken, 1970). Similar changes have been reported for amphibian oocytes (Spiegel, Spiegel, and Meltzer, 1970). Histone changes during chick development have been reported by Agrell and Christensson (1965). They observed a gradual quantitative change in lysine-rich and slightly lysine-rich fractions. By contrast, Kischer, Gurley, and Shepherd (1966) observed no substantial changes during early development.

Histone changes have also been reported for developing plant systems. Srivastava (1971) reported changes in the ratio of histone fractions from different tissues in tobacco. Fambrough, Fujimura, and Bonner (1968) reported quantitative changes but no qualitative changes in embryonic tissues of the pea plant. McDaniel (1972b) reported quantitative variations between different tissues in barley during development. More recently, Spiker and Krishnaswamy (1973) conducted an electrophoretic study of histones from various tissues of wheat and found no differences in mobility or relative quantity of the various histone types.

Studies of Insect Histones

Most insects undergo drastic morphological and biochemical changes during metamorphosis. These changes would seem to necessitate equally abrupt variations in patterns of genetic activity and would make them ideal systems for correlating changes in histone composition with variations in genetic expression. In spite of this and the fact that some insects, such as *Drosophila*, have been extensively utilized in studies of gene function, their histone complement during the various stages of their life cycle has not been extensively studied.

Agrell and Lindh (1966) extracted basic nuclear proteins from blowflies but were unable to clearly identify them as histones. Dick and Johns (1969) extracted and characterized histones from *Drosophila melanogaster*. They reported four major fractions, three of which were similar in electrophoretic mobility to those reported by Johns (1964) for calf thymus histones. The fraction corresponding to histone f1 was

not detected. Comparative studies of histones extracted from different tissues of *Drosophila* were conducted by Cohen and Gotchel (1971). Identical histone patterns were observed from polytene and nonpolytene nuclei. These histones differed considerably from those of sea urchins, birds, and mammals. The lysine-rich fraction was found to have a slower electrophoretic mobility than that of the other organisms.

Williams (1971) conducted an electrophoretic study of *Drosophila* histones during embryogenesis and development. Histones were not detected until after the tenth cleavage stage in *Drosophila* eggs. The pattern consisted of four types and was similar to that obtained from adult flies. A unique fraction was detected in the larvae, however.

Oliver and Chalkley (1972a,b) conducted a similar study with two species of *Drosophila* but reported five major histone fractions from adult flies. Four were similar in electrophoretic mobility to those reported for calf thymus. The f1 fraction had a lower mobility in agreement with Cohen and Gotchel (1971). Qualitative and quantitative differences between larvae and adult histones were found. A larval specific fraction with an electrophoretic mobility slightly greater than the f1 fraction was also observed.

Comings (1967) studied the histone composition of euchromatin and heterochromatin in *Drosophila* and mealy bugs. In male mealy bugs one haploid set of chromosomes is heterchromatic whereas in females both sets are euchromatic (Brown and Nur, 1964). Electrophoretic patterns of the histones were not different, however. He also analyzed the histone patterns of constitutive heterochromatin from XO and XYY *Drosophila*. No significant differences were observed in either larvae or adult flies.

Age-Related Variations in Histones

A number of theories of cellular aging based on changes in genetic regulation have been proposed (reviewed by Hahn, 1970). Orgel (1963) hypothesized that synthesis of functional proteins required not only the correct DNA sequence but also proper regulation. Medvedev (1964) described cellular aging as a "chaotic continuation of morphogenesis" and suggested that similar basic metabolic processes might be involved but that age-related changes resulted from faulty genetic regulation.

Hahn (1966) proposed a model of cellular aging to account for the reduction in ability to synthesize proteins with advanced age. He suggested that such a reduction could be the result of failure of the regulation mechanism at the gene level. Age-related losses in RNA and protein synthesis were thought to result from irreversible repression of the genome (reviewed by Hahn, 1971). The histones are believed to have a key role in this repression.

Age-related structural interactions of histones and DNA have been discussed by Hahn (1966). Local unwinding of the DNA double-helix is required for transcription; therefore, changes in binding could be expected to affect transcription. It is assumed that a gradual increase in histone:DNA binding intensity occurs in post-mitotic cells with advanced age. The end result is a progressive reduction in RNA and protein synthesis, reduced homeostasis, and ultimately death (Hahn, 1971).

The nature of the histone:DNA binding and how it changes with advanced age is not clearly understood. It is thought (Hahn, 1966) that

in chromatin from young cells, weak ionic bonds are involved, whereas in chromatin from old cells, covalent bonds are formed.

Evidence for the above hypothesis includes thermal denaturation studies of double-stranded DNA from bovine thymus (Hahn and Verzar, 1963; Hahn, 1965) and rats (Hahn and Fritz, 1966). Thermal stability of the DNA:histone complex increased with advanced age. Furthermore, Hahn (1971) demonstrated that, when histones were enzymatically removed from chromatin extracted from senescent mice, the thermal stability of the DNA decreased. Pÿhtila and Sherman (1968) also reported age-related changes in thermal denaturation of DNA in the presence of histones but not in their absence.

Quantitative age-related differences in protein:DNA ratios have also been reported (Hahn, 1971). Pÿhtila and Sherman (1968) detected an increase in protein:DNA ratio in bovine thymus from old animals. A similar increase was reported for mouse brain (Kurtz and Sinex, 1967). However, a decrease was reported for senescent rat liver and kidney (Pÿhtila and Sherman, 1969).

Materials and Methods

Experimental Material

Drosophila inbred and hybrid stocks utilized in electrophoretic studies of histone were maintained as described in the preceding chapter.

Collection of Larvae

To obtain early third-instar larvae of relatively uniform age, 25 fertilized females were placed in each culture bottle. Egg deposition

was allowed for 2 hours and then the females were transferred to fresh bottles. Subsequent transfers were made until eggs had been deposited in five culture bottles, and then the females were discarded. The eggs were incubated under normal laboratory conditions (22°-24°C). Early third-instar larvae were collected using a modification of the method described by Mead (1964). Culture bottles were filled with 2-molar sucrose solution, and the larvae floated to the top while contaminating food particles sunk to the bottom. The contents of all bottles were pooled into a 1000-ml beaker. The larvae were collected off the top, washed with distilled water to remove small adhering particles, placed on paper towels to remove excess moisture, and frozen at -20°C until used for histone extraction. This procedure was followed until 15 g of larvae were collected for each cross and parent.

Collection of Adult Flies

Adult flies were collected from culture bottles at 24-h intervals and either frozen immediately, if one-day-old flies were required, or placed in aging cages and aged for the appropriate period and then frozen. Collections were made until 15 g of flies were collected for each cross and control at each age interval. The flies were kept frozen at -20°C until used for histone extraction.

Isolation of Nuclei and Nucleohistone

Nuclei were isolated by a modification of the methods described by Oliver and Chalkley (1972a) and Chery (1972). The method is outlined in Fig. 13. All procedures were carried out on ice between 0° and 5°C.

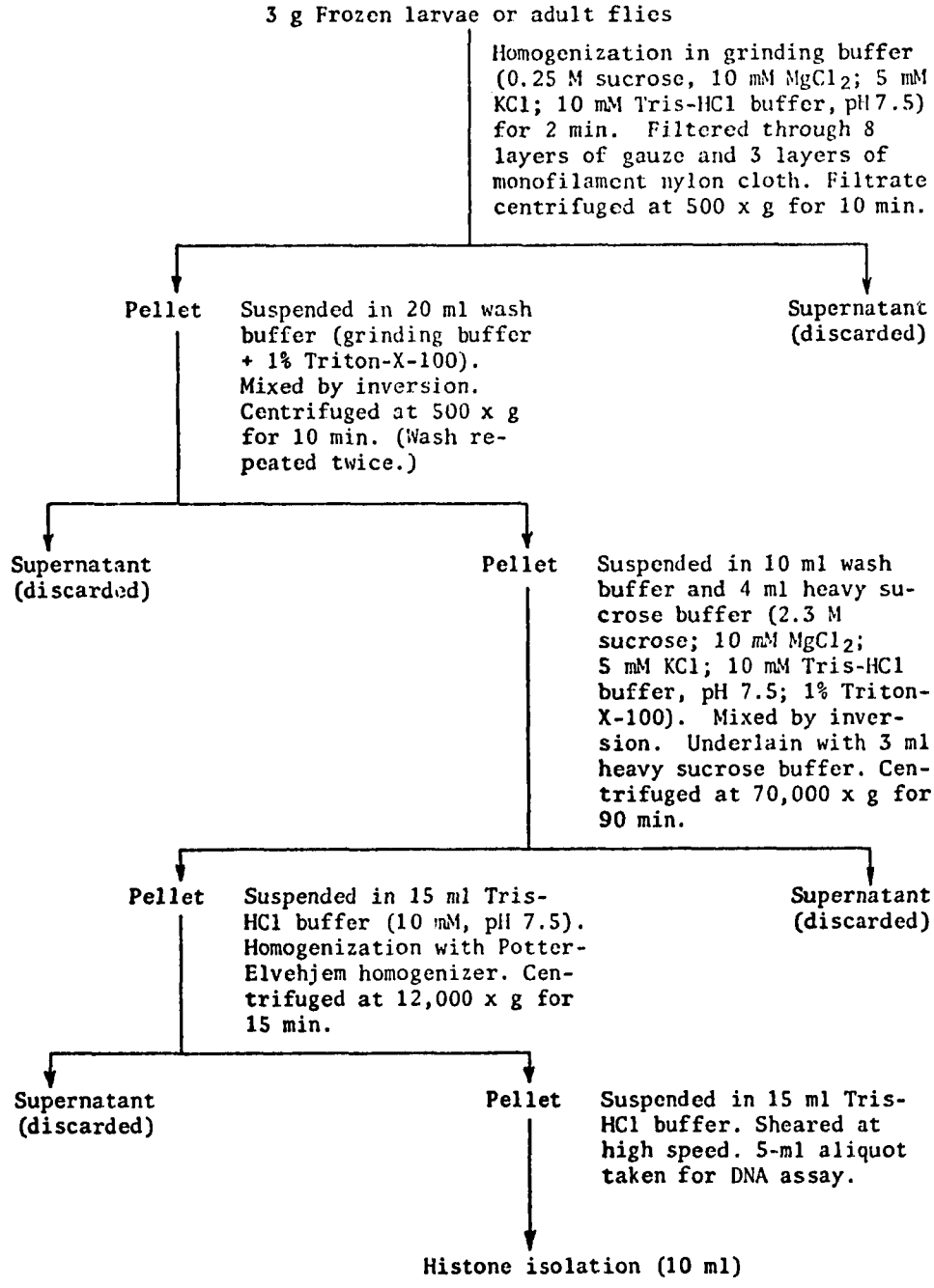

Fig. 13. Flow Sheet for Extraction and Purification of Nuclei and Nucleohistone.

All operations are carried out at 0-5°C.

The resulting homogenate was centrifuged at 12,000 x g for 15 min. This procedure was repeated twice. The final pellet was resuspended in 15 ml Tris-HCl buffer, the chromatin gel sheared at high speed with a variable speed laboratory motor (Tri-R Instrument, Inc.) and 10 ml used in histone isolation. The remaining 5 ml was used for determination of DNA content in the sample using the method of Burton (1956). Calf thymus DNA (Sigma, Type V) was used as a standard.

Isolation of Histone

Histone was extracted from chromatin as outlined in Fig. 14.

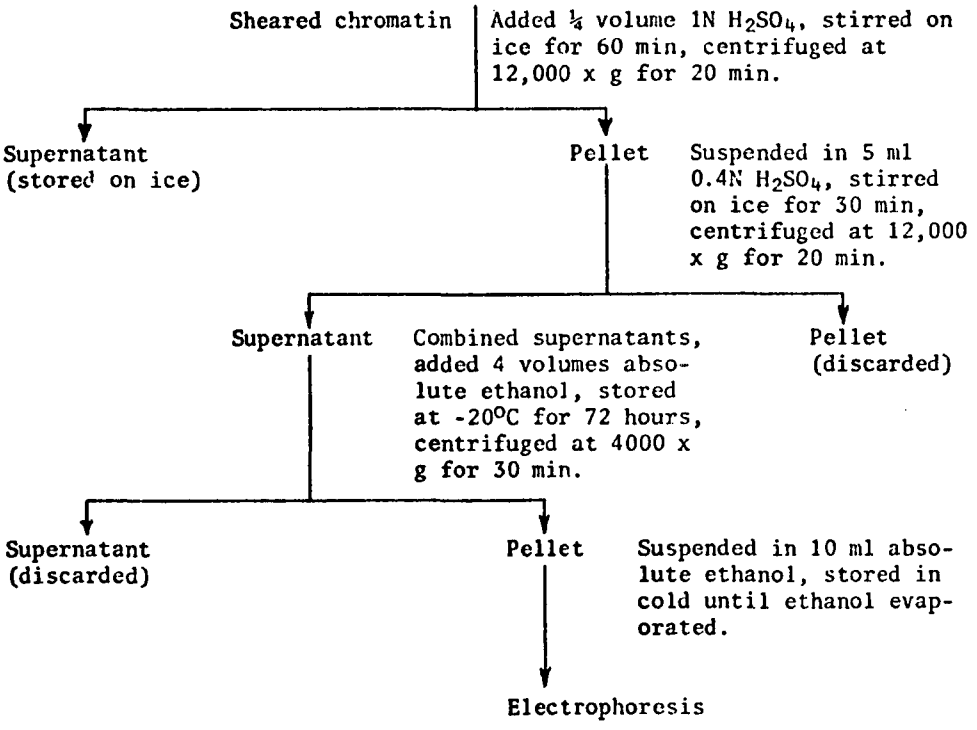

Fig. 14. Flow Sheet for Isolation of Histone from Chromatin.

One-fourth volume 1 N sulfuric acid was added to the chromatin and stirred on ice for 1 hour. The chromatin-acid mixture was centrifuged at 12,000 x g for 20 min. The resulting supernatant was stored on ice. The pellet was resuspended in 5 ml 0.4 N sulfuric acid and stirred on ice for an additional 30 min. This was followed by another centrifugation at 12,000 x g for 20 min. The supernatants containing histone/sulfates were pooled. Histone was recovered by precipitation with four volumes absolute ethanol for 72 hours at -20°C. The ethanol-histone solution was centrifuged at 4,000 x g for 30 min. The resulting pellet was resuspended in 10 ml absolute ethanol and placed in a walk-in cold room until the ethanol evaporated. The dried histone was then dissolved in 1 ml buffer I (0.8 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 40 mM Tris-base buffer, pH 2.9) and a 0.1-ml aliquot was used for determination of histone content by Chaykin's modification of Lowry's protein assay. All samples were adjusted to a histone concentration of 0.5 mg/ml and the desired concentration was applied to 15% polyacrylamide gels using a 0.2-ml measuring pipette.

Electrophoretic Fractionation of Whole Histone

The procedure used for fractionation of histone by electrophoresis was based on a modification of the methods described by Fambrough and Bonner (1966) and Panyim and Chalkley (1969). Glass columns, 6.5 cm long and 0.6 cm inside diameter were used as supports for polymerization of polyacrylamide gels. The gels were prepared as described by McDaniel (1972b) by dissolving 5.7 g urea in 8 ml solution C and 6 ml solution A. Solution C consisted of 30.0 g of acrylamide and 0.40 g of bisacrylamide

made to 100 ml with deionized water and adjusted to pH 2.9. Solution A consisted of 71 ml of glacial acetic acid and 1.55 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) made to 100 ml with deionized water and adjusted to pH 2.9. After the urea was completely dissolved, 2 ml of 1.4% ammonium persulfate in deionized water, adjusted to pH 2.9, was added to the solution, which was then applied to the glass columns using a Pasteur pipette. The acrylamide solution was then layered with deionized water with a 25-µl Hamilton syringe and allowed to polymerize under normal laboratory conditions. After polymerization had been effected, the appropriate histone concentration was applied to each column. The solution of acrylamide and urea was diluted 1:1 with deionized water, carefully layered over the histone, and allowed to polymerize. Electrophoresis was carried out with fresh tray buffer (0.37 M glycine in 0.1 N glacial acetic acid, pH 4.0) in a refrigerator at 3 ma per column until the methylene blue marker band was 1 cm from the bottom of the column.

At the termination of electrophoresis, the gels were removed from the glass columns by rimming the edges with water with a syringe fitted with a 22-gauge hypodermic needle and transferred to 15-cm by 0.8-cm glass columns. Gels were stained for 30 min in 1% Amido-Schwartz 7% acetic acid solution. The gels were rinsed with water, placed in the destaining chamber containing 7% acetic acid, and stirred until excess stain was removed from them (approximately 12 hours).

Stained gels were scanned at 600 nm with a Densicord recording electrophoresis densitometer (Photovolt Corp.) equipped with an Integraph automatic integrator (model 49, Photovolt Corp.). The relative area of histone fractions was determined by counting the number of marks made by the integrator under each peak.

Preparation and Characterization of
Drosophila DNA and Chromatin

DNA was extracted from purified chromatin by the technique described by Marmur (1961). The DNA was purified by addition of crystalline bovine pancreas ribonuclease (Calbiochem) at a concentration of 50 µg/ml and incubated for 1 hour at 37°C. Following this, pronase (Calbiochem) was added at a concentration of 1 mg/ml and also incubated for 1 hour at 37°C. The DNA was precipitated with 1 vol. of 95% ethanol and suspended in .1 x SSC (.015 M sodium chloride, .0015 M sodium citrate).

The chromatin and DNA were characterized by ultraviolet absorbance on a Beckman-DBG spectrophotometer over the range 230 to 300 nm. Representative absorption spectra of chromatin and DNA are presented in Fig. 15. Spectral ratios of 260/280 and 260/230 were used as indications for DNA purity.

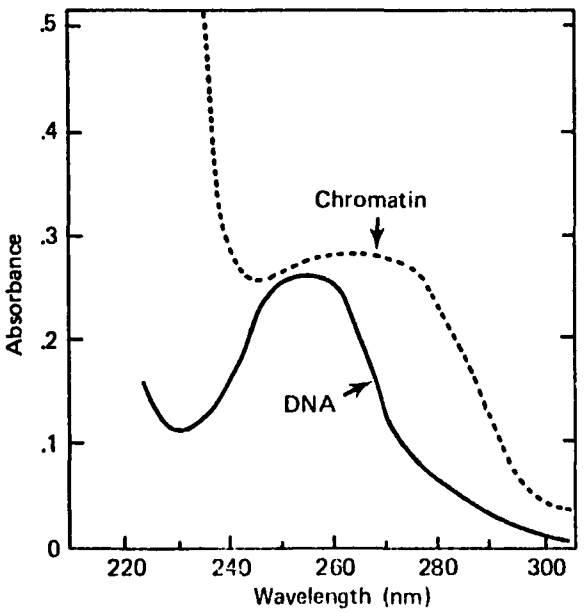

Fig. 15. Ultraviolet Absorption Spectra of *Drosophila* Chromatin and DNA.

Thermal denaturation of DNA was carried out at 260 nm in a Gilford Multiple Sample Recording Spectrophotometer by the technique described by Bonner et al. (1968). A representative thermal denaturation curve of DNA is presented in Fig. 16.

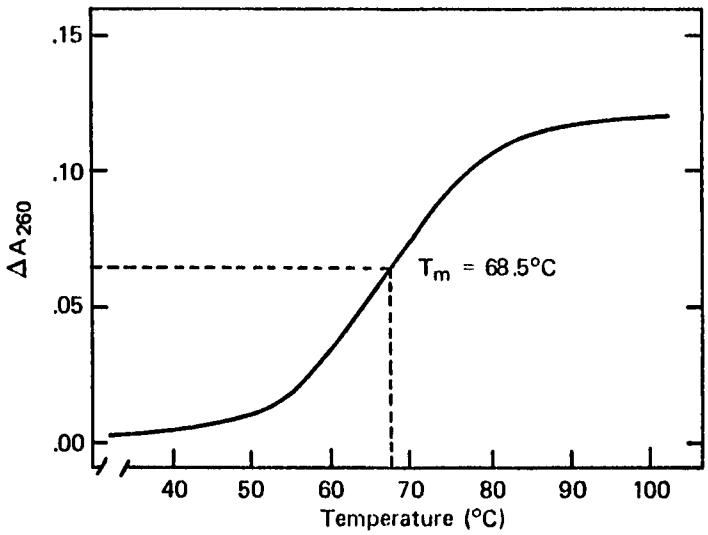

Fig. 16. Thermal Denaturation Curve of *Drosophila* DNA in .1 x SSC pH 7.5.

Results and Discussion

Characterization of *Drosophila* Histones

The electrophoretic pattern of *Drosophila* histone extracted from one-day-old adult flies is presented in Fig. 17A, gel D. A polyacrylamide gel of calf thymus histone (Sigma, Type II) is included as a standard (gel CTH).

The calf thymus histone was fractionated into at least four and sometimes five types. These types were identified as described by Johns

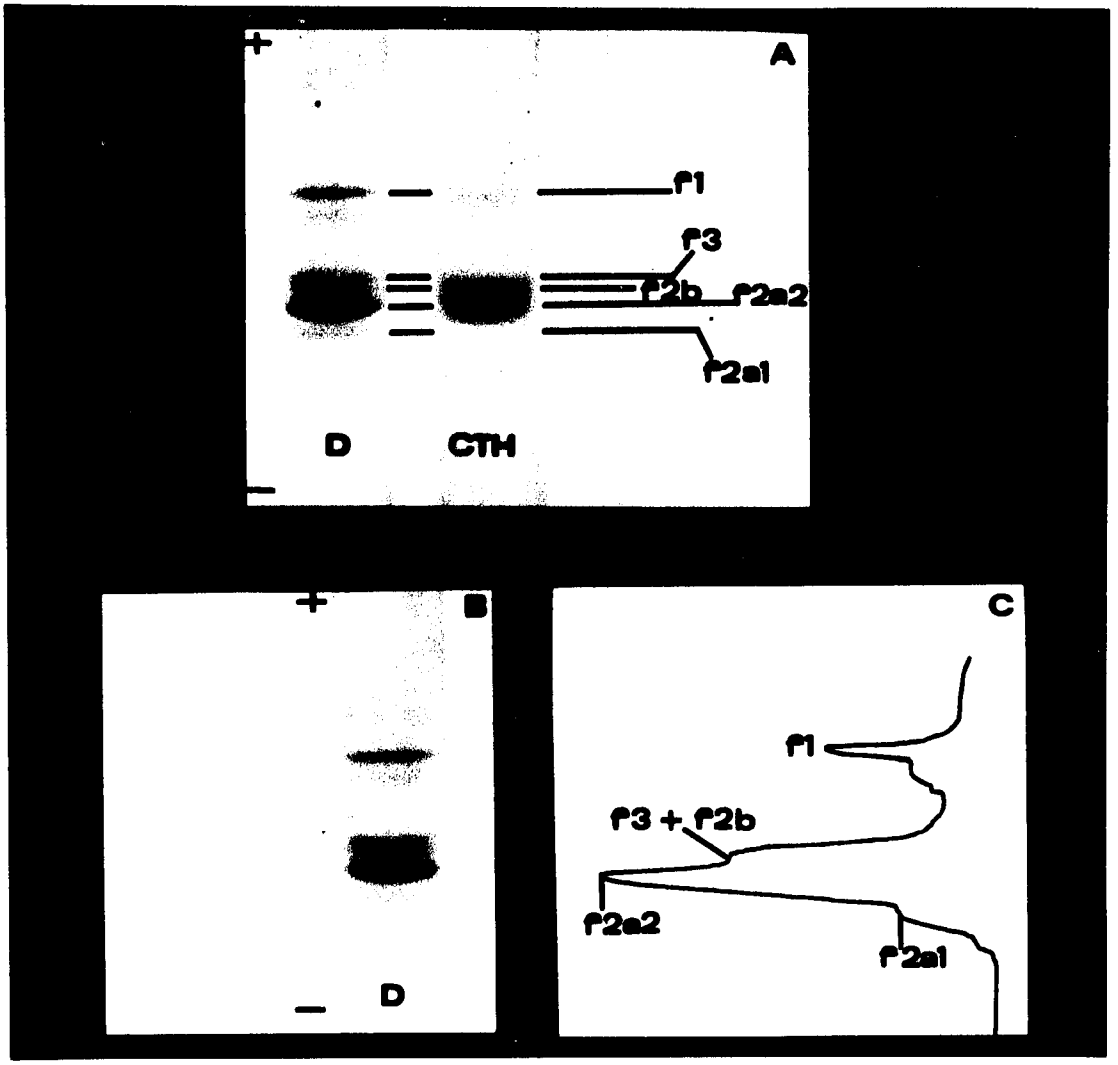

Fig. 17. Electrophoretic Patterns and Characterization of Adult *Drosophila* Histone.

A. Comparison of histone patterns from *Drosophila* (D) and calf thymus (CTH). The five major histone fractions are designated.

B. Typical gel of *Drosophila* adult histone.

C. Densitometric profile of *Drosophila* histone, showing the quantitative relationship between band intensity and peak height.

(1964): f1 (lysine-rich) histone, f3 (arginine-rich) histone + f2b (lysine-rich) histone, f2a2 (slightly lysine-rich) histone, and f2a1 (arginine-rich) histone, in order of increasing mobility.

Characterization of *Drosophila* histone is based solely on the similarity of electrophoretic pattern to that of calf thymus histone. Identification of each fraction was made on the basis of electrophoretic mobility assuming the same order of migration. The fractionation of five major types in short gels (6.5-cm columns) is clearly demonstrated. These fractions are labeled in Fig. 17A.

An electrophoretic comparison between the two diverse organisms demonstrates that four of the five (f3, f2b, f2a2, and f2a1) fractions correspond quite closely in electrophoretic mobility. The most apparent difference is in the f1 (lysine-rich) fraction, which in *Drosophila* generally has a slower electrophoretic mobility in agreement with Cohen and Gotchel (1971) and Oliver and Chalkley (1972a). This difference is depicted more clearly in Fig. 18A (page 70).

The quantitative distribution of *Drosophila* adult histone is shown in the densitometer tracing (Fig. 17C) of a typical gel (Fig. 17B). It should be noted that not all major histone fractions are distinctly resolved into separate curves but instead appear as shoulders of other curves.

As shown in Fig. 17A, minor faint bands migrating slower than the f1 fraction are also detected in *Drosophila*. These bands probably represent contamination of other basic proteins, possibly of cytoplasmic origin, although they could also represent histone degradation from acid extraction. Johns (1964) reported that calf thymus histone is subject

to degradation during acid extraction. Still another possibility for at least one of these bands is dimerization of f3 histone as a result of oxidation during extraction.

The fact that the f1 histone from *Drosophila* has a slower electrophoretic mobility than the corresponding histone from calf thymus may indicate that they differ in chemical properties and/or in amino acid composition. Upon chemical characterization, Cohen and Gotchel (1971) found that the f1 fraction from *Drosophila* had a lower proportion of basic amino acids and also a higher molecular weight than in other organisms. Either of these differences may explain the observed decrease in mobility. Oliver and Chalkley (1972a) reported similar findings but concluded that the reduction in mobility was primarily a function of charge density as a result of lower basicity and only secondarily was due to an increase in molecular weight.

Comparison of Larval and Adult Histone Patterns

As noted previously, in *Drosophila* the genetic constitution of almost every cell is thought to be identical. However, judging by extreme differences in morphology between the various developmental stages, substantial differences in genetic activity must be present. It is reasonable to assume that metamorphic changes in holometabolous insects such as *Drosophila* should be accompanied by drastic qualitative and quantitative changes in genetic expression. A gradual increase in differentiation takes place during development until the fully differentiated stage of the mature adult is attained. One would expect a corresponding gradual increase in genetic activity to accompany the

process of differentiation. If this interpretation is correct, a mechanism that allows for activation and repression of the genome must be operating in eucaryotic cells. It is possible that such a mechanism involves the histones, for it is well recognized that these basic proteins influence DNA-dependent RNA synthesis. Changes in histone pattern may reflect changes in genetic activity.

Electrophoretic patterns of larval and adult histone are compared in Fig. 18A, gels 1 and 3, respectively. A gel of calf thymus histone is included as a standard (gel 2). Comparisons are made from visual examination of banding patterns as well as from quantitative data obtained from densitometric profiles. It is assumed that band intensity is a direct measurement of histone content. This is borne out by densitometer tracings (Figs. 18B and 18C), which indicate that the curves are proportional to band intensity under a constant wavelength.

These electrophoretic patterns demonstrate that both one-day-old adult flies and early third instar larvae contain the five histone types described in the previous section. The electrophoretic mobility of these fractions appears to be very similar in both growth stages and is not substantially different from calf thymus histone, with the exception previously noted of the f1 histone. However, striking quantitative differences and a possible qualitative difference between the two stages are present. These variations are clearly depicted in the photograph shown in Fig. 18A as well as in the densitometric profiles of larval (Fig. 18B) and adult (Fig. 18C) histone.

One of the most obvious quantitative differences occurs in the f1 (lysine-rich) fraction, which in the larval pattern appears as a

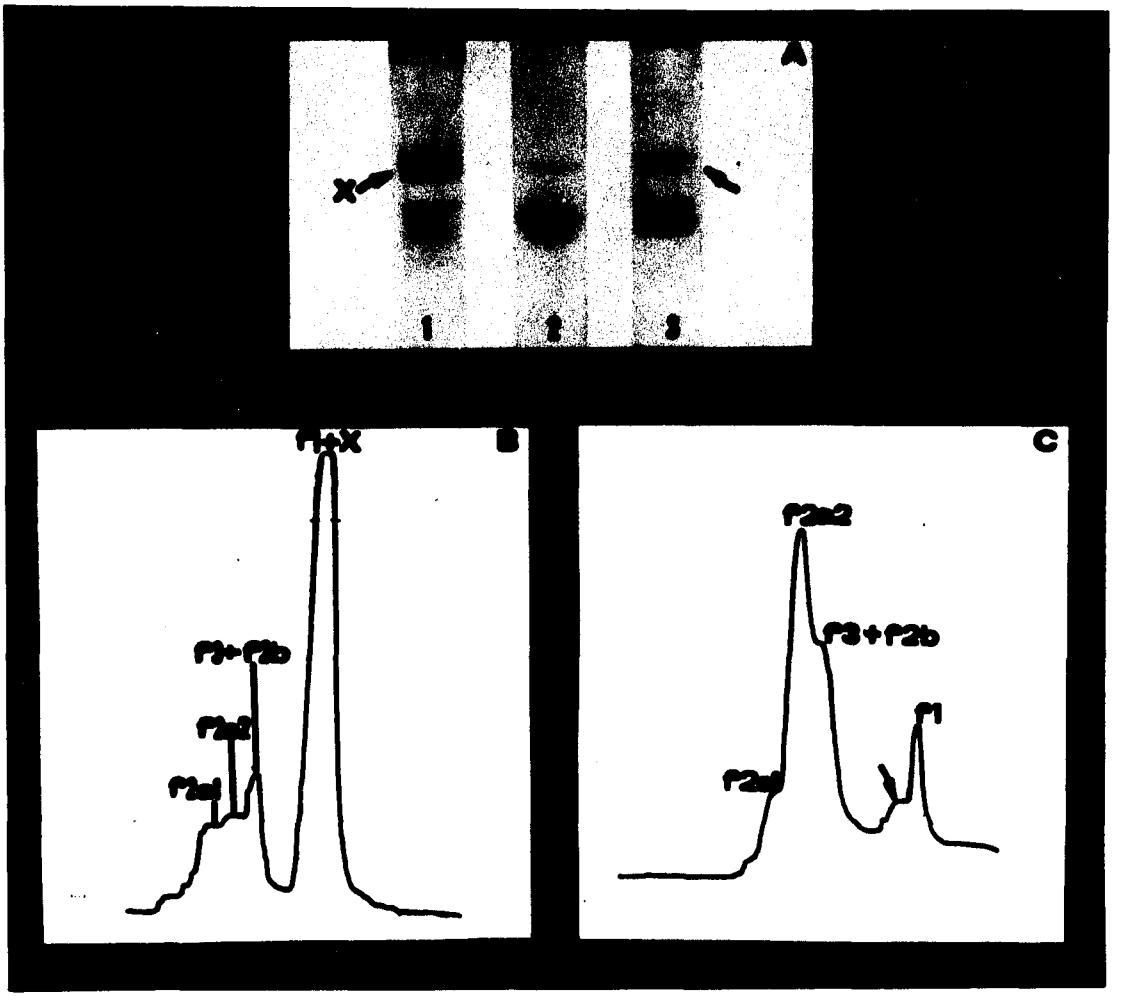

Fig. 18. Electrophoretic Comparison of Histone from Early Third Instar Larvae and One-Day-Old Adult *Drosophila*.

A. Comparison of larval (1), calf thymus (2), and adult (3) histone patterns. The symbol X denotes a possible larval-specific fraction. The arrow denotes a faint band in the adult pattern that may correspond to fraction X.

B. Densitometric profile of larval histone.

C. Densitometric profile of adult histone.

heavily stained band. A marked decrease in intensity of this band is observed in the adult histone pattern.

Another striking quantitative and possibly qualitative difference is the presence in larvae of a very intense band (labeled X in Fig. 18A) with an electrophoretic mobility slightly greater than that of the f1 fraction. A fraction corresponding to fraction X has been reported in *Drosophila* larvae of different species by other investigators (Williams, 1971; Oliver and Chalkley, 1972b). A corresponding fraction in the adult histone pattern was not detected, however. Thus, these investigators concluded that this fraction was specific to the larval state. Furthermore, based on studies of solubility properties of this fraction, Oliver and Chalkley (1972b) suggested that this fraction might not be a histone and might not be complexed directly with DNA but might instead be complexed with histone. The results obtained in this study indicate that a faint band (indicated by an arrow in Figs. 18A and 18C) with similar mobility to that of band X is also present in the adult pattern. This band is sometimes extremely faint and may not be detected in photographs and densitometric profiles. It is possible that the two fractions are identical, the only difference being one of quantity instead of quality. However, it is also possible that this faint band detected in the adult pattern represents a contaminating basic protein, possibly of cytoplasmic origin. Further work, including studies with different procedures of histone extraction and purification as well as chemical characterization of the two fractions in question, is needed in order to determine whether they are histones and also their similarity.

Quantitative changes of other histone fractions are also detected as indicated by differences in band intensities (Fig. 18A) and relative content (Figs. 18B and 18C). Substantial increases in the proportions of fractions f3+f2b and f2a2 occur in the adult pattern. The proportion of fraction f2a1 is about the same in both growth stages, however.

Table 11 lists the mean proportion of histone based on two different preparations. Fractions were pooled into three categories since they were not always completely resolved into distinct peaks in densitometric profiles. These categories are (1) f1+X, (2) f1+faint band and f2+f2b+f2a2+f2a1, designated as $f_{(pooled)}$ in subsequent discussions, and (3) a ratio of f1+X to $f_{(pooled)}$ for larvae and f1+faint band to $f_{(pooled)}$ for adults. This last category is included as a convenient indicator of gross changes in proportions; these ratios are different in the two stages, indicating that quantitative changes have occurred in histone composition.

Table 11. Relative Proportion of Histone Fractions in Early Third Instar Larvae and One-Day-Old Adults.

Each value represents the average of three extractions.

Ore	Histone fraction		Ratio of
	f_1	$f_{(pooled)}$[a]	$f_1 : f_{(pooled)}$[a]
Larvae	45.10[b]	54.90	0.82
Adults	35.24[c]	64.76	0.54

[a] $f_{(pooled)}$ includes fractions f3, f2b, f2a2, and f2a1.
[b] Larval f_1 proportion includes fraction X.
[c] Adult f_1 proportion includes fraction corresponding to fraction X.

Fractions f1+X make up 45% to 55% of the total histone extracted from larvae. However, f1+faint band makes up only 30% to 35% of the total histone in adult flies. This suggests that the $f_{(pooled)}$ fraction increases relative to fraction f_1 in the adult flies. Specific fractions that account for this increase were discussed previously.

Earlier investigations (Williams, 1971; Oliver and Chalkley, 1972b) failed to show any quantitative differences between larval and adult histone. One possible explanation for this might be that larvae of a different state of differentiation were used, late as opposed to early third instar larvae. The precise meaning of these quantitative differences in relation to genetic activity cannot be determined from this experiment. One can only speculate using facts previously established.

If histones do function as generalized repressors, as has been suggested, one might expect to find quantitative changes in histone during the course of development, reflecting steps in the process of differentiation. Such quantitative variations are detected between *Drosophila* larvae and adults.

In early third instar larvae, which should be undergoing differentiation, one would expect a more "temporary" type of genetic repression in order to facilitate sequential activation of structural genes at later stages of development, while in the fully differentiated adult fly whose somatic tissues are post-mitotic and no longer dividing one would expect a more "permanent" type of repression. The results obtained in this study indicate that specific fractions are involved in this quantitative change. This may suggest that specific histone fractions may be

involved in genetic repression in different growth stages. Furthermore, the type or degree of repression elicited by particular fractions may also be different, such as "temporary" opposed to more "permanent" repression. Increase in the ratio of arginine-rich to lysine-rich histones on differentiation of embryonic plant cells has been reported by Srivastava (1971). Indications are that arginine-rich histones bind more strongly to DNA and are better inhibitors of DNA-dependent RNA synthesis (Allfrey and Mirsky, 1963). A "permanent" repression of genes specific to earlier metamorphic stages could be expected in fully differentiated cells.

By contrast, Srivastava (1971) also found a higher proportion of f1 (lysine-rich) histone in young tissues. Similar findings have been reported by Agrell and Christensson (1965) for chicken embryos. It is thought (Jensen and Chalkley, 1968) that f1 histone forms crosslinks between DNA strands. This interaction is believed to be weak and easily broken upon chemical modification of f1 histone. These results suggest that f1 histone may be involved in a more "temporary" type of repression such as would be expected in tissue undergoing differentiation. However, these changes could also be attributable to phosphorylation of f1 histone, which is known to occur during development. The possibility that the observed changes merely reflect metabolic changes should not be overlooked. Further studies attempting to correlate RNA-synthesizing activity, template activity of the chromatin, and histone composition should provide more insight as to the significance of these observed quantitative changes in histone composition and their influence on genetic expression.

Determination of Optimal Histone Concentration for Polyacrylamide Gel Electrophoresis

Figure 19A shows polyacrylamide gel separation of *Drosophila* histone extracted from adult flies and which contain an increasing amount of basic protein as determined by Chaykin's (1966) modification of Lowry's protein assay. It should be noticed that the same basic pattern is obtained at all concentrations tested. From these results it was determined that a histone concentration of 60 µg/gel would be an adequate amount to produce good resolution of the major histone fractions. The same basic pattern is obtained from different lines at this concentration as is indicated in Fig. 19B. This is the amount of histone that was applied to each gel in all subsequent studies to be discussed.

Comparison of Histone Composition of F_1 Hybrids and Inbred Controls During Development and Aging

The histone composition of *D. melanogaster* F_1 reciprocal hybrids and inbred controls at various stages of their life cycle was investigated. The electrophoretic patterns are presented in Figs. 20 and 21. Five major histone fractions are recognized in all genotypes at all stages examined. Visual examination of these patterns gives the indication that substantial differences in total histone content, as reflected by over-all differences in staining intensities may be present between hybrids and inbred controls. However, this may be attributable to analytical procedures rather than to true quantitative differences.

The larval histone patterns obtained from hybrid and inbred strains are similar (Figs. 20A and 21A). The data presented in Table 12 indicate that similar proportions of pooled fractions are found in all

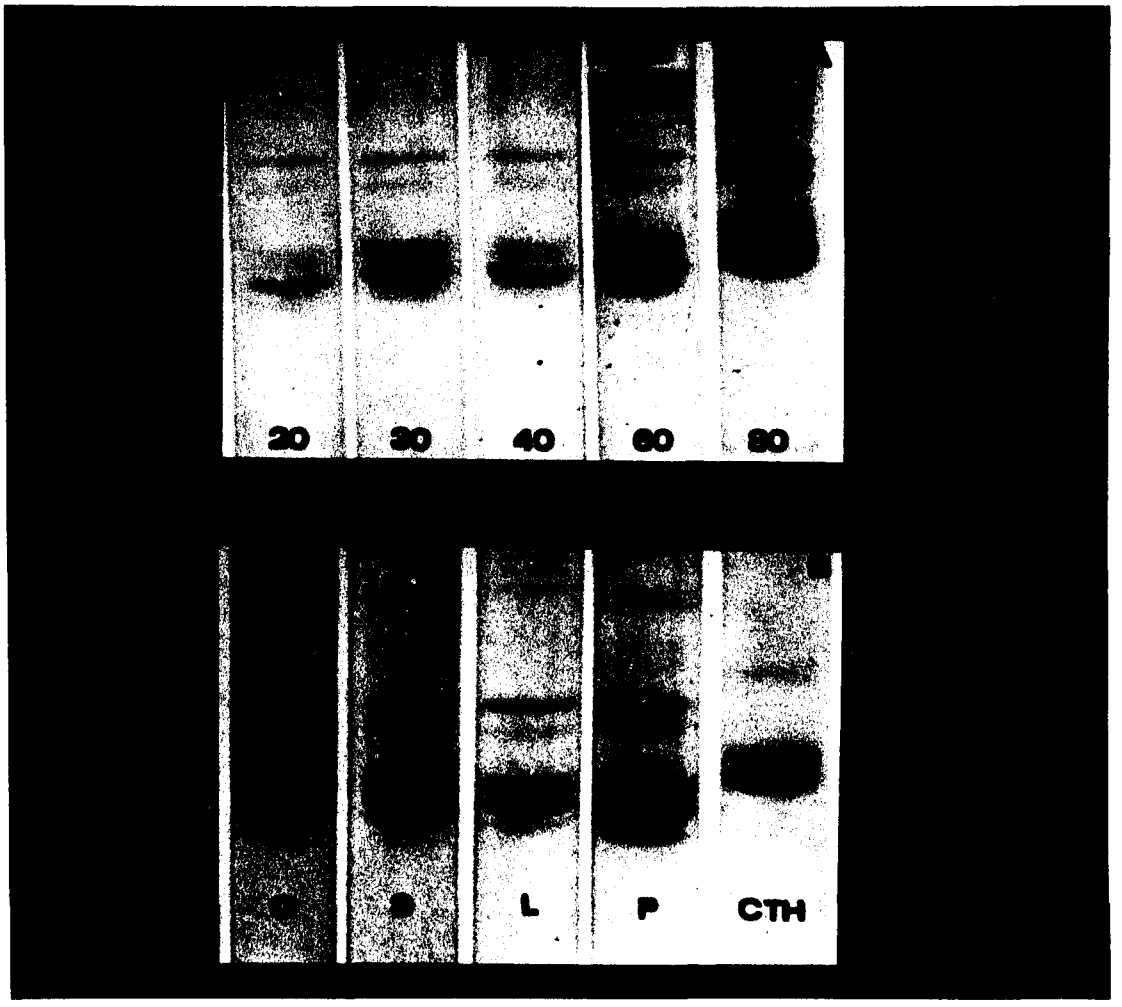

Fig. 19. Electrophoretic Patterns of Adult *Drosophila* Histone at Different Histone Concentrations and from Different Lines.

A. Different concentrations of histone per gel. The number below each gel designates the concentration in micrograms.

B. Histone patterns from different lines at a constant concentration of histone per gel (60 µg). The letter below each gel designates the strain of *Drosophila* or standard used for comparison purposes (O = Ore-RC, S = Swe-C, L = Lau, P = Para^{ts}, CTH = calf thymus histone).

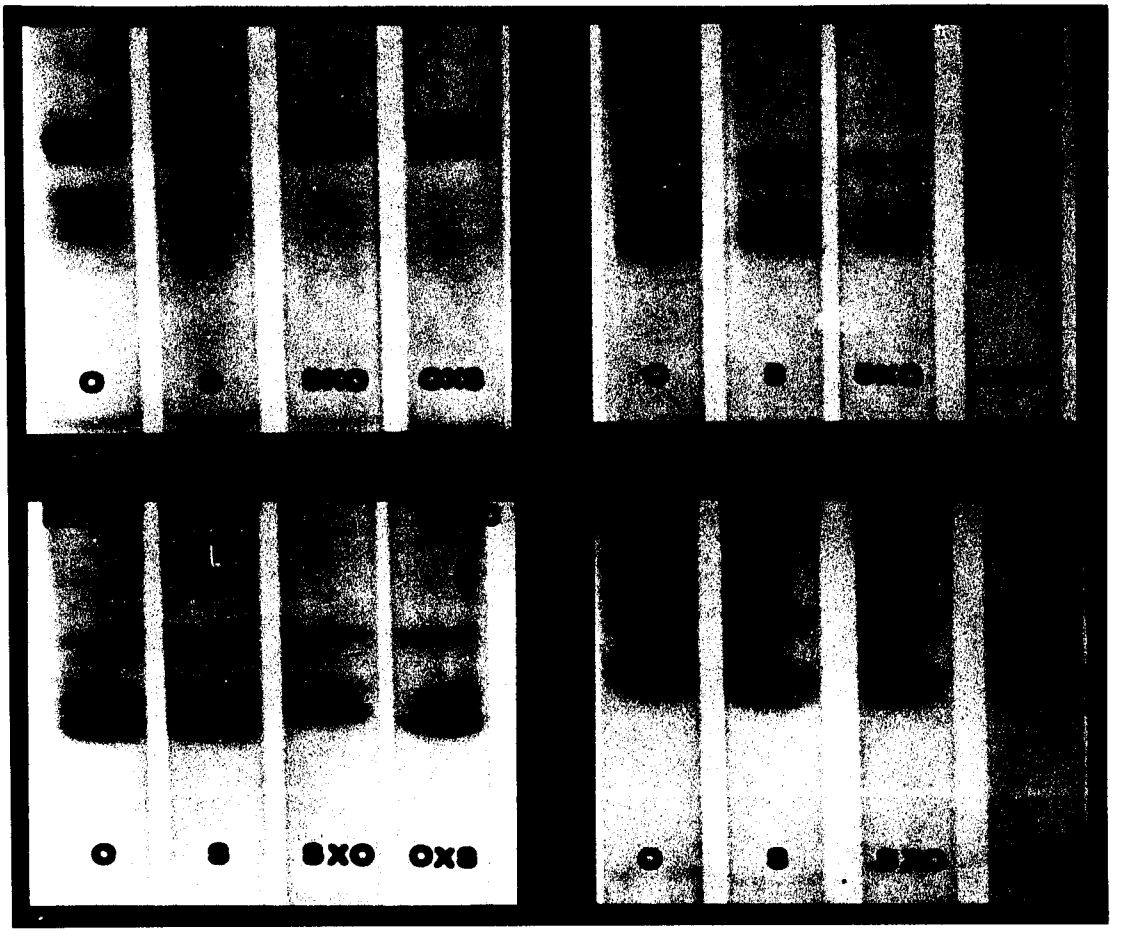

Fig. 20. Electrophoretic Patterns of Inbred Parents, Ore-RC and Swe-C, and Reciprocal Hybrids, SxO and OxS, at Various Stages of Their Life Cycle.

A. Larvae.

B. One-day-old adults.

C. Twenty-one-day-old adults.

D. Thirty-five-day-old adults.

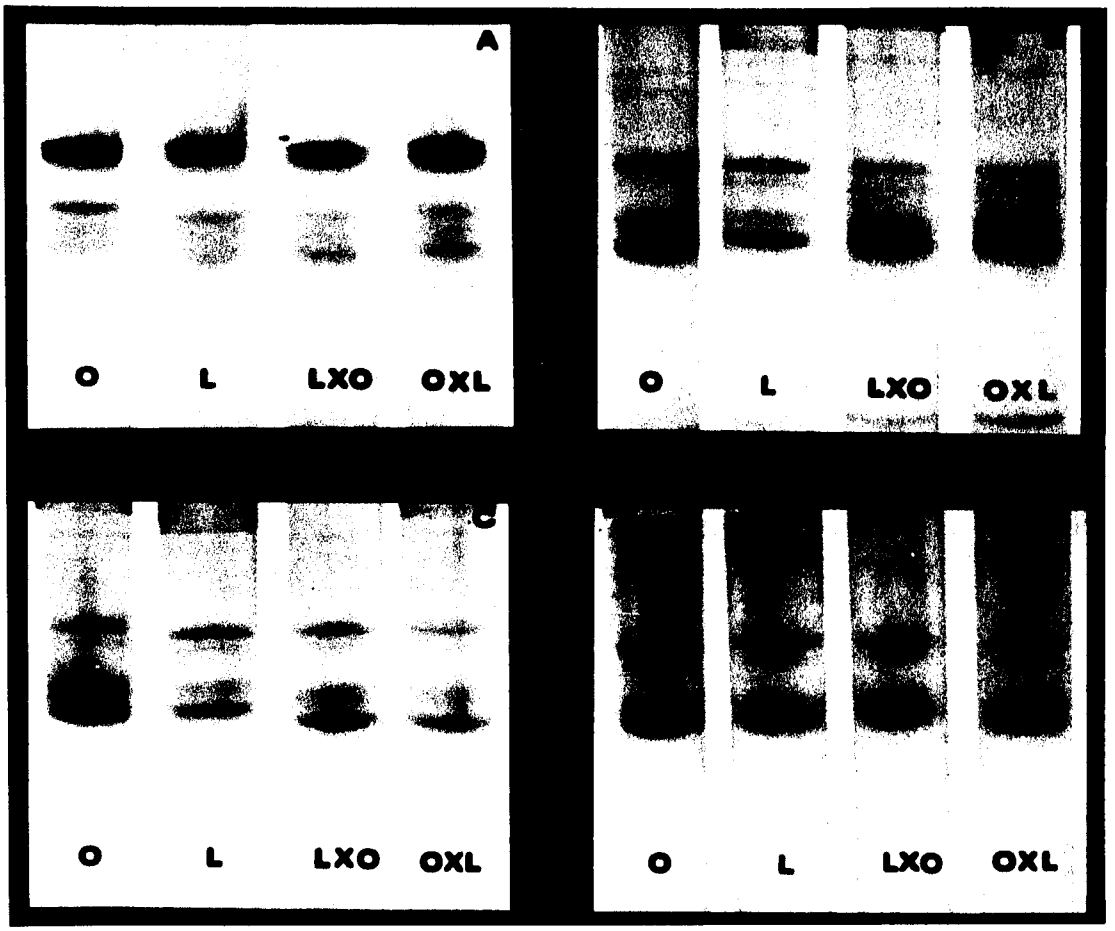

Fig. 21. Electrophoretic Patterns of Inbred Parents, Ore-RC and Lau, and Reciprocal Hybrids, LxO and OxL, at Various Stages of Their Life Cycle.

A. Larvae.

B. One-day-old adults.

C. Twenty-one-day-old adults.

D. Thirty-five-day-old adults.

Table 12. Relative Proportions of Histone Fractions in F_1 Hybrids and Inbred Controls at Different Stages in Their Life Cycle.

Each value represents an average of three extractions.

Genotype	Fraction[a]	Larvae[b]	Adults[c] (age in days) 1	21	35
Ore	f1	45.10	33.33	23.81	25.00
	$f_{(pooled)}$	54.90	66.67	76.19	75.00
	Ratio	0.82	0.50	0.31	0.33
Swe	f1	48.31	37.50	23.72	26.86
	$f_{(pooled)}$	51.69	62.50	76.28	73.14
	Ratio	0.93	0.60	0.31	0.37
Lau	f1	52.06	36.36	23.53	25.00
	$f_{(pooled)}$	47.94	63.64	76.47	75.00
	Ratio	1.08	0.57	0.30	0.33
SxO	f1	46.00	30.92	31.81	32.55
	$f_{(pooled)}$	54.00	69.08	68.19	67.45
	Ratio	0.85	0.44	0.46	0.48
OxS	f1	50.00	34.93	31.70	31.57
	$f_{(pooled)}$	50.00	65.07	68.30	68.43
	Ratio	1.00	0.54	0.46	0.46
LxO	f1	45.93	33.33	23.94	26.50
	$f_{(pooled)}$	54.07	66.67	54.06	74.50
	Ratio	0.83	0.50	0.31	0.36
OxL	f1	47.54	34.54	25.50	24.62
	$f_{(pooled)}$	52.40	65.46	74.50	75.38
	Ratio	0.91	0.53	0.33	0.32

[a] $f_{(pooled)}$ includes fractions f3, f2b, f2a2, and f2a1.
[b] Larval f1 proportion includes fraction X.
[c] Adult f1 proportion includes fraction corresponding to fraction X.

genotypes. Similar quantitative differences in histone composition, as discussed in the previous section, between larvae and adult flies occur in both hybrids and inbreds.

Electrophoretic patterns of histone extracted from adult flies at different age intervals are depicted in Figs. 20B, C, and D and 21B, C, and D. Quantitative differences between heterotic hybrid (SxO and OxS) and inbred adult patterns are indicated by the data in Table 12. The f1:$f_{(pooled)}$ ratio is considerably higher in the heterotic hybrids at 21 and 35 days. This indicates that the proportions of $f_{(pooled)}$ and f1 fractions changed only slightly from day 1 in these flies.

Heterotic hybrids used in this experiment demonstrated heterosis in terms of greater longevity and greater body weight (Table 1). The genetic nature of heterosis has not been adequately explained. The functional significance of the greater f1:$f_{(pooled)}$ ratios found in the heterotic hybrids at 21 and 35 days may be related to heterosis. One possibility is that these variations may reflect differences in genetic expression between heterotic hybrid and inbred flies. If, as suggested previously, some specific fractions bind more intensely to DNA and are better inhibitors of DNA-dependent RNA synthesis, a higher proportion of these fractions should produce more repression. On the other hand, weak DNA:f1 histone bonds should be more easily broken upon interaction with the RNA polymerase enzyme or upon chemical modification. If these interpretations are correct, one should expect quantitative and possibly qualitative differences in genetic activity. The results observed in this study appear to be compatible with this view.

Age-correlated quantitative differences in histone composition within each line and between heterotic hybrids and inbred flies are also indicated by the data in Table 12. The same trend, namely a decrease in the $f1:f_{(pooled)}$ ratio between day 1 and day 21, is exhibited by all genotypes. However, this decline is not as great in heterotic flies. This decline is probably attributable to an increase in the $f_{(pooled)}$ fraction and a corresponding decrease in f1 histone. No substantial change in the ratio is found between 21 and 35 days within each line. The $f1:f_{(pooled)}$ ratio for heterotic hybrids is still greater than that of the inbred controls at 35 days.

Table 13 lists mean histone:DNA ratios for larvae and adult flies. These ratios are based on two separate preparations. Statistical analysis to determine whether the differences are significant was not carried out because these data were too variable to make such comparisons meaningful.

Table 13. Histone/DNA Ratios for F_1 Reciprocal Hybrids and Inbred Parents at Various Age Intervals.

Each value represents the average of two extractions.

Genotype	Larval	Adult (age in days) 1	21	35
Ore	1.15	2.00	2.28	1.76
Swe	1.63	1.13	2.31	2.28
Lau	1.79	1.32	2.00	2.04
$Para^{ts}$	1.46	1.69	1.59	1.52
SxO	1.67	1.71	1.90	1.48
OxS	1.31	2.40	2.69	1.92
LxO	0.95	1.39	2.59	1.88
OxL	1.64	1.48	3.90	1.95

The ratios ranged from 0.95 to 3.90, indicating high variability. The fact that some ratios are close to the theoretical ratio of 1.0-1.3 (Bonner et al., 1968) suggests that this variation is probably attributable to analytical procedures during DNA and histone assays, although this does not necessarily mean that DNA ratios that deviate considerably from the theoretical ratio cannot occur *in situ*. It is possible that histone and DNA synthesis do not proceed simultaneously or that there may be no direct connection between their synthesis. In both cases, a histone:DNA ratio deviating from the theoretical ratio would not be unlikely.

It is known that differentiation involves changes in the relative activities of different genes. Aging could be a consequence of such changes. For example, in *Drosophila* adults all somatic cells are post-mitotic and may lose the capacity to synthesize essential proteins because no further messenger-RNA can be produced owing to increased repression of the genome. Such "permanent" repression could lead to decreased cellular homeostasis, cell death, and eventually death of the adult organism because these cells are not replaced. Hahn and Verzar (1963) have suggested that aging takes place in the chromatin of cells and that it appears to involve the structural relationship of DNA with histone. The exact nature of this relationship and how it might change with advanced age is not known. The data of Hahn and Fritz (1966) shows an increased stability of chromatin with advanced age that suggests that a change in the binding intensity of histone to DNA might take place. This change could result from quantitative changes of specific fractions such as an increase in histone fractions that bind more strongly

to DNA. The data obtained in this experiment appear to be compatible with this line of thought. A decrease in weakly interacting f1 histone and a corresponding increase in strongly interacting fractions may occur. One possibility for the longer life span of heterotic hybrids could be a delay in the synthesis of the latter types of histones, which would be reflected in less "permanent" repression of the genome during the period in question. However, it must be realized that these are only speculations based on previously established evidence. Further work, including *in vitro* studies of template activity of chromatin from aging hybrid and inbred flies should provide a better understanding of what these differences mean in terms of histone function as well as their relation to the "heterosis concept" and the "aging process."

Comparison of Histone Patterns from Para^{ts} Mutants at Various Stages of Their Life Cycle

Electrophoretic patterns of histone extracted from the paralytic-temperature-sensitive mutant Para^{ts} at different stages of development and maturation are presented in Fig. 22. The same basic histone pattern and quantitative changes between larvae and adult flies as observed for wild-type strains are detected. No qualitative changes in adult histone composition that could be correlated with either advanced aging or the mutation are evident.

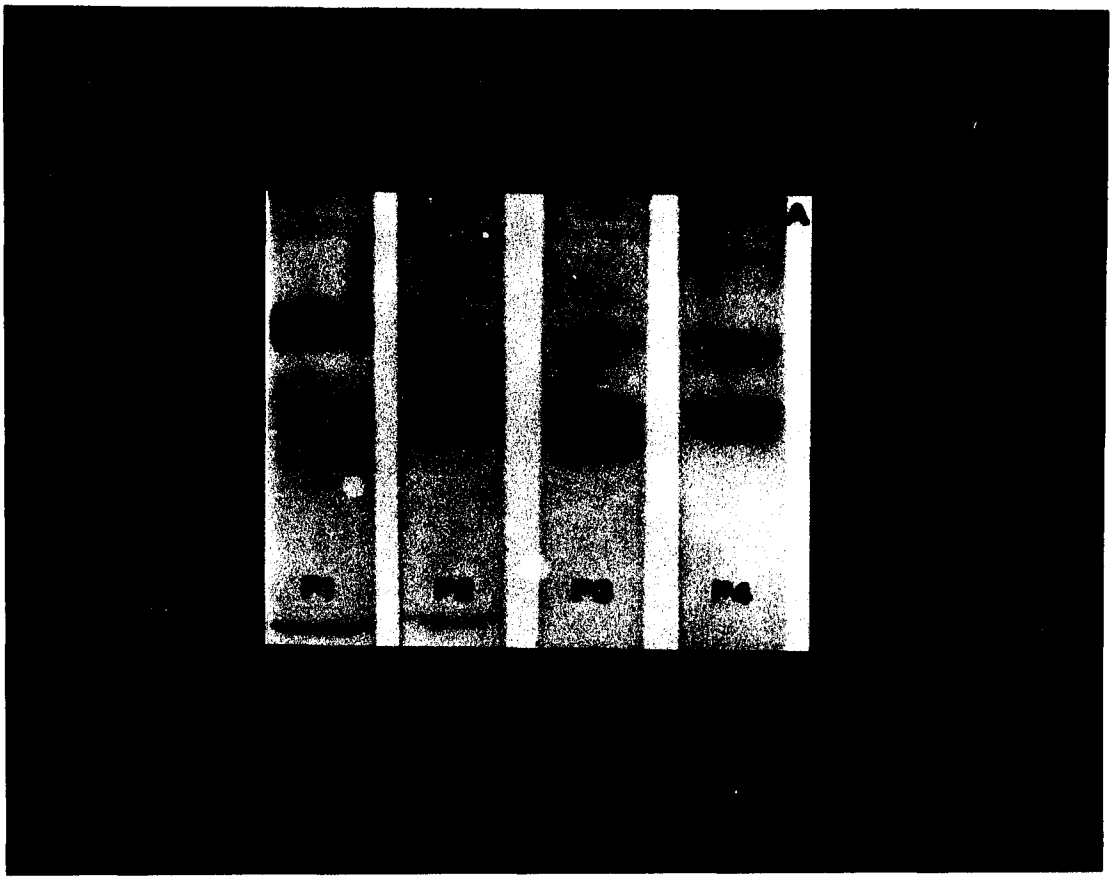

Fig. 22. Electrophoretic Patterns of Para^{ts} Histone, at Various Stages of Their Life Cycle.

The designation below each gel indicates the stage (P1 = larvae, P2 = 1-day-old adults, P3 = 21-day-old adults, P4 = 35-day-old adults).

SUMMARY

Differences and temporal changes of mitochondrial activity and histone composition were investigated in *D. melanogaster* F_1 reciprocal hybrids and inbred controls.

Mitochondria were isolated from intact flies and used in biochemical studies. Mitochondria of *Drosophila* oxidized pyruvate and alpha-glycerophosphate quite readily. However, acetate was oxidized more slowly with little evidence of coupling. These results appear to indicate that pyruvate and alpha-glycerophosphate may be important substrates in the respiratory metabolism of adult flies and that they readily penetrated the mitochondrial membrane. ADP:O ratios higher than the theoretical ratio were observed with pyruvate. The higher ratios were probably the result of oxidation of endogenous substrates.

The addition of exogenous magnesium ions to the reaction medium resulted in lower efficiency of phosphorylation (ADP:O) with both pyruvate and alpha-glycerophosphate. Respiratory control was also decreased with pyruvate. These changes were related to enhanced steady-state levels of ADP resulting from increased Mg^{2+}-ATP-ase activity.

Heterosis in terms of longer life spans and heavier body weights was exhibited by one reciprocal cross. One of the hybrids also exhibited enhanced mitochondrial activity over the theoretical midparent (mitochondrial heterosis). Furthermore, *in vitro* mixtures of the parental lines that produced the heterotic hybrid also exhibited higher mitochondrial efficiency than the midparent average (mitochondrial

complementation). The reciprocal hybrid did not show consistent mitochondrial heterosis, however. Nonheterotic hybrids and 1:1 mitochondrial mixtures of their inbred parents did not show enhanced mitochondrial activity. Efficiency of energy conservation (ADP:O) of the heterotic hybrid, SxO, and the parental mitochondrial mixture, OxS, over all age intervals was significantly higher than the highest parent. These results were significant with respect to the functional mechanism of mitochondrial heterosis, mitochondrial polymorphism, and mitochondrial inheritance.

Aging effects were observed in all parameters measured (ADP:O, respiratory control, and State 3 oxidation). However, hybrids and parents behaved similarly with respect to any particular parameter although substantial differences in the rates of change of some of these parameters occurred. These results were consistent with genetic control of respiratory metabolism, mitochondrial damage, and ability to oxidize substrate during aging.

Electrophoretic patterns of *Drosophila* histone extracted from two different growth stages and from adult flies at various age intervals were compared. Five major fractions were identified from adult flies. The histone pattern of adult histone was similar, with the exception of histone f1, to the pattern observed from calf thymus histone, which was used as a standard.

Substantial quantitative differences and a possible qualitative change were observed between early third instar larval and adult histone. The significance of these differences with respect to genetic activity and genetic regulation were discussed.

Age-related quantitative changes were detected in both hybrids and parents. However, no qualitative changes in the histone pattern were observed with advanced aging. Differences in the proportional changes of specific fractions were observed between heterotic hybrids and inbred parents. Histones were also extracted from a paralytic temperature-sensitive mutant. No qualitative changes in histone pattern correlated with the mutation were detected.

APPENDIX A

SUPPLEMENTARY TABLES

Table A-1. ADP:O Values of Inbred Parents and Reciprocal F_1 Hybrids at Different Age Intervals.

Genotype	Age in days					
	1	7	14	21	28	35
Ore	3.05	3.32	3.22	3.28	3.58	3.18
Swe	3.18	2.88	3.13	3.54	3.61	3.45
Urb	3.11	2.97	3.00	3.72	3.65	3.35
Lau	3.49	3.47	3.54	3.79	3.64	3.21
SxO	3.49	3.48	3.33	3.56	3.29	3.46
OxS	3.22	3.39	3.17	3.35	3.11	3.55
LxO	3.19	3.04	3.27	3.42	3.26	2.80
OxL	3.25	3.10	3.19	3.07	3.19	3.02
UxO	3.03	3.03	3.11	3.24	3.04	3.12
OxU	2.90	3.16	3.17	3.14	3.21	3.00
LxS	3.34	3.36	3.20	3.43	3.32	3.30
SxL	2.62	2.86	3.19	3.01	3.09	3.06
UxS	2.70	2.81	3.15	3.21	2.83	3.01
SxU	3.04	2.93	3.16	3.09	3.29	3.33
LxU	3.29	3.18	3.04	2.90	3.33	3.22
UxL	2.98	3.12	3.15	3.17	3.05	3.06

Table A-2. Analysis of Variance of ADP:O Ratios from Representative Polarograph Data from Experiments of Mitochondrial Heterosis and Complementation in *Drosophila melanogaster*.

Source	Deg. of freedom	ADP:O ratios	
		Mean square	F value
Replication (R)	2	.2766	1.50
Genotype (G)	8	.3146	1.70
Error A (R x G)	16	.1842	--
Cycles (C)	4	2.6337	79.57*
Cycle x Genotype (C x G)	32	.0528	1.59
Error B (RC + RCG)	72	.0331	--

Significance of F value is indicated by *, 1% level. Replication = each mitochondrial preparation. Cycle = each transition from State 3 to 4.

REFERENCES

Adelman, R. C., 1972. Age-dependent control of enzyme adaptation, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 4, pp. 1-24.

Agrell, I. P. S., and E. G. Christensson, 1965. Changes of histone composition in the developing chick embryo. Nature 207:638-640.

Agrell, I. C. S., and N. O. Lindh, 1966. Changes in basic proteins during the metamorphosis of the fly *Calliphora erythrocephala*. Comp. Biochem. Physiol. 19:691-698.

Akinrimisi, E. O., J. Bonner, and P. O. P. T'so, 1965. Binding of basic proteins to DNA. J. Mol. Biol. 11:128-136.

Allfrey, V. G., R. Faulkner, and A. E. Mirsky, 1964. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc. Nat. Acad. Sci. U.S. 51:786-794.

Allfrey, V. G., and A. E. Mirsky, 1963. Mechanisms of synthesis and control of protein and RNA synthesis in the cell nucleus. Cold Springs Symp. Quant. Biol. 28:247-262.

Andrew, W., 1964. Changes in the nucleus with advancing age of the organism, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 1, pp. 87-108.

Angelova-Gatava, P., 1969. Tissue respiration and glycolysis in quadriceps femoris and heart of rats of different ages during hypodynamia. Exptl. Gerontol. 4:177-187.

Auer, G., 1972. Cytochemical properties of nuclear chromatin as demonstrated by the colloidal iron binding technique. Exptl. Cell. Res. 75:237-244.

Bakerman, S., 1969. *Aging Life Processes*. Springfield, Ill., Charles C. Thomas, Pub.

Balboni, E. R., 1967. The respiratory metabolism of insect flight muscle during adult maturation. J. Insect Physiol. 13:1849-1856.

Bartley, J., and R. Chalkley, 1970. Further studies of a thymus nucleohistone-associated protease. J. Biol. Chem. 245:4286-4308.

Bekhor, I., G. M. Kung, and J. Bonner, 1969. Sequence-specific interactions of DNA and chromosomal protein. J. Mol. Biol. 39:351-364.

Bender, A. D., C. G. Kormendy, and R. Powell, 1970. Pharmacological control of aging. Exptl. Gerontol. 5:97-129.

Benttinen, L. C., and D. G. Comb, 1971. Early and late histones during sea urchin development. J. Mol. Biol. 57:355-358.

Blumenthal, H. T., and A. W. Berns, 1964. Autoimmunity in aging, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 1, pp. 289-342.

Boardman, N., A. W. Linnane, and R. M. Smillie, eds., 1971. *Autonomy and Biogenesis of Mitochondria and Chloroplasts*. Amsterdam, North-Holland Publishing Co.

Bonner, J., G. R. Chalkley, M. Dahmus, D. Fambrough, F. Fujimura, R. C. Huang, J. Haberman, R. Jensen, K. Marushige, H. Ohlenbusch, B. Olivera, and J. Widholm, 1968, in *Methods in Enzymology*, L. Grossman and K. Moldave, eds., New York, Academic Press, Vol. 12, pp. 3-65 (nucleic acids).

Bonnier, G., 1961. Experiments on hybrid superiority in *Drosophila melanogaster*. II. Rate of development from egg hatching to eclosion. Genetics 46:85-91.

Brewbaker, J. L., 1964. *Agricultural Genetics*. Englewood Cliffs, N.J., Prentice Hall.

Brown, S. W., and U. Nur, 1964. Heterochromatic chromosomes in the coccids. Science 145:130-136.

Bulos, B., S. Shukla, and B. Sacktor, 1972. Bioenergetic properties of mitochondria from flight muscle of aging blowflies. Arch. Biochem. Biophys. 149:461-469.

Burton, K., 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323.

Busch, H., 1965. *Histones and Other Nuclear Proteins*. New York, Academic Press.

Caspari, E., 1956. The inheritance of the difference in the composition of the liver mitochondria between mouse strains. Genetics 41: 107-117.

Chance, B., 1961. The interaction of energy and electron transfer reactions in mitochondria. III. Substrate requirements for pyridine nucleotide reduction in mitochondria. J. Biol. Chem. 236:1555-1561.

Chance, B., and G. R. Williams, 1955. A simple and rapid assay of oxidative phosphorylation. Nature 175:1120-1121.

Chaykin, S., 1966. *Biochemistry Laboratory Techniques*. New York, John Wiley and Sons, Inc.

Chen, J. C., J. B. Warshaw, and D. R. Sanadi, 1972. Regulation of mitochondrial respiration in senescence. J. Cell. Physiol. 80:141-148.

Cheng, E. Y., and L. K. Cutkomp, 1972. Ageing in the honey-bee, *Apis mellifera*, as related to brain ATPases and their DDT sensitivity. J. Insect Physiol. 18:2285-2291.

Chery, M., 1972. University of Arizona, Department of Agronomy and Plant Genetics, personal communication to the author.

Childress, C. C., and B. Sacktor, 1966. Pyruvate oxidation and the permeability of mitochondria from blowfly flight muscle. Science 154:268-270.

Clark, A. M., 1964. Genetic factors associated with aging, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 1, pp. 207-256.

Clark, A. M., and M. Rockstein, 1964. Aging in insects, in *Physiology of Insecta*, New York, Academic Press, Vol. I, pp. 227-281.

Cohen, L. H., and B. V. Gotchel, 1971. Histones of polytene and nonpolytene nuclei of *Drosophila melanogaster*. J. Biol. Chem. 246: 1841-1848.

Comfort, A., 1956. *The Biology of Senescence*. New York, Rinehart and Company, Inc.

Comings, D. E., 1967. Histones of genetically active and inactive chromatin. J. Cell Biol. 35:699-708.

Comings, D. E., 1972. The structure and function of chromatin, in *Advances in Human Genetics*, H. Harris and K. Hirschhorn, eds., New York, Plenum Press, Vol. 3, pp. 237-431.

Crompton, M., and L. M. Birt, 1967. Changes in the amounts of carbohydrates, phosphagen, and related compounds during the metamorphosis of the blowfly, *Lucilia cuprina*. J. Insect Physiol. 13: 1575-1592.

Curtis, H. J., 1971. Genetic factors in aging, in *Advances in Genetics*, E. W. Caspari, ed., New York, Academic Press, Vol. 16, pp. 305-326.

D'Costa, M. A., and L. M. Birt, 1966. Changes in the lipid content during the metamorphosis of the blowfly, *Lucilia*. J. Insect Physiol. 12:1377-1394.

DeLange, R., and E. Smith, 1971. Histones: structure and function. Ann. Rev. Biochem. 40:279-314.

DeLange, R. J., E. L. Smith, D. M. Fambrough, and J. Bonner, 1968. Amino acid sequence of histone. IV. Presence of ε-N-acetyllysine. Proc. Nat. Acad. Sci. U.S. 61:1145-1146.

Dick, C., and E. W. Johns, 1969. Histone from *Drosophila melanogaster*. Comp. Biochem. Physiol. 31:529-533.

Duvick, D. N., 1965. Cytoplasmic pollen sterility in corn. Adv. Genet. 13:1-56.

East, E. M., 1936. Heterosis. Genetics 21:375-397.

Edwards, P. A., and K. V. Shooter, 1969. The interaction of fraction f2b from calf thymus histone with deoxyribonucleic acid. Biochem. J. 114:54P.

Elgin, S. C. R., S. C. Froehner, J. E. Smart, and J. Bonner, 1971. The biology and chemistry of chomosomal proteins, in *Advances in Cell and Molecular Biology*, E. J. DuPraw, ed., New York, Academic Press, Vol. 1, pp. 1-57.

Ernster, L., and Z. Drahota, eds., 1969. *Mitochondria Structure and Function.* FEBS Symposium, Vol. 17, New York, Academic Press.

Fambrough, D. M., and J. Bonner, 1966. On the similarity of plant and animal histones. Biochemistry 5:2563-2570.

Fambrough, D. M., F. Fujimura, and J. Bonner, 1968. Quantitative distribution of histone components in the pea plant. Biochemistry 7:575-584.

Fincham, J. R. S., 1966. *Genetic Complementation.* New York, W. A. Benjamin.

Georgiev, G. P., 1969. Histone and control of gene action. Ann. Rev. Genet. 3:155-180.

Gold, P. H., M. V. Gee, and B. L. Strehler, 1968. Effect of age on oxidative phosphorylation in the rat. J. Gerontol. 23:509-512.

Gowen, J. W., 1952a. Hybrid vigor in *Drosophila*, in *Heterosis*, J. W. Gowen, ed., Ames, Iowa, Iowa State College Press, pp. 474-493.

Gowen, J. W., 1952b. *Heterosis.* Ames, Iowa, Iowa State College Press.

Green, D. E., and H. Baum, 1970. *Energy and the Mitochondrion.* New York, Academic Press.

Green, D. E., and R. Harris, 1969. Conformational basis of energy transduction in mitochondria. FEBS Letters 5:241-245.

Green, D. E., W. F. Loomis, and V. H. Auerbach, 1948. Studies on the cyclophorase system. I. The complete oxidation of pyruvic acid to carbon dioxide and water. J. Biol. Chem. 172:389-403.

Gregory, D. W., R. W. Lennie, and L. M. Birt, 1967. An electron microscope study of flight muscle development in the blowfly *Lucilia cuprina.* J. Roy. Microsc. Soc. 88:151-175.

Greville, G. D., 1969. A scrutiny of Mitchell's chemiosmotic hypothesis of respiratory chain and photosynthetic phosphorylation. Curr. Topics Bioenerg. 3:1-78.

Grimwood, B. G., 1972. Mitochondrial respiration, energy conservation and heterosis in *Cucurbita* and *Drosophila.* Doctoral dissertation, University of Arizona.

Gusseck, D. J., 1972. Endocrine mechanisms and aging, in *Advances in Gerontological Research,* B. L. Strehler, ed., New York, Academic Press, Vol. 4, pp. 105-166.

Hagihara, B., 1961. Techniques for the application of polarography to mitochondrial respiration. Biochim. Biophys. Acta 46:134-141.

Hahn, H. P. von, 1965. Stabilization of DNA structure by histones against thermal denaturation. Experientia 21:90-91.

Hahn, H. P. von, 1966. A model of "regulatory" aging of the cell at the gene level. J. Gerontol. 21:291-294.

Hahn, H. P. von, 1970. The regulation of protein synthesis in the aging cell. Exptl. Gerontol. 5:323-334.

Hahn, H. P. von, 1971. Failures of regulation mechanisms as causes of cellular aging, in *Advances in Gerontological Research,* B. L. Strehler, ed., New York, Academic Press, Vol. 3, pp. 1-34.

Hahn, H. P., von, and E. Fritz, 1966. Age-related alterations in the structure of DNA. III. Thermal stability of rat liver DNA, related to age, histone content, and ionic strength. Gerontologia 12:237-250.

Hahn, H. P. von, and F. Verzar, 1963. Age-dependent thermal denaturation of DNA from bovine thymus. Gerontologia 7:104-107.

Hanson, J. B., R. H. Hageman, and M. E. Fisher, 1960. The association of carbohydrases with the mitochondria of corn scutellum. Agron. J. 52:49-52.

Harman, D., 1972. The biological clock: the mitochondrion. J. Am. Geriat. Soc. 20:145-147.

Herold, R. C., 1965. Development and ultrastructural changes of sarcosomes during honey bee flight muscle development. Develop. Biol. 12:269-286.

Herold, R. C., and H. Borei, 1963. Cytochrome changes during honey bee flight muscle development. Develop. Biol. 8:67-79.

Hnilica, L. S., M. E. McClure, and T. C. Spelsberg, 1971. Histone biosynthesis and the cell cycle, in *Histones and Nucleohistones*, D. M. P. Phillips, ed., New York, Plenum Press, pp. 187-240.

Hobson, G. E., 1971. A study of mitochondrial complementation in wheat. Biochem. J. 124:10P, abstr.

Hogeboom, G. H., W. C. Schneider, and G. H. Palade, 1948. Isolation of intact mitochondria from rat liver: some biochemical properties of mitochondria and submicroscopic particulate matter. J. Biol. Chem. 172:619-635.

Huang, R. C. C., and J. Bonner, 1962. Histone, a suppressor of chromosomal RNA synthesis. Proc. Nat. Acad. Sci. U.S. 48:1216-1221.

Hull, F. H., 1945. Recurrent selection for specific combining ability in corn. J. Am. Soc. Agron. 37:134-145.

Irwin, M. R., 1971. The evolution of two gene systems and of associated hybrid substances in Columbidae. Genetics 68:509-526.

Jensen, R. H., and R. Chalkley, 1968. The physical state of nucleohistone under physiological ionic strength. The effect of interaction with free nucleic acids. Biochemistry 7:4388-4395.

Johns, E. W., 1964. Studies on histones. 7. Preparative methods for histone fractions from calf thymus. Biochem J. 92:55-59.

Johns, E. W., and S. Forrester, 1970. Interactions between histone fractions and DNA. Biochim. Biophys. Acta 209:54-57.

Johnson, F. M., and C. Denniston, 1964. Genetic variation of alcohol dehydrogenase in *Drosophila melanogaster*. Nature 204:906-907.

Jones, D. F., 1917. Dominance of linked factors as a means of accounting for heterosis. Genetics 2:466-479.

Jones, D. F., 1952. Plasmagenes and chromogenes in heterosis, in *Heterosis*, J. W. Gowen, ed., Ames, Iowa, Iowa State College Press, pp. 224-235.

Keilin, D., and E. F. Hartree, 1938. Cytochrome oxidase. Proc. Roy. Soc. (London) Ser. B 125:171-186.

Kischer, C. W., L. R. Gurley, and G. R. Shepherd, 1966. Nuclear histones and early embryogenesis of the chick. Nature 212:304-306.

Kleinman, L., and R. C. C. Huang, 1971. Binding of actinomycin D to calf thymus chromatin. J. Mol. Biol. 55:503-521.

Kormendy, C. G., and Bender, A. D., 1971. Experimental modification of the chemistry and biology of the aging process. J. Pharm. Sci. 60:167-180.

Kurtz, D. I., and F. M. Sinex, 1967. Age related differences in the association of brain DNA and nuclear protein. Biochim. Biophys. Acta 145:840-842.

Langridge, J., 1962. A genetic and molecular basis for heterosis in *Arabidopsis* and *Drosophila*. Am. Nat. 96:5-27.

Lardy, H. A., and S. M. Ferguson, 1969. Oxidative phosphorylation in mitochondria. Ann. Rev. Biochem. 38:991-1034.

Lardy, H. A., and H. Wellman, 1952. Oxidative phosphorylations: rôle of inorganic phosphate and acceptor systems in control of metabolic rates. J. Biol. Chem. 195:215-224.

Larsen, W. J., 1970. Genesis of mitochondria in insect fat body. J. Cell Biol. 47:373-383.

Lehninger, A. L., 1960. Oxidative phosphorylation in submitochondrial systems. Fed. Proc. 19:952-962.

Lehninger, A. L., 1965. *The Mitochondrion*. New York, W. A. Benjamin, Inc.

Lehninger, A. L., 1970. *Biochemistry*. New York, Worth Publishers, Inc.

Lehninger, A. L., 1971. *Bioenergetics*. Menlo Park, California, W. A. Benjamin, Inc.

Leng, M., and G. Felsenfeld, 1966. The preferential interactions of polylysine and polyarginine with specific base sequence in DNA. Proc. Nat. Acad. Sci. U.S. 56:1325-1332.

Lennie, R. W., and L. M. Birt, 1967. Aspects of the development of flight-muscle sarcosomes in the sheep blowfly, *Lucilia cuprina*, in relation to changes in the distribution of protein and some respiratory enzymes during metamorphosis. Biochem. J. 102:338-350.

Lennie, R. W., D. W. Gregory, and L. M. Birt, 1967. Changes in the nucleic acid content and structure of the thoracic mitochondria during development of the blowfly, *Lucilia cuprina*. J. Insect Physiol. 13:1745-1756.

Lints, F. A., 1971. Life span in *Drosophila*. Gerontologia 17:33-51.

Littau, V. C., C. J. Burdick, V. G. Allfrey, and A. E. Mirsky, 1965. The role of histones in the maintenance of chromatin structure. Proc. Nat. Acad. Sci. U.S. 54:1204-1212.

Manwell, C., and C. M. A. Baker, 1970. *Molecular Biology and the Origin of Species*. Seattle, Washington, University of Washington Press.

Marmur, J., 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218.

Marushige, K., and J. Bonner, 1966. Template properties of liver chromatin. J. Mol. Biol. 15:160-174.

Maynard Smith, J., 1966. Theories of aging, in *Topics in the Biology of Aging*, P. L. Krohn, ed., New York, John Wiley and Sons, pp. 1-35.

McClure, M. E., and L. S. Hnilica, 1972. Nuclear proteins in genetic restriction. III. The cell cycle. Sub-Cell. Biochem. 1:311-332.

McDaniel, R. G., 1967. Biochemical studies of mitochondrial heterosis and complementation. Doctoral dissertation, West Virginia University.

McDaniel, R. G., 1971. Mitochondrial heterosis in barley, in *Barley Genetics*, R. A. Nilan, ed., Pullman, Washington, Washington State University Press, Vol. II, pp. 323-337.

McDaniel, R. G., 1972a. Mitochondrial heterosis and complementation as biochemical measures of yield. Nature 236:190-191.

McDaniel, R. G., 1972b. University of Arizona, Department of Agronomy and Plant Genetics, personal communication to the author.

McDaniel, R. G., 1973. Genetic factors influencing seed vigor--biochemistry of heterosis. Seed Sci. Technol. 1:25-50.

McDaniel, R. G., and B. G. Grimwood, 1971. Hybrid vigor in *Drosophila*: respiration and mitochondrial energy conservation. Comp. Biochem. Physiol. 38B:309-314.

McDaniel, R. G., and I. V. Sarkissian, 1966. Heterosis: complementation by mitochondria. Science 152:1640-1642.

McDaniel, R. G., and I. V. Sarkissian, 1968. Mitochondrial heterosis in maize. Genetics 59:465-475.

McDaniel, R. G., and I. V. Sarkissian, 1970. Kinetics of mitochondrial complementation. Physiol. Plantarum 23:335-342.

Mead, C. G., 1964. A deoxyribonucleic acid associated ribonucleic acid from *Drosophila melanogaster*. J. Biol. Chem. 239:550-554.

Medvedev, Zh. A., 1964. The nucleic acids in development and aging, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 1, pp. 181-202.

Medvedev, Zh. A., 1967. Molecular aspects of ageing, in *Aspects of the Biology of Ageing*, H. W. Woolhouse, ed., Cambridge, University Printing House, Vol. 21, pp. 1-28.

Mehlman, M. A., and R. W. Hanson, eds., 1972. *Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria*. New York, Academic Press.

Milkman, R. D., 1967. Heterosis as a major cause of heterozygosity in nature. Genetics 55:493-495.

Oliver, D., and R. Chalkley, 1972a. An electrophoretic analysis of *Drosophila* histones. I. Isolation and identification. Exptl. Cell Res. 73:295-302.

Oliver, D., and R. Chalkley, 1972b. An electrophoretic analysis of *Drosophila* histones. II. Comparison of larval and adult histone patterns in two species of *Drosophila*. Exptl. Cell Res. 73:303-310.

Ord, M. G., and L. A. Stocken, 1970. Changes in the composition of acid extracts of purified echinus nuclei during cleavage stages and from early embryos. Biochem. J. 120:671-672.

Orgel, L. E., 1963. The maintenance of the accuracy of protein synthesis and its relevance to ageing. Proc. Nat. Acad. Sci. U.S. 49:. 517-521.

Packer, L., 1957. Coupled phosphorylation in rat heart muscle sarcosomes. Arch. Biochem. Biophys. 70:290-293.

Panyim, S., and R. Chalkley, 1969. High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. 130:337-346.

Pittenger, T. H., 1956. Synergism of two cytoplasmically inherited mutants in *Neurospora crassa*. Proc. Nat. Acad. Sci. U.S. 42:747-752.

Pullman, M. E., and G. Schatz, 1967. Mitochondrial oxidations and energy coupling. Ann. Rev. Biochem. 36:539-610.

Pÿhtila, M. J., and F. G. Sherman, 1968. Age-associated studies on thermal stability and template effectiveness of DNA and nucleoproteins from beef thymus. Biochem. Biophys. Res. Commun. 31:340-344.

Pÿhtila, M. J., and F. G. Sherman, 1969. Influence of age on rat liver and kidney chromatin. Gerontologia 15:321-327.

Racker, E., 1965. *Mechanisms in Bioenergetics*. New York, Academic Press.

Reznikoff, W. S., 1972. The operon revisited. Ann. Rev. Genet. 6:133-156.

Rockstein, M., 1966. Biology of aging in insects, in *Topics in the Biology of Aging*, P. L. Krohn, ed., New York, John Wiley and Sons, pp. 43-61.

Rockstein, M., and G. T. Baker, eds., 1972. *Molecular Genetic Mechanisms in Development and Aging*. New York, Academic Press.

Rockstein, M., and P. L. Bhatnagar, 1965. Age changes in size and number of the giant mitochondria in the flight muscle of the common housefly (*Musca domestica* L.). J. Insect Physiol. 11:481-491.

Rockstein, M., and D. E. Gutfreund, 1961. Age changes in adenine nucleotides in flight muscle of male housefly. Science 133:1476-1477.

Sacher, G., 1956. On the statistical nature of mortality with special reference to chronic radiation. Radiobiology 67:250-257.

Sacktor, B., 1965. Energetics and respiratory metabolism of muscular contraction, in *The Physiology of Insects*, M. Rockstein, ed., New York, Academic Press, Vol. 2, pp. 484-580.

Sacktor, B., and Y. Shimada, 1972. Degenerative changes in the mitochondria of flight muscle from aging blowflies. J. Cell Biol. 52: 465-477.

Sarkissian, I. V., and R. G. McDaniel, 1967. Mitochondrial polymorphism in maize. I. Putative evidence for *de novo* origin of hybrid-specific mitochondria. Proc. Nat. Acad. Sci. U.S. 57:1262-1266.

Sarkissian, I. V., and H. K. Srivastava, 1969. High efficiency, heterosis, and homeostasis in mitochondria of wheat. Proc. Nat. Acad. Sci. U.S. 63:302-309.

Schwartz, D., 1965. Genetic studies on mutant enzymes in maize. VI. Elimination of allelic isozyme variation by glyceraldehyde treatment. Genetics 52:1295-1302.

Shih, T. Y., and J. Bonner, 1970. Template properties of DNA-polypeptide complexes. J. Mol. Biol. 50:333-344.

Shull, G. H., 1952. Beginnings of the heterosis concept, in *Heterosis*, J. W. Gowen, ed., Ames, Iowa, Iowa State College Press, pp. 14-48.

Slater, E. C., 1969. Energy-conservation mechanisms of mitochondria. FEBS Symp. 17:205-217.

Slater, E. C., 1971. The coupling between energy-yielding and energy-utilizing reactions in mitochondria. Quart. Rev. Biophys. 4: 35-71.

Slater, E. C., J. M. Tager, S. Papa, and E. Quagliariello, eds., 1968. *Biochemical Aspects of the Biogenesis of Mitochondria*. Bari, Adriatica Editrice.

Smart, J. E., and J. Bonner, 1971. Studies on the role of histones in relation to the template activity and precipitability of chromatin at physiological ionic strengths. J. Mol. Biol. 58:675-684.

Spiegel, M., E. S. Spiegel, and P. S. Meltzer, 1970. Qualitative changes in the basic protein fraction of developing embryos. Develop. Biol. 21:73-86.

Spiker, S., and L. Krishnaswamy, 1973. Constancy of wheat histones during development. Planta 110:71-76.

Srivastava, S., 1971. Relationships of histones to RNA synthesis in plant tissues. Physiol. Plantarum 24:27-33.

Stedman, E., and E. Stedman, 1950. Cell specificity of histones. Nature 166:780-781.

Stedman, E., and E. Stedman, 1951. The basic proteins of cell nuclei. Phil. Trans. Roy. Soc. London, Ser. B 225:565-595.

Steele, M. W., W. J. Young, and B. Childs, 1969. Genetic regulation of glucose-6-phosphate dehydrogenase activity in *Drosophila melanogaster*. Biochem. Genetics 3:359-370.

Stellwagen, R. H., and R. D. Cole, 1969. Chromosomal proteins. Ann. Rev. Biochem. 38:951-990.

Strehler, B. L., 1962. *Time, Cells, and Aging*. New York, Academic Press.

Strehler, B. L., 1972. The understanding and control of the aging process, in *Challenging Biological Problems*, J. A. Behnke, ed., London, Oxford University Press, pp. 133-147.

Suzuki, D. T., 1970. Temperature-sensitive mutation in *Drosophila melanogaster*. VI. Temperature effects on development of sex-linked recessive lethals. Develop. Biol. 23:492-509.

Tanguay, R., and K. D. Chaudhary, 1970. Studies on mitochondria. I. Mitochondrial DNA in relation to morphological development of sarcosomes in thoracic muscles of the African desert locust (*Schistocerca gregaria* Forsk). Can. J. Biochem. 49:358-367.

Tantawy, A. O., 1961. Developmental homeostasis in populations of *Drosophila pseudoobscura*. Evolution 15:132-144.

Tarjan, E., and R. W. Von Korff, 1967. Factors affecting the respiratory control ratio of rabbit heart mitochondria. J. Biol. Chem. 242:318-324.

Thaler, M. M., C. L. Malcolm, and C. A. Villee, 1970. Histones in early embryogenesis: developmental aspects of composition and synthesis. J. Biol. Chem. 245:1479-1483.

Tribe, M. A., 1967. Changes taking place in the respiratory efficiency of isolated flight muscle sarcosomes, associated with the age of the blowfly, *Calliphora erythrocephala*. Comp. Biochem. Physiol. 23:607-620.

Tupper, J. T., and H. Tedeschi, 1969. Microelectrode studies on the membrane properties of isolated mitochondria. II. Absence of a metabolic dependence. Proc. Nat. Acad. Sci. U.S. 63:713-717.

Van Dam, K., and A. J. Meyer, 1971. Oxidation and energy conservation by mitochondria. Ann. Rev. Biochem. 40:115-160.

Van den Bergh, S. G., and E. C. Slater, 1962. The respiratory activity and permeability of housefly sarcosomes. Biochem. J. 82:362-371.

Von Korff, R. W., 1965. Metabolic characteristics of isolated rabbit heart mitochondria. J. Biol. Chem. 240:1351-1359.

Vorobyev, V. I., 1969. The effect of histones on RNA and protein synthesis in sea urchin embryos at early stages of development. Exptl. Cell Res. 55:168-170.

Vorobyev, V. I., A. A. Gineitis, and I. A. Vinofradova, 1969. Histones in early embryogenesis. Exptl. Cell Res. 57:1-7.

Wainio, W. W., 1970. *The Mammalian Mitochondrial Respiratory Chain*. New York, Academic Press.

Walford, R. L., 1967. The general immunology of aging, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 2, pp. 159-204.

Walker, A. C., and L. M. Birt, 1969. Development of respiratory activity and oxidative phosphorylation in flight muscle mitochondria of the blowfly, *Lucilia cuprina*. J. Insect Physiol. 15:305-317.

Weinbach, E. C., and J. Garbus, 1959. Oxidative phosphorylation in mitochondria from aged rats. J. Biol. Chem. 234:412-417.

Welch, J. P., 1967. Somatic mutations and the aging process, in *Advances in Gerontological Research*, B. L. Strehler, ed., New York, Academic Press, Vol. 2, pp. 1-36.

Wilhelm, J. A., T. C. Spelsberg, and L. S. Hnilica, 1971. Nuclear proteins in genetic restriction. I. The histones. Sub-Cell. Biochem. 1:39-65.

Williams, G. C., 1957. Pleiotropy, natural selection and the evolution of senescence. Evolution 11:398-411.

Williams, S. H., 1971. A comparative study of histone present during development in the fruitfly, *Drosophila*, the snail, *Biomphalaria glabrata*, and the frog, *Rana pipiens*. Doctoral dissertation, University of Michigan.

Woolhouse, H. W., ed., 1967. *Aspects of the Biology of Ageing*. Society for Experimental Biology Symposium, Vol. XXI. Cambridge, University Printing House.