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Title BIOCHEMICAL STUDY OF AGING IN SEEDS

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A biochemical study of aging was conducted on mitochondria from the embryo axes of germinating new and old soybean seeds. Differential phosphorylative efficiency, an average P/O ratio of 3.03 of the new material compared to 1.44 of the old, was obtained when all the co-factors and substrate were provided. No difference in oxygen consumption of both materials was observed.

In an attempt to delineate some of the changes that took place in the mitochondria of naturally aged soybean seed, the effects of serum albumin, naF, and 2, 4-dinitrophenol as well as the permeability of mitochondria membranes under different sucrose osmotic concentrations were examined. The highest P/O ratios of mitochondria from new and old materials were obtained in the presence of serum albumin. The effect of albumin was mainly in increasing phosphorylation with no change in oxygen uptake. The addition of 0.013M NaF per flask produced no change in oxygen

consumption and phosphorylation on washed mitochondria of both materials. However, the same concentration of NaF did improve the P/O ratio in the unwashed mitochondria in a preliminary experiment of the embryo axes of the new material. 2,4-dinitrophenol used at the concentration of 0.001M per flask reduced slightly the oxygen uptake of the old but had no effect on that of the new material. The P/O ratios of both materials were reduced practically to the same level; namely, to 1.09 and 0.95 in the new and old, respectively. The conductivity of the leachate from the old material, based on any known quantity of mitochondria, was always higher than that of the new in 0.25M, 0.50M and 0.75M sucrose concentrations. Maximum difference in conductivity measured in μmhos between the new and old was obtained in the 0.5M osmotic concentration.

The experimental results indicate that mitochondrial phosphorylative efficiency of old but germinative soybeans are reduced to 48 percent of the new. This reduction is not because of higher content of adenosine triphosphatase in old material, but because of degradation in the integrity of mitochondrial membrane. This kind of degradation might result in uncoupling of electron transporting and phosphorylative systems.

BIOCHEMICAL STUDY OF AGING IN SEEDS

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BIOCHEMICAL STUDY OF AGING IN SEEDS

INTRODUCTION

Seeds of different genera may vary in their life span. Those of willows and poplars may live for a few days, whereas some leguminous seed may survive for hundreds of years. Many investigators have been concerned with and are still interested in the preservation of seed viability for economic reasons. Factors affecting longevity and preservation have been widely studied but very little work has been reported as to the basic changes underlying aging and deterioration of seeds. Aging, nevertheless, is an universal phenomenon occurring in every living organism after its maturity. The rate of aging varies with the genetic make-up of the species and the environmental conditions under which the individual is growing or existing. An understanding of the aging process in biochemical terms is, thus, imperative in developing scientific methods to reduce the rate of aging or to preserve the organism.

In well kept but aged seed, germinability is not impaired, but the vigor or the growth rate of seedlings is often markedly reduced. This lack of vigor is probably due to a limitation either at substrate and/or enzymes levels for growth or of energy supplies. The substrates are usually stored as food reserves in the seed and are not likely to be the limiting factor. The synthesis of enzymes for degradation of food reserves and anabolic processes of growth involves

many steps; first, the inherited macromolecules of deoxyribonucleic acid send proper messenger-ribonucleic acids which in turn relay the information for synthesizing specific protein of an enzyme; second, under a suitable micro-chemical and physical environment, the enzyme catalyzes proper reactions; and third, with a precise combination and coordination of all the substrates, enzymes, and conditions, growth occurs. It would be difficult to locate the site or link responsible for reduction of growth. Therefore, the energy supply of new and aged seed was investigated.

The effect of aging on mitochondrial activities has been chosen as the criteria of this biochemical study owing to the significance of mitochondria in the production of biological energy necessary in cellular metabolism. These organelles contain the organized enzyme systems of the Krebs citric acid cycle, the electron transport, and oxidative phosphorylation. Through their coordinated actions, most of the foodstuff's energy is trapped in chemical bonds of adenosine triphosphate, the main source of energy supply for all the endergonic reactions. Growth is an energy requiring process, and it could be limited by a defective phosphorylation mechanism in mitochondria. Thus, this study concentrates on the phosphorylative efficiency of mitochondria isolated from actively growing embryo axes. Photosynthetic phosphorylation was not investigated as the material used was dark-grown seedlings.

REVIEW OF LITERATURE

Although biologists were aware of the aging phenomenon in plants and animals prior to 1950, very little was known about its precise nature excepting vague generalities and postulates. A great interest, however, has been instigated during the past decade towards a basic understanding of the nature of underlying changes observed in aging processes.

Strehler (79 p. 11) refers to aging as the changes which occur in the post reproductive stage and which result in a decreased survival capacity in the organism. Using different terms, Comfort (14 p. 12) defines it as a biological deteriorative process which renders the organism more susceptible to disease. According to Medawar (52 p. 12) aging is referred to as that change in the bodily faculties, sensibilities and energies that progresses the organism towards death. Owing to the similarities in some basic factors of aging in both animals and plants and to the fact that more investigation on aging has been done with animals than with plants, the following survey of literature thus includes information on aging problems in both animals and plants.

Aging in General

Many "theories" which have been put forward to describe biological aging have been reviewed in a number of recent publications

(15 p. 687, 70 p. 489-506, 77 p. 261-272). These theories may be grouped into three main categories: (a) Those which relate aging to the accumulation of deleterious products of metabolism. Crocker (18 p. 151) in 1948 attributed aging of seeds to accumulation of toxic substances, degeneration of enzymes and accumulation of chromosome aberrations. Lespeschkin (49 p. 167-177) believes that natural death is caused by a gradual poisoning of the cells and a spontaneous decomposition of the principal compounds. It is believed, however, that the accumulation of poisonous products is a result of aging rather than its cause. (b) The "Wear and Tear" theory, as summarized by Comfort (15 p. 687), proposed that organisms wear out as they are subjected to various stresses during their life span. Even though, Stelye and Prioreschi (77 p. 261-272) consider stresses as an important factor in aging, there is no direct experimental evidence to support the validity of this theory as Curtis (20 p. 687-688), using different kinds of stresses on rats, had failed to induce aging phenomenon. (c) The mutation theory which relates aging to the accumulation of deleterious genes by somatic mutation. Bender and Gooch (4 p. 44-53), Curtis (19 p. 104, 20 p. 686-694) and many others argue in support of the view that aging is a result of random somatic mutations or genetic shift. In contrast to the two theories mentioned above, there is a great deal of indirect evidences in support of the mutation theory. Such evidences are listed in the

following: Andrew (1 p. 1402) observed abnormal cell division and abnormal nuclei in senescent animals. Jacobs et al (40 p. 1178-1181) reported increasing abnormal numbers of chromosomes with age in human blood cells. Using pollen abortion as an index of mutation rate, Cartledge and Blakeslee (8 1. 523) observed pollen abortion in less than one percent of plants produced from one year old seed in contrast to ten percent of plants from 7-8 year old seeds when stored under room conditions. When seed of the same species were buried in the soil for twenty two years, a very low rate of pollen abortion was observed (9 p. 492-493). Peto (58 p. 261-262) seems to favor the concept that environment plays an important role in influencing the rate of mutation, since barley seeds exposed to artificial aging by high temperature and humidity show an increasing number of chromosomal aberrations. Chromosomal aberrations were observed in the root tips of 6 and 7 year old seeds of Crepis tectorum L. (54 p. 436). The nuclei of old nerve cells also showed a high frequency of chromosomal abnormality (5 p. 1402). Avery (3 p. 36-37) reported 11 different phenotypes caused by mutation in the progeny of 315 plants grown from 7 to 10 year old seeds. Harrison and Mcleish (35 p. 593-594) found that the loss of viability in lettuce seed is directly correlated with chromosomal breakage observed in the primary roots one day after the onset of germination. No such correlation, however, was observed in onion. In fact, very little

breakage was observed in onion at all levels of germination. This led the authors conclude that chromosomal breakage may be the result rather than the cause of aging.

The strong support of the mutation theory is the fact that radiation "accelerates aging" and is also known as a potent mutagenic agent. Navashin (54 p. 436) believes that mutation is caused by some abnormal intracellular process and may be created or accelerated by x-rays. The changes produced by ionizing radiation are in many cases similar to those observed during natural aging as exemplified by the observations of Sinex (74 p. 1402-1405). He estimated about 30 percent decrease in the total number of viable cells in certain regions of the brain under conditions of normal aging. He also reported a decrease in the number of viable cells as a consequence of radiation injury. In most cases, radiation increases the death rate of animals. The author also reported that ionizing radiation induces aging in mice similar to natural aging and that the shortening of the life span of the irradiated animals is proportional to the amount of radiation absorbed. The sensitivity of mice to x-rays irradiation was found to increase with age (65 p. 1039-1040). In addition to the shortening effect of the life span of the irradiated animals, the longevity of their descendents are also diminished (35, p. 593-594, 64 p. 324-329). This is probably due to harmful mutation induced in the irradiated parents and manifested in the F_1 generation offspring. In an attempt to establish

the similarities between shortening of life by radiation and natural death due to aging, Lindop and Rotblat (51 p. 645-648) observed that non-irradiated and irradiated mice differ in relative age of the onset of diseases associated with aging and death. Consequently, they concluded that radiation-induced aging and natural aging are not identical. It is generally believed that exposure of living organisms to non-lethal doses of radiation accelerates natural aging. On the basis of a detailed analysis of the possible sites of major radiation effects, however, Strehler concluded the following (78 p. 139):

1. "The primary sensitive site of radiation damage is the genetic apparatus of dividing cells, whereas, the principal changes associated with aging appear to take place in non-dividing cells.
2. Certain organisms which show marked age changes are remarkably insensitive to radiation. It, therefore, appears unlikely that they age by somatic mutation processes.
3. Radiation exposure does not produce in certain connective tissue elements (collagen) changes that are analogous to normal age changes.
4. The radiation dose required to double the rate of "aging" of rats (as measured by the rate of increase of mortality rate) should produce about twenty-five times the basal number of mutations (assuming equal effectiveness of radiation on the germ line and soma) rather than a simple doubling; that is, the life-shortening

and mutational efficiencies are apparently quite different."
He summarized from the above conditions that natural aging is more complex in its origin and mechanisms than radiation life-shortening and that somatic mutation can not be considered an important part in the cause of aging.

Recently, errors in protein synthesis have been considered by Medvedev (53 p. 255-265) as possible means that may lead to aging. Curtis (20 p. 690-693) postulated that mutations in the non-dividing cells of living organism are the site of aging, since any mutation (DNA damage) in dividing cells will be eliminated by direct death of the cell or through its struggle for existence with the normal neighboring cells. Wulff et al (93 p. 1373-1375) believes that the basic problem in aging is the formation of faulty messenger RNA, by a damaged DNA, which causes the synthesis of defective enzymes. On the other hand, Oerin and Tanase (55 p. 273-288) postulate that the main cause of errors in the biosynthesis of proteins is due to the changes that occur in the free and total amino acids with age. This was concluded from the investigation carried on 200 children (aged from 9 to 11) versus 200 aged people (between 60 to 93 years). It was found in another investigation on 400 young and old rats that aging is associated with impairment in the biochemical process involving methylation and sulfur metabolism (55 p. 273-279). Demethylation or decrease in methionine concentration and accumulation of

disulfide groups appear to take place with age. The administration of cysteine, vitamin B_6, B_{12}, or folic acid to old animals restored the level of methionine and cysteine and re-established a proper biochemical equilibrium similar to those found in young animals (55 p. 273-279).

Irreplaceable changes in structural configuration and conformation of macromolecules may be considered as another possible factor that contributes to aging. In a number of experiments carried out by many investigators (87 p. 189-190, 71 p. 171, 44 p. 760-762, 62 p. 753-754, 86 p. 410-411) on hemoglobin from young and old, it has been found that its electronegativity increases on aging. It was further reported by Walter (86 p. 410-411) that its oxygen dissociation and its ability to bind chromium decreases as it gets older. He suggested structural changes such as gain or loss of certain functional groups, oxidation of sulfhydryl groups, partial unfolding, conversion of one amino acid to another within the protein molecule, etc., as possible changes that could happen with time and environmental stresses. Due to the complexity of the macromolecules, it is very likely that aging may result in structural changes that will in turn affect their physiological role. Heat may be considered as an important agent in exerting a continuous influence for the alteration of the structure of macromolecules. Collagen of connective tissue, bone, tendon and blood vessels was found to alter its

elasticity when exposed to different temperatures (72 p. 196). Furthermore, the observed changes at normal physiological range, i.e., 38°C, resemble those occurring during senescence. Sinex (73 p. 15-18) also found that temperatures above 38°C resulted in hydrolysis of certain amide groups in callogen. The significance of this hydrolytic process under normal conditions in vivo is not known.

Harman (32 p. 298-300) and Sinex (72 p. 190-197) attributed aging in part to the accumulation of deleterious side effects of free radicals that are produced in metabolic processes and induced by ionized radiation (76 p. 650). The short-lived free radicals like ROO$^{\cdot}$, RO$^{\cdot}$, $^{\cdot}$OH and R$^{\cdot}$, which could be produced from the direct decomposition of peroxides during lipid peroxidation, can be very damaging to proteins, enzymes and other biological compounds in their proximity. Tappel (82 p. 126-127) demonstrated that free radicals of lipid peroxides damage cytochrome C by changing its physical and catalytic properties. He presumed that the free radicals attach themselves to the protein by covalent linkages. Autoxidation that proceeds by a free radical mechanism could be prevented by the use of specific antioxidants (33 p. 267, 82 p. 131-132). Kinetin at the concentration of 10^{-5}M restored the germination of artificially-aged lettuce seed by heat from 11 percent to 90 percent (59 p. 70-71). The author considered that kinetin may act as antioxidant in this instance.

Aging in the Mitochondria

Mitochondria are dynamic labile subcellular structures in that their number, size, shape and complexity, may change in response to physiological and pathological conditions (25 p. 632-633, 21 p. 305-310). Early in 1918, Cowdry (17 p. 307) observed under the light microscope a considerable decrease in number of mitochondria in aged cells. About 30 years later, Payne (57 p. 77-91) reported that mitochondria of the pituitary and adrenal become swollen with age and are converted into pigmented granules. Using the electron microscope, this finding has been confirmed by Andrew (2 p. 167) who observed granulous and short rod types with vesicles in old mice neuron mitochondria in contrast to filamentous types in the young ones. Similarly, Weiss and Lansing (89 p. 460-465) observed enlarged and vacuolated mitochondria in the pituitary of old mice accompanied with membranous fragments.

The behavior of mitochondria as osmometers has been observed by many workers (83 p. 52-67, 39 p. 65-70, 81 p. 336-338, 90 p. 79-91, 56 p. 218-219). The rapid swelling and shrinking behavior is related in part to mitochondria activity and give significant information regarding the permeability of mitochondria to various solutes and co-factors. Many chemicals such as Ag^{+}, Hg^{++}, Ca^{++}, thyroxine, reduced glutathione (GSH), cysteine and others have been shown

to cause swelling of mitochondria (46 p. 946-950, 45 p. 109-116, 81 p. 336-338). GSH in concentrations equivalent to those found in fresh intact rat liver was reported by Lehninger (45 p. 109-116) to cause maximum swelling effect, suggesting that GSH may be an intracellular factor concerned in the balance between swelling and "active contraction of mitochondria. Considerable amounts of proteins and nucleotides were lost from the GSH-induced swollen mitochondria into the suspending medium (47 p. PC8). It is suggested that this factor which is a soluble protein may be concerned in energy coupling, in addition to its effect in the swelling-contraction cycle. Comparable to these observations, Lehninger and Remmert (48 p. 2459-2464) found that artificial aging of mitochondria by incubation at 37°C resulted in the loss of the ability to couple phosphorylation and the simultaneous appearance of an isooctane-extractable uncoupling agent. This agent, designated as "u" factor, stimulates ATPase activity and induces swelling of the rat liver mitochondria.

Kielley and Kielley (42 p. 497-499) have observed a latent ATPase in liver mitochondria which increases in activity upon aging. In another study on the mitochondria of the house fly, Sacktor (69 p. 386) reported a much greater ATPase activity in the finely dispersed mitochondria particles than in the intact ones. From these two findings, it is suggested that the primary effect of aging results in disintegration of mitochondria, and the disintegration in

turn brings about a higher activity of ATPase. This is in parallel with the concept that has been demonstrated and stressed by Keilin and Hartree (41 p. 214-217) and Harman and Feigelson (34 p. 521-524) that any change in the physical structure of the mitochondria will modify its overall activities. Worthy of note in this connection is the finding of Sacktor (69 p. 386) that the high activity of the ATPase in the finely dispersed particles of the house fly mitochondria was not inhibited by fluoride but was by azide and chloromercuribenzoate.

The addition of 2, 4-dinitrophenol (DNP) to freshly prepared mitochondria of rat liver and sarcosomes of house flies induces ATPase activity. Washing the sarcosome pellets, however, results in the complete loss of the DNP induced ATPase activity (11 p. 399-406). The loss of ATPase activity induced by DNP has been observed also during aging of the mitochondrial suspension. The cause of this loss by aging is attributed to the accumulation of saturated and unsaturated fatty acids (12 p. 239-243, 10 p. 1941-1944). According to Bos and Emmelot (7 p. 21-29) and Borst et al (6 p. 509-518), the addition of oleic acid to freshly prepared mitochondria of rat liver inhibits the DNP-induced ATPase activity similar to that by aging. Chefurka (11 p. 399-406) found that the loss of the DNP-induced ATPase activity during aging can be attenuated by the addition of ethylenediamine tetraacetic acid (EDTH) and/or bovine albumin. The effect of albumin is probably due to the binding of fatty acids

whereas EDTA, according to Hulsman et al (37 p. 267-276) acts here by inhibiting the production of fatty acids.

The ability of EDTA to stabilize mitochondria and maintain their activities during aging has been found by many investigators (13 p. 497-499, 75 p. 118-119, 85 p. 308-310) who attribute this action mainly to the chelating power of EDTA to metal ions and particularly to Ca^{++}. Hunter et al (38 p. 697-698), Gallaher (23 p. 162-164) and Lester and Hatefi (50 p. 110-112) believe that the effect of EDTA in maintaining the activity of mitochondria is by preventing the loss of pyridine nucleotides to "leak" out either by maintaining the semi-permeable character of the membrane or by inhibiting the release of pyridine nucleotides from mitochondrial enzyme systems. Gallaher also suggests the possibility that EDTA may exert its influence by complexing with structural cations and altering the charge on the mitochondrial membrane or at the site of binding of pyridine nucleotides (23 p. 163).

Human and bovine serum albumins stimulate oxidative phosphorylation in the larval mitochondria of the wax moth (92 p. 277-289). Similarly, the addition of serum albumin to insect flight muscles' mitochondria improved oxidative phosphorylation and respiration markedly (68 p. 1233-1235). In both cases, it has been shown that albumin exerts this effect by actually binding and removing uncoupling compounds from the mitochondrial preparations (92 p. 277-289,

68 p. 1233-1235). By means of paper chromatography the uncoupling compounds were identified as fatty acids; namely, palmitic, stearic, oleic, linoleic, and linolenic (92 p. 277-289). The swelling and uncoupling actions of the isooctane-extractable "u" factor that developed upon aging of liver mitochondria and has the properties of a higher fatty acid, are also abolished by the presence of serum albumin (48 p. 2464). According to Weinbach et al (88 p. 561-567) serum albumin is both specific and obligatory for the restoration of oxidative phosphorylation in swollen and uncoupled rat liver mitochondria. ATP is also needed to help induce contraction of swollen mitochondria by probably interacting with contractile proteins in the membranes (88 p. 561-567, 42 p. 497-499). Morphologically distorted rat mitochondria due to the action of pentachlorophenol, showed a considerable capacity for coupled phosphorylation in the presence of serum albumin (88 p. 561-567). This is in accordance with many observations that membrane fragments of mitochondria can catalyze coupled reactions of oxidative phosphorylation (60 p. 355-360).

It has also been suggested that Tocopheral or vitamin E, which is known as a strong anti-oxidant, protects mitochondria from the harmful effects of peroxidation of unsaturated fatty acids normally present in these subcellular particles (16 p. 157-158).

Aging rat liver mitochondria at 30^{o}C for 90 minutes brought about uncoupled oxidative phosphorylation with a simultaneous

decrease in mitochondrial phospholipids. The addition of phosphorylcholine, phosphorylserine and phosphorylethanolamine to the incubation medium by Rossi et al (63 p. 170-175) protected the mitochondria from decay of oxidative phosphorylation efficiency whereas serum albumin proved ineffective. This is in agreement with the view of Green and Fleischer (26 p. 554) that the phospholipid pattern in the protein-phospholipid structure of the mitochondria is essential for a normal oxidative phosphorylation.

In 1959, Hanson (27 p. 1303-1306) found that incubation of corn, soybean and rat liver mitochondria in the pancreatic RNAase will uncouple phosphorylation and impair respiration. The author observed a loss of RNA and an increase in acid-soluble nucleotides after the incubation. In a later study on respiration and ion incubation by corn and soybean root tissue, Hanson (28 p. 377-379) found that exogenous RNAase impairs respiration, ion retention and phosphate accumulation probably through its action on RNA. He suggested that RNA is implicated in ion accumulation, solute retention and oxidative phosphorylation and that its metabolism could be governed by $K^{+}/Ca^{++} + Mg^{++}$ ratio. In another experiment on corn shoot mitochondria, the same effect of RNAase was attributed to its binding ability as a basic protein to negatively charged phosphorylation sites on the mitochondrial membrane (29 p. xxxii). The addition of protamine, a basic protein, to the Warburg vessel decreased oxidative

phosphorylation with very little effect on respiration. This inhibition effect of protamine was reversed by potassium ions (61 p. 318-321). No explanation was given for the action of K^+ in reversing the P/O inhibition. Hanson and Swanson (30 p. 442-446) believe that the decrease of respiration and oxidative phosphorylation associated with aging of corn scutellum mitochondria is due to the formation of ribonuclease and/or other basic proteins that bind themselves to the mitochondrial membranes. Pretreatment of inactive aged scutellum mitochondria with chymotrypsin or the use of high pH (7.5 - 8.0 instead of 6.8 as commonly used in the Warburg vessel) improved oxidation and P/O ratio. Two ribonuclease fractions (A and B) of corn seedling extracts were separated by Wilson (91 p. 177-184) by means of column chromatography. It is suggested by the author that these RNAases have different functions. RNAase "A" was found to be highly inhibitory to oxidative phosphorylation of corn scutellum mitochondria and according to Hanson et al (31 p. xiii) it is the type that increases in aging, and thus accounts for the decrease in mitochondrial activity. Both RNAases are found to be very abundant in dried and dead lower leaves of the corn plant. In conclusion, the authors believe that senescence in plant tissues such as damaged cell membranes may be attributed in part to the accumulation of inhibitory proteins, such as RNAase "A".

The influence of growth regulators on the permeability of

protoplasmic membranes has been given some consideration by few investigators (84 p. 308, 67 p. 752-758, 66 p. 1199-1200). Sacher (66 p. 1199-1200) believes that one of the functions of auxin is to maintain the selective permeability of membranes thereby preventing the loss of essential cellular substances into the intercellular spaces. Any alteration in the semipermeability of membranes will accelerate aging. In addition to their influence on maintaining membranes integrity, Switzer (80 p. 42-44) and Key et al (43 p. 177-183) found that 2, 4-dichlorophenoxyacetic acid, as an auxin, has a formative and growth effect on membrane structures such as mitochondria. This finding emphasizes the importance of the unsettled problems of biogenesis and degradation of subcellular organelles and their role in biological aging.

In summary, mitochondria become less in number, swollen, vesicular, pigmented and fragmented in old organisms. In aged, isolated mitochondria, loss of selective permeability of their membrane, accompanies swelling. Increase of ATPase and reduction of phosphorylative activity are observed. These functional changes induced in vitro could be repaired partly or in whole by addition of unsaturated fatty acids, phospholipids, chelating agents, (EDTA), and fatty acid-binding compounds (albumin). Binding of basic proteins or RNAase on mitochondria in aged corn scutellum tissue was found to impair oxygen uptake and phosphorylation. This type of

inhibition could be restored by the addition of potassium ion or incubation with chymotrypsin *in vitro*.

MATERIALS AND METHODS

Plant Material

Old and new soybeans, Glycine max L., selection M-316G were used in the experiments. The old lot was obtained from eastern Oregon at the Ontario Agricultural Experiment Station, and stored under room conditions for about three years. At the time of these experimentations, the seed lot showed a high percentage of short and abnormal seedlings even though the total germination percentage was high. The new seeds were freshly hand harvested from the field in June, 1964, from the same Station, air-dried to 7.2 percent moisture, and then kept in air-tight glass jars at about $3^{\circ}C$ to maintain their high viability during the period of the experiment. Very small seed, diseased, immatured, and those with broken seed coat were removed from both old and new lots prior to storage.

Seeds were soaked in one percent suspension of improved Ceresan (DuPont Semesan Co., Wilmington 98, Delaware, Active Ingredient - Ethyl Mercury Phosphate) for two minutes to retard fungal growth, washed in tap water, and then germinated in moistened paper towels placed in plastic boxes at alternating daily temperatures of $20^{\circ}C$ for 8 hours and $30^{\circ}C$ for 16 hours in the dark. Additional water was added to the paper towels as necessary during the period of germination. After four and a half days, the seedlings from the

new seeds reached an average length of 20 cm., while those from the old seeds were only 10 cm. in length. In order to eliminate the intermediate sizes, seedlings longer than 20 cm. from the new material and shorter than 10 cm. from the old were chosen for study. Diseased and moldy seedlings were discarded. Owing to the major activity of catabolism prevailing in cotyledons, only embryo axes were used for comparison of their mitochondrial activity. Embryo axes (shoot and root) were obtained by removing cotyledons from the seedlings and were placed on moist paper towel on crushed ice.

Preparation of Mitochondria

Preliminary experiments on the yield of mitochondria, based on mg N, obtained from a certain fresh weight with identical procedures showed that old material produced 30-50 percent more mitochondria pellet than the new. Consequently, the fresh weight of embryo axes used from the old was about 40 percent less than that of the new to yield an approximately equal quantity of mitochondria from both materials in each experiment. The material was cut into pieces smaller than one half cm. in length by means of a sharp razor blade in a walk-in cold room, held at 4° to 10°C, where other preparation work, such as, homogenization, suspension, and preparation of the reaction mixtures were carried out. The cut material was ground in a chilled grinding medium in an ice-cold High Speed Omni Mixer at 6,000

r.p.m. for 30 seconds. The ratio of the grinding medium to the material, on volume to weight basis, was one-and-a-half to one. The grinding medium contained 0.5M sucrose, 0.1M potassium phosphate at pH 7.4 and 0.002M ethylenediamine tetraacetic acid (EDTA). The whole homogenate was strained through four layers of cheesecloth and then centrifuged at 1,100 xg for ten minutes to remove tissue pieces, cell debris, nuclei, starch granules, etc. The supernatant was centrifuged at 20,000 xg for ten minutes to isolate mitochondria. The pellet was dispersed in 15 ml. suspension medium (0.5M sucrose and 0.125M potassium phosphate, pH 7.4) and centrifuged at 20,000 xg for ten minutes. This suspending and recentrifuging is referred to as the washing procedure. The washed mitochondria pellet was then suspended in a volume of suspending medium enough for Warburg respirometer study and nitrogen determination, usually about five milliliters. This suspension is referred to as "mitochondria preparation." All the centrifugation was done at 0° to -5°C in a Servall Automatic Refrigerated Centrifuge, Type RC-2. The amount of nitrogen in the "mitochondria preparation" was determined by the micro-Kjeldahl method.

Oxygen Uptake and Oxidative Phosphorylation

Oxygen consumption by the mitochondria preparation was measured in a Warburg respirometer with air as the gas phase. The

water bath temperature was kept constant at 30°C in all experiments.

The total volume of the reaction mixture in each Warburg vessel was 3.0 ml. and 0.2 ml. of 20 percent KOH was added to the center well with a 2 x 2 cm. filter paper serving as a wick. Except as noted otherwise, the molar concentrations of different solutes in the reaction mixture of each vessel were as follows: 0.3M sucrose; 0.0067M magnesium; 0.05M potassium phosphate buffer; 0.04M glucose; 0.00072M adenosine 5'-triphosphate (ATP), crystalline disodium salt from Equine muscle; 0.0026M B-nicotinamide adenine dinucleotide (DPN); 0.00036M thiamine pyrophosphate chloride (TPP); 0.000044M coenzyme A (Co. A); 0.0000026M cytochrome C; 0.005M α-keto-glutaric acid, crystalline monopotassium salt; 0.013M sodium fluoride if used; 150 units of hexokinase; 0.5 percent Bovine serum albumin. ATP, DPN, TPP, cytochrome C, α-ketoglutaric acid, hexokinase and albumin are products of Sigma, St. Louis, Missouri, but coenzyme A was obtained from Calbiochem, Los Angeles, California.

The solutions of ATP, Co. A, DPN, cytochrome C, TPP and hexokinase were prepared fresh for each experiment. Hexokinase was dissolved in glucose. All other solutions were prepared weekly and were kept between 0° to 5°C when not in use. All the chemicals were kept chilled in ice before they were added to the Warburg vessels. The vessels were also placed in an ice pan during and after the preparation of the reaction mixtures. A definite time schedule

was followed throughout all the operations in all the experiments as much as possible. All the reactants were added to the main compartment except hexokinase which was added to the side arm and was tipped in at zero time after seven minutes of temperature equilibration. When 2, 4-dinitrophenol (DNP) was used, it was placed in the side arm and the hexokinase added into the main compartment. The reaction periods were 30 minutes. Two flasks were used for each treatment, and in almost all cases experiments were repeated once.

Oxygen consumption was expressed as $QO_2(N)$, μl of O_2 used per hr. per mg of mitochondria N; and oxidative phosphorylation as the ratio: μ moles of inorganic phosphorus converted to organic phosphorus/μg atom oxygen consumed. The amount of inorganic phosphorus esterified to ATP was estimated by the difference of the "initial" and "final" amounts to inorganic phosphorus remaining in the reaction mixture before and after the incubation period. The "initial" measurement was obtained after equilibration from the flask containing the reaction mixture and the "final" reading was obtained from the flasks used for oxygen consumption at the end of the incubation period. In determining the inorganic phosphorus, the reaction was stopped by adding three milliliters of 0.4M trichloroacetic acid (TCA). The TCA-treated reaction mixtures were then centrifuged at 5,000 xg for eight minutes to sediment the mitochondria, and two milliliters aliquot of the supernatant were diluted to 100 milliliters

for each sample for inorganic phosphorus determination by the method of Fiske and SubbaRow.

Conductivity Test

Separate experiments were conducted on the permeability of the mitochondria membrane by means of testing the conductivity of leachate in 0.25M, 0.50M, and 0.75M sucrose concentrations from the mitochondria preparation of new and old material. In order to correlate the electrolytes leached out and the quantity of mitochondria as measured by mg N, usually 0.4, 0.6, 0.8, and 1.0 milliliter aliquots of the mitochondria preparation were used in each experiment. The conductivity in μ mhos was measured by means of the conductivity bridge Type RC 16B2 from Industrial Instruments, Inc., N. J. All the readings were taken at room temperature and at zero time, i.e., directly after adding the mitochondria preparation to the sucrose solution since no significant change in conductivity has been observed with longer time of leaching.

RESULTS AND DISCUSSION

In preliminary experiments, unwashed mitochondria preparations were studied. The oxygen uptake from the embryo axes of four and a half day old seedlings from new and old material is shown in Tables 1 and 2, respectively. Little difference in respiration rates as measured by $QO_2(N)$ was shown between new and old. These low respiration rates indicate a relatively inactive mitochondria according to Howell (36 p. 95) where he found $QO_2(N)$ of 115 and less in seedlings of five to nine day old soybeans with pyruvate as substrate. This relatively inactive condition is possibly due to the presence of some inhibitors for the electron transport system in the unwashed mitochondria preparations or contamination of N-compounds in the preparation. From the same materials, the phosphorylative efficiency shows a big contrast between the new and old (Table 2). A low P/O ratio of 1.37 was obtained from the old material compared to a very high one of 3.73 of the new. The difference in P/O ratios is attributed exclusively to the difference in the activity of the particle-bond enzyme systems in esterifying inorganic phosphate (Pi) into high energy phosphate (~P), i.e., adenosine triphosphate. Nitrogen content of these mitochondria preparations has no direct effect on the P/O ratio as the ratio is calculated from the results obtained from each flask. The amount of phosphorus esterified per flask of the new was 26.24 μ mole compared to 10.94 μ mole of the old, whereas

Table 1. Oxygen consumption of unwashed mitochondria of soybean seedlings from new and old seed.

Material (Fresh wt.)	Expt. No.	μl O_2/1/2 hr./Flask		mg N Flask	QO_2(N)		
		I	II		I	II	Average
New (65g)	a	76.95	77.52	1.37	112.34	113.17	112.75
New (68g)	b	78.30	82.96	1.43	109.51	116.03	112.77
							112.76
Old (45g)	a	86.31	91.79	1.77	103.72	97.53	100.62
Old (38g)	b	86.31	91.79	1.59	108.57	115.46	112.02
							106.32

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-ketoglutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

Table 2. Oxidative phosphorylation (P/O ratios) by unwashed mitochondria of soybean seedlings from new and old seed.

Material	Expt. No.	μg Atom O			μ mole Pi Esterified			P/O
		I	II	Ave.	I	II	Ave.	
New	a	6.86	6.92	6.89	27.10	26.19	26.65	3.87
New	b	6.98	7.40	7.19	26.84	24.84	25.84	3.59
							26.24	3.73
Old	a	8.20	7.70	7.95	9.03	8.13	8.58	1.08
Old	b	7.70	8.18	7.94	14.19	12.42	13.30	1.67
							10.94	1.37

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-ketoglutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

very little difference in oxygen uptake per flask was obtained between the two materials.

Washing of the mitochondria with homogenizing medium is a conventional procedure usually followed to remove contaminants, inhibitors, soluble metabolites, etc., in isolating mitochondria; thus, the major experiments of this study involved use of once-washed mitochondria preparation. The oxygen consumption was increased after washing in both materials with a relatively less increase in the old (70 percent) compared to the new (208 percent) (Table 3).

From the data in Tables 1 and 3, it is obvious that the less the quantity of mitochondria, as measured by mg N, per flask, the more the oxygen uptake was observed. This suggests that perhaps the co-factors, the ADP regenerating system, the co-enzymes, the protectants, the substrates, all of which were added into each flask in identical quantity, or other unknown factors are limiting. To verify this indication, a separate experiment was conducted by using an approximate quantity of mitochondria for both new and old materials. The results which are summarized in Figure 1 clearly indicate no difference in $QO_2(N)$ between new and old.

Differential phosphorylative efficiency between new and old, found in the unwashed material, was also observed after washing of the mitochondria as shown in Table 4. An average P/O ratio of 3.03 of the new material and 1.44 of the old were obtained. The

Table 3. Oxygen consumption of mitochondria of soybean seedlings from new and old seed.

Material (Fresh wt.)	Expt. No.	μl O_2/1/2 hr./Flask		mg N/ Flask	QO_2(N)		
		I	II		I	II	Average
New (73g)	a	80.52	92.48	0.64	251.62	289.00	270.31
New (90g)	b	79.06	88.32	0.85	186.02	207.81	196.92
							233.62
Old (73g)	a	92.46	---	1.10	168.11	---	168.11
Old (90g)	b	89.10	102.00	1.06	168.11	192.45	180.28
							174.20

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-ketoglutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

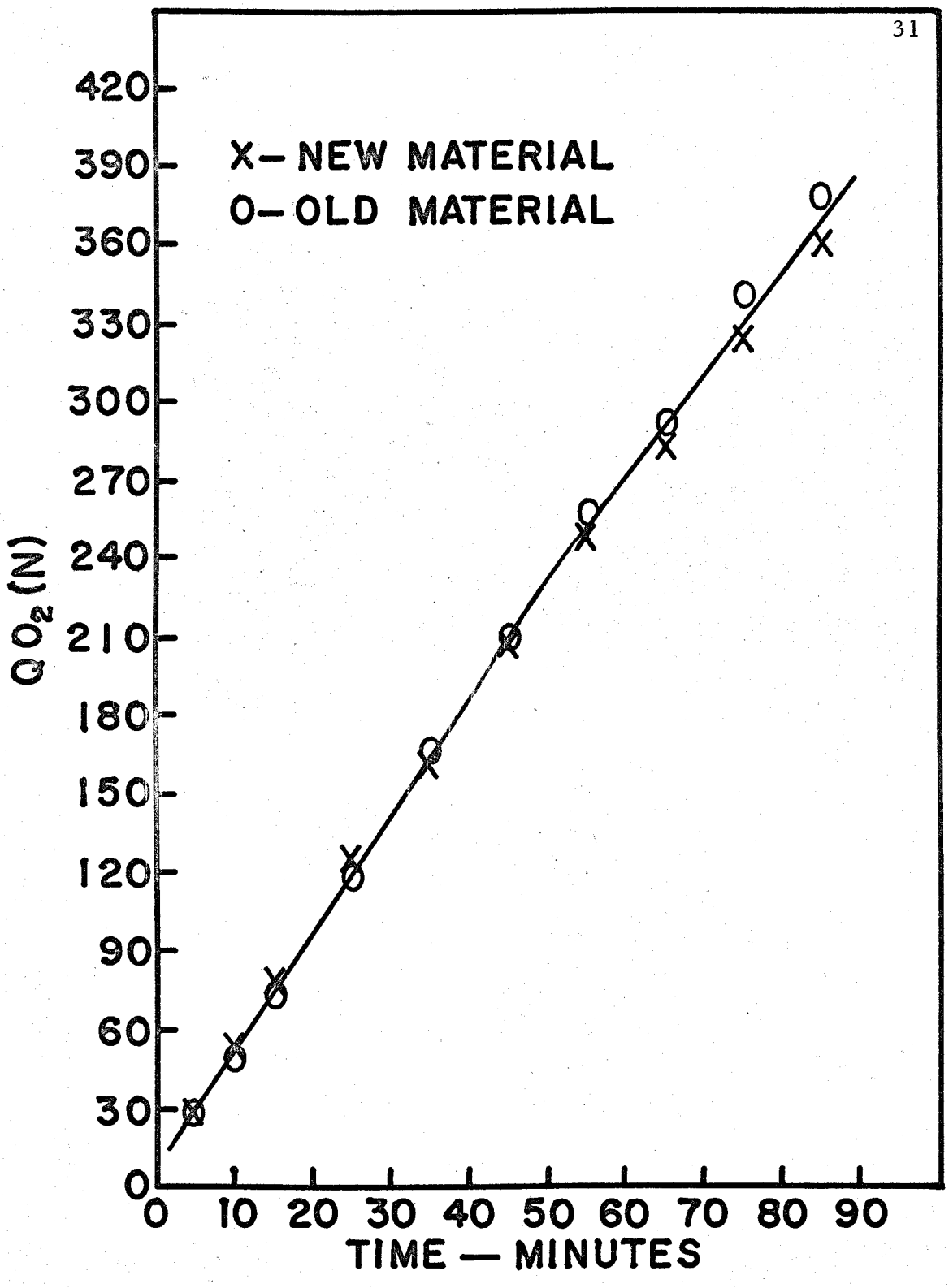

Figure 1. Oxygen uptake of mitochondria from embryo axes of new and old seeds.

Table 4. Oxidative phosphorylation (P/O ratios) by mitochondria of soybean seedlings from new and old seed.

Material	Expt. No.	μg Atom O			μ mole Pi Esterified			P/O
		I	II	Ave.	I	II	Ave	
New	a	8.25	7.38	7.81	23.44	22.52	22.98	2.94
New	b	7.05	7.88	7.47	12.42	14.19	23.30	3.12
							23.14	3.03
Old	a	8.25	---	8.25	15.17	---	15.17	1.84
Old	b	7.95	9.10	8.52	7.98	9.76	8.87	1.04
							11.77	1.44

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-ketoglutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

amount of phosphorus esterified by the new material was about twice that of the old. Considerably different P/O ratios are observed between experiments of the old material. The variation could be partly due to the fact that only one flask was left in one of the experiments when the center well of the other was accidentally broken while adding the mitochondria preparation. This kind of difference in mitochondrial activity often observed between repeated experiments in this aging study was probably due to biological variation of the material and to varied proportions of the chemicals and co-factors added to the reaction mixture in relation to the quantity of mitochondria from different preparations. Nevertheless, the trend between the old and new is clearly indicated and the magnitude is significantly improbable of belonging to the same population.

It is interesting that the results of this in vivo aging agree with the findings of Lehninger and Remmert (48 p. 2459-2464), Rossi et al (63 p. 170-175) and Hanson et al (31 p. XIii), on artificial aging of mitochondria preparations in vitro. Recently, Geronimo and Beevers (24 p. 786-792) observed a decrease of P/O ratio in mitochondria preparation of pea leaves with developmental age of the plant. They also found sub- or super-optimum growth temperatures reduced the P/O ratio of the mitochondria. This is one of the original reports which relate developmental status and environmental condition to functions of subcellular particles. A parallelism of the

present study to that report is apparent except for the relation of seed age to seedling vigor.

Therefore, one may conclude that natural aging of soybean seed, stored under room conditions, brought about a decrease in the phosphorylative efficiency of mitochondria from embryo axes of germinated seedling. This decrease in activity or in the production of chemical energy in the form of ATP may be responsible for the production of weak and abnormal seedlings during germination as the energy necessary for germination and growth in the dark is solely dependent on the energy produced from mitochondria. The importance of mitochondrial supply of energy after the appearance of chlorophyll will be minimized, but the energy required to bring about a healthy, vigorous plant to the green stage must be provided by the mitochondria.

In an attempt to delineate some of the changes that took place in the mitochondria of naturally aged soybean seed, the effects of serum albumin, fluoride ions and 2, 4-dinitrophenol as well as the permeability of the mitochondria membrane to electrolytes under different osmotic conditions were studied.

Effect of Serum Albumin

It was found by Lehninger and Remmert (48 p. 2459-2464) that artificial aging of rat liver mitochondria resulted in the loss of ability

to couple phosphorylation and the simultaneous appearance of an isooctane-extractable, uncoupling agent. The uncoupling effect, associated with mitochondrial swelling, was abolished by the addition of serum albumin via its ability to bind fatty acids. In the present study, the effect of bovine albumin on respiration and P/O ratios are shown in Tables 5 and 6, respectively. The average $QO_2(N)$ values show little difference that may be attributed to the presence or absence of albumin in either the new or old.

It is to be noted, however, that the highest P/O ratios in the new and the old were obtained in the presence of serum albumin. The effect of albumin on P/O ratio (Table 6) was mainly in increasing phosphorylation with no change in oxygen consumption (Table 5). This increase was about 28 percent in the old material compared to 12 percent in the new. It is reasonable to expect a relatively higher improving effect of albumin in phosphorylation in the old material that have suffered damage due to aging. But the effect of albumin at the quantity of 0.5 percent per total reaction mixture in restoring the phosphorylative efficiency of the old material did not bring it up to the level of the new material. It is, therefore, assumed that the damage caused by aging in mitochondria must have been more than simply the release of swelling uncoupling agents of long chain fatty acid in nature (Lehninger and Remmert; 48 p. 2459-2464). A possibility still exists, however, that an albumin concentration more

Table 5. Effect of albumin on respiration of mitochondria in soybean seedlings from new and old seed.

Material (Fresh wt.)	Treatment	Expt. No.	$\mu l\ O_2$/1/2 hr./Flask		mg N/ Flask	QO_2(N)		
			I	II		I	II	Average
New (125g)	+ Albumin	a	92.20	108.80	0.637	289.48	341.60	315.55
New (115g)	"	b	85.05	93.84	0.506	336.20	370.90	353.35
								334.55
New (125g)	- Albumin	a	104.12	104.12	0.637	326.90	326.90	326.90
New (115g)	"	b	94.53	95.90	0.506	373.64	379.04	376.34
								351.62
Old (90g)	+ Albumin	a	114.54	102.00	0.722	317.28	241.27	279.30
Old (75g)	"	b	122.82	110.16	0.501	490.28	439.76	465.02
								372.16
Old (90g)	- Albumin	a	103.36	112.34	0.722	286.30	311.18	298.70
Old (75g)	"	b	111.52	121.93	0.501	445.18	486.74	465.46
								382.08

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-keto-glutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 0.3 ml. of 5 percent bovine serum albumin (0.3 ml. of glass distilled water was used in flasks with no albumin); 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

Table 6. Effect of albumin on phosphorylation (P/O ratios) by mitochondria of soybean seedlings from new and old seed.

Material	Treatment	Expt. No.	μg Atom O			μ mole Pi Esterified			P/O
			I	II	Average	I	II	Average	
New	+ Albumin	a	8.23	9.79	8.92	20.32	20.32	20.32	2.28
New	"	b	7.59	8.37	7.98	21.22	22.13	21.67	2.71
								20.99	2.49
New	- Albumin	a	9.29	9.29	9.29	15.63	18.39	17.01	1.83
New	"	b	8.44	8.56	8.50	21.15	22.06	21.60	2.54
								19.30	2.18
Old	+ Albumin	a	10.22	9.10	9.66	18.06	12.58	15.32	1.59
Old	"	b	10.96	9.83	10.39	22.13	20.32	21.22	2.04
								18.27	1.86
Old	- Albumin	a	9.22	10.02	9.62	10.57	12.41	11.49	1.19
Old	"	b	9.95	10.88	10.41	13.77	16.55	15.16	1.46
								13.32	1.37

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-keto-glutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ moleDPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 0.3 ml. of 5 percent bovine serum albumin (0.3 ml. of glass distilled water was used in flasks with no albumin); 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

than 0. 5 percent in the reaction mixture or less mitochondria material used per each flask may give a different result.

Effect of Fluoride

Two separate experiments were conducted to study the effect of fluoride on the mitochondrial activity of new and old material. Only one concentration of NaF, namely, 0.013M, was used in the two experiments. The data in Table 7 show no effect of fluoride on oxygen consumption per unit-nitrogen basis in new and old material. The results also demonstrate the inverse relationship between the amount of mitochondria per flask and oxygen uptake similar to the one observed before in Tables 3 and 5, and to the results reported in Table 10. The presence or absence of NaF showed no influence at all on phosphorylation (μ mole Pi esterified) and the P/O ratios in either the new or old (Table 8). The usual effect of fluoride is to inhibit the activity of ATPase and show the true reduction of Pi in reaction mixture. The fact that the amount of fluoride did not change phosphorylative efficiency of the old material, then, precludes the possibility of high ATPase activity being responsible for the low phosphorylative efficiency observed. This conclusion does not agree with the finding of Kielley and Kielley (42 p. 497-499) on artificially aged liver mitochondria, in which high ATPase was found. The concentration of 0.013M NaF that was used in this study should be

Table 7. Effect of fluoride on respiration of mitochondria of soybean seedlings from new and old seed.

Material (Fresh wt.)	Treatment	Expt. No.	$\mu l\ O_2$/1/2 hr./Flask		mg N/ Flask	QO_2(N)		
			I	II		I	II	Average
New (140g)	+ Fluoride	a	94.50	112.88	0.910	207.69	248.08	227.89
New (135g)	"	b	99.90	106.08	0.910	219.56	233.14	226.35
								227.12
New (140g)	- Fluoride	a	111.52	109.60	0.910	245.08	240.86	242.87
New (135g)	"	b	102.38	115.05	0.910	222.80	252.92	237.86
								240.36
Old (80g)	+ Fluoride	a	121.44	125.12	0.592	410.28	422.70	416.49
Old (75g)	"	b	102.12	99.28	0.531	382.62	373.94	378.28
								397.38
Old (80g)	- Fluoride	a	112.88	117.82	0.592	381.35	398.04	389.70
Old (75g)	"	b	114.24	115.08	0.531	430.28	433.64	431.96
								410.83

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-ketoglutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg ml.); 0.2 ml. of 0.2M NaF (0.2 ml. of glass distilled water was used in flasks with no NaF); 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

Table 8. Effect of fluoride on phosphorylation (P/O ratios) by mitochondria of soybean seedlings from new and old seed.

Material	Treatment	Expt. No.	μg Atom O			μ mole Pi Esterified			P/O
			I	II	Average	I	II	Average	
New	+Fluoride	a	8.44	10.07	9.25	---	21.29	21.29	2.30
New	"	b	8.91	9.46	9.18	22.18	23.16	22.62	2.46
								21.95	2.38
New	- Fluoride	a	9.94	9.78	9.86	20.40	20.40	20.40	2.07
New	"	b	9.05	10.27	9.66	23.18	23.06	23.12	2.39
								21.76	2.23
Old	+ Fluoride	a	10.84	11.16	11.00	15.08	17.74	16.41	1.49
Old	"	b	9.11	8.86	8.98	14.19	14.19	14.19	1.58
								15.30	1.53
Old	- Fluoride	a	10.08	10.51	10.25	14.19	16.85	15.52	1.51
Old	"	b	10.19	10.27	10.23	15.97	13.31	14.64	1.43
								15.08	1.47

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-keto-glutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF (0.2 ml. of glass distilled water was used in flasks with no NaF); 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

reasonably high enough to show at least some kind of inhibition of ATPase activity if the enzyme were present since the same concentration of NaF used on unwashed mitochondria of the new material in a preliminary experiment, improved the P/O ratio by decreasing oxygen uptake and increasing phosphorylation as shown in Table 9. The reason this effect was not repeated after the mitochondria pellets were resuspended and washed once may indicate the possibility that ATPase was eliminated upon washing. Alternatively, the activity of this enzyme in soybean mitochondria preparations could not be inhibited by fluoride. This possibility could be exemplified by the finding of Sacktor (69 p. 386) who failed to inhibit the activity of ATPase by NaF at concentrations of 5×10^{-4}M, 1×10^{-3}M, 5×10^{-3}M and 1×10^{-2}M in the finely dispersed fragmented mitochondria whereas azide and chloromercuribenzoate were inhibitory.

Effect of 2, 4-dinitrophenol (DNP)

The expected effect of DNP as an uncoupling agent is to increase oxygen uptake and decrease phosphorylation; thus, lowering the P/O ratio. As shown in Table 10, mitochondria from the embryo axes of germinating new soybean seed exhibited no significant change in oxygen consumption as a response to DNP at the concentration of 1×10^{-3}M. The result from the old material, however, shows a 10 percent decrease in oxygen uptake due to DNP. This unclassical

Table 9. Effect of fluoride on phosphorylation (P/O ratios) by unwashed mitochondria of soybean seedlings from new seed.

Treatment	μl O_2/1/2 hr./Flask	QO_2(N)	μg. Atom O	μ mole Pi Esterified	P/O
+ Fluoride	86.03	155.01	7.67	25.50	3.32
- Fluoride	101.82	183.46	9.10	21.00	2.31

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-ketoglutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF (0.2 ml. of glass distilled water was used in flasks with no NaF); 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation (1.11 mg N) containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.0 ml. plus 0.2 ml. of 20 percent KOH in the center well. The incubation was carried at 30°C for 30 minutes.

Table 10. Effect of DNP on respiration by mitochondria of soybean seedlings from new and old seed.

Material (Fresh wt.)	Treatment	Expt. No.	$\mu l\ O_2$/1/2 hr./Flask		mg N/ Flask	QO_2(N)		
			I	II		I	II	Average
New (100g)	+ DNP	a	92.87	87.95	0.455	408.22	386.60	397.41
New (120g)	"	b	84.80	75.77	0.661	256.58	229.26	242.92
								320.16
New (100g)	- DNP	a	82.23	100.08	0.455	361.80	430.80	400.30
New (120g)	"	b	73.70	90.17	0.661	223.00	272.82	247.91
								324.10
Old (70g)	+ DNP	a	93.80	104.96	0.455	412.30	461.36	436.83
Old (90g)	"	b	96.84	92.82	0.772	250.88	240.46	245.67
								341.25
Old (70g)	- DNP	a	112.06	129.29	0.455	492.56	565.30	530.43
Old (90g)	"	b	94.71	113.62	0.772	245.36	294.34	269.85
								400.14

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-keto-glutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08$_3\mu$ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 1×10^{-3} M DNP; 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitocnondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.3 (glass distilled water was used in the absence of DNP) plus 0.2 ml. of 20 percent KOH in the center well. The incubation period was carried at 30°C for 30 minutes.

response to DNP has been also reported by Howell (36 p. 93-94) at concentrations of 6.7×10^{-7}, 6.7×10^{-6}, and 6.7×10^{-4} on cotyledons from germinating soybean seed with the tendency of the DNP-induced decrease in oxygen uptake to increase as the seedlings grew older. On the other hand, a decrease in phosphorylation from 19.03 to 8.33 μ mole Pi, or 55.7 percent in the new; and from 15.94 to 8.03 μ mole Pi, or 50 percent in the old, was obtained due to the effect of DNP (Table 11). As for the P/O ratios, the reduction in the new was from 2.47 to 1.09 or 55.8 percent which corresponds to the reduction in phosphorylation . The reduction in the P/O ratios of the old was from 1.59 to 0.95 or only 40.2 percent which is about 10 percent less than the reduction in phosphorylation. With respect to the latter, the decrease was attributed to the 10 percent reduction in O_2 consumption. Even though the reduction in P/O ratios due to the uncoupling action of DNP was not proportional between new and old, interesting enough, however, is the finding that the P/O ratios of both materials were reduced practically to the same level; namely, to 1.09 and 0.95 in the new and old, respectively. This perhaps indicates that natural uncoupling agents or disorganized molecular conformative status were produced by aging and brought about the reduction of P/O ratio in the old material. An addition of DNP merely reduces the oxidative phosphorylation to a ground level for both materials. The ground level or P/O of 1 is probably DNP

Table 11. Effect of DNP on phosphorylation (P/O ratios) by mitochondria of soybean seedlings from new and old seed.

Material	Treatment	Expt. No.	μg Atom O			μ mole Pi Esterified			P/O
			I	II	Average	I	II	Average	
New	+ DNP	a	8.29	7.85	8.07	9.52	9.52	9.52	1.18
New	"	b	7.57	6.76	7.16	6.67	7.61	7.14	1.00
								8.33	1.09
New	- DNP	a	7.34	8.93	8.13	18.08	19.98	19.03	2.34
New	"	b	6.58	8.05	7.31	17.13	20.94	19.03	2.60
								19.03	2.47
Old	+ DNP	a	8.37	9.36	8.36	5.71	5.71	5.71	0.68
Old	"	b	8.64	8.28	8.46	12.35	8.35	10.35	1.22
								8.03	0.95
Old	- DNP	a	10.00	11.54	10.77	14.27	19.03	16.65	1.55
Old	"	b	8.45	10.14	9.30	13.35	17.13	15.24	1.64
								15.94	1.59

Additions per flask were as follows: 300 μ mole sucrose; 20 μ mole magnesium; 15 μ mole α-keto-glutaric acid (monopotassium salt); 120 μ mole glucose; 2.87 μ mole ATP; 7.94 μ mole DPN; 1.08 μ mole TPP; 0.132 coenzyme A; 0.1 ml. of cytochrome C (1 mg/ml.); 0.2 ml. of 0.2M NaF; 1×10^{-3} M DNP; 0.3 ml. of 5 percent bovine serum albumin; 150 units of hexokinase; 1.3 ml. of the mitochondria preparation containing approximately 600 μ mole sucrose and 150 μ mole potassium phosphate buffer (pH 7.4). The liquid volume in each flask was 3.3 (glass distilled water was used in the absence of DNP) plus 0.2 ml. of 20 percent KOH in the center well. The incubation period was carried at 30°C for 30 minutes.

insensitive. The variation between replications, shown in Table 11, is probably due to differences in DNP concentration in the reaction mixture. Some precipitation of the chemical was observed in the side arm of the Warburg flask during temperature equilibration and before tipping. Even though DNP was added just before attaching the vessels to the manometers, part of the chemical in solution precipitated and stuck to the wall of the side arm due to its low solubility at low temperature.

Permeability of Mitochondria Membrane

Many workers (Jackson and Pace, 39 p. 65-70; Tapley, 81 p. 336-338; and Packer, 56 p. 218-219) have reported the behavior of mitochondria as osmometers and have emphasized the relationship of mitochondrial activity to the permeability of its membranes to various solutes and co-factors. Kunkel and Schneider (45 p. 109-116) attributed the reduced activity of glutathione (GSH) -induced swollen mitochondria to proteins and nucleotides he found in the leachate. In this study, the conductivities of mitochondria suspensions of old and new were compared in 20 milliliter sucrose solutions of different osmotic concentrations. The conductivity values for different concentrations of mitochondria based on mg nitrogen in 0.25, 0.50 and 0.75M sucrose solutions are summarized in Figure 2. In all cases, a linear relationship was obtained between

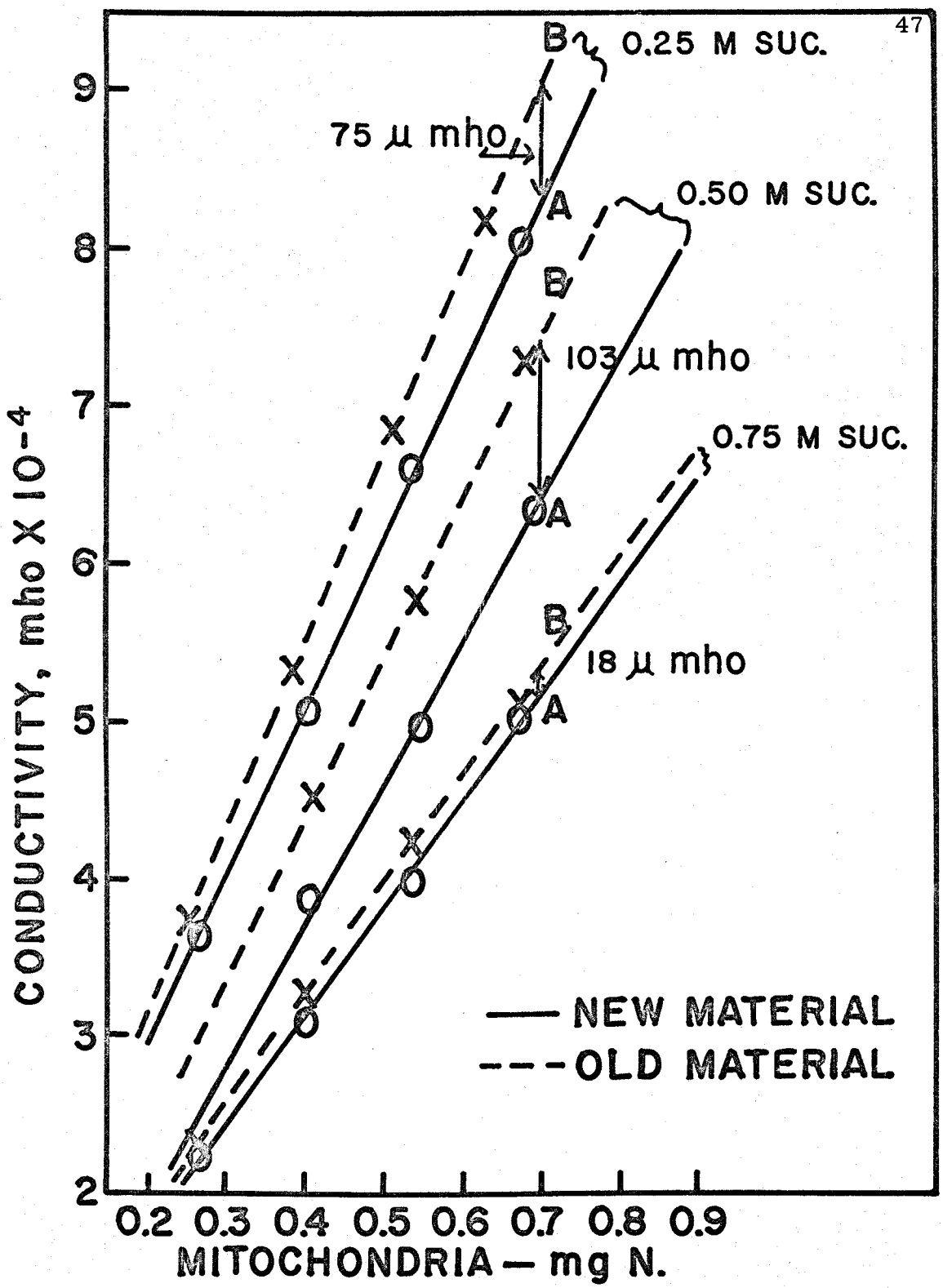

Figure 2. Conductivity of leachate from mitochondria isolated from embryo axes of old and new seeds in different osmotic concentrations of sucrose.

the amount of mitochondria (mg N) and conductivity (μ mhos). Based on any known quantity of mitochondria, the conductivity of leachate from the old material was always higher than that of the new under the three osmotic conditions studied. This is based on the supposition that the old and new material have the same amount of protein nitrogen per mitochondrion. If, however, the mitochondria from the old material have acquired an extra layer of protein around them as a consequence of aging (Hanson and Swanson, 30 p. 442-446), the conductivity values of the old material will be even higher than the new material on a per mitochondrion basis. The two points A and B on the graphs of the new and old, respectively, were chosen as points of reference for comparing the conductivity of the same amount of mitochondria (0.7 mg N) between the two materials in the three osmotic concentrations. The conductivity values of A in 0.25, 0.50 and 0.75M sucrose concentrations were 825, 637 and 515 μ mhos, respectively, which shows an inverse relationship between osmotic concentration and conductivity. This would indicate that swelling of mitochondria was produced in inverse proportion to the tonicity of the media and, consequently, the amount of solutes and co-factors (electrolytes) that "leaked" out was proportional to the magnitude of swelling. The same relationship existed in the old material except that the conductivity values were higher (see B) or more electrolytes leached out. This could be due to poor membrane structure and/or

dissolution in the mitochondria as a consequence of aging. Disintegration of artificially aged mitochondria has been suspected by Sacktor (69 p. 386) to take place. Based on the same level of mitochondria, i.e., 0.7 mg N, maximum difference between old and new of 103 μ mhos was obtained under an osmotic condition of 0.5M, compared to 75 in 0.25M and 18 in the 0.75M. This behavior pattern is to be expected if one assumes the in situ microenvironment to be around 0.5M in osmotic concentration and maximum activity of the mitochondria in vitro would be expressed under that condition. Any defects that may have resulted in the integrity of the mitochondria membrane by aging will be manifested during its swelling and "active" contraction under the most sensitive condition.

Effect of Concentration of Mitochondria on Oxygen Uptake

It has been observed throughout this study that there was an inverse relationship between the amount of mitochondria and oxygen uptake per flask in both old and new material. This relationship is well illustrated in Figure 3 where the amount of mitochondria (mg N) per flask is plotted against oxygen uptake, $QO_2(N)$. Such a relationship was not linear because it was obtained from a variety of experiments where many factors were involved, but it does show clearly a consistent trend in both old and new materials. A similar inverse relationship was found by Freebain (22 p. 59-61) between

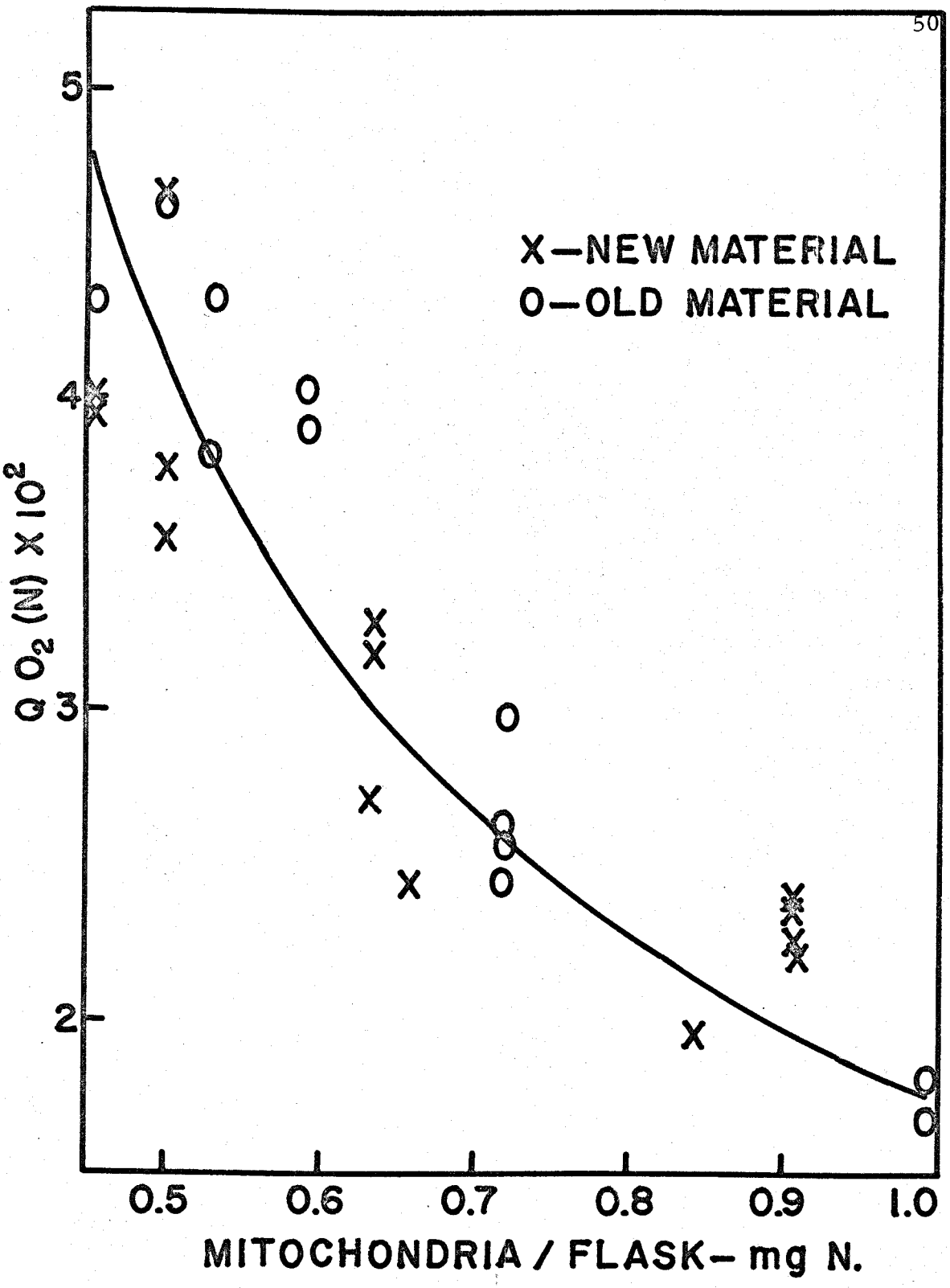

Figure 3. Effect of mitochondria quantity per flask on oxygen uptake.

mitochondria concentration and P/O ratio to which the author gave no explanation. An attempt made to relate the quantity of each co-factor and substrate added to the reaction medium and the quantity of mitochondria failed to give a clear explanation. Apparently, combined competitive inhibition limits the oxygen uptake at higher concentrations of mitochondria flask.

SUMMARY

Mitochondria from the embryo axes of germinating new and old seeds were isolated by homogenization and differential centrifugation, and their oxygen consumption and P/O ratios were studied. The effects of serum albumin, NaF, and DNP, as well as the permeability of the mitochondria membranes under different osmotic concentrations were examined. The following results were obtained:

1. The phosphorylative efficiency of the new material was always higher than that of the old. Washing the mitochondria did not change the P/O ratio of the old but did lower slightly that of the new.

2. No difference in oxygen consumption was found between the new and old material. The effect of the quantity of mitochondria per flask was discussed. An inverse relationship was observed between quantity of mitochondria and oxygen uptake in both materials at the level of added co-factors.

3. The highest P/O ratios in the new and old material were obtained in the presence of serum albumin. The effect of albumin was mainly in increasing phosphorylation while causing no change in oxygen uptake.

4. The addition of 0.013M NaF per flask produced no change in oxygen consumption and phosphorylation of washed mitochondria; however, the same concentration of NaF did improve the P/O ratio in the unwashed mitochondria from the embryo axes of the new material.

5. 2, 4-dinitrophenol (1×10^{-3} M) reduced slightly the oxygen uptake of the old but had no effect on that of the new. The P/O ratios of both materials were reduced practically to the same level; namely, to 1.09 and 0.95 in the new and old, respectively.

6. The conductivity of the leachate from the old material, based on any known quantity of mitochondria, was always higher than that of the new in 0.25M, 0.50M and 0.75M sucrose concentrations. Maximum difference in conductivity (μ mhos) between the new and old was observed in the 0.5M osmotic concentration. This behavior pattern is to be expected if one assumes the in situ microenvironment to be around 0.5M osmotic concentration and maximum activity in vitro would be expressed under that condition.

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