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LOW MITOCHONDRIAL FREE RADICAL PRODUCTION PER UNIT O_2 CONSUMPTION CAN EXPLAIN THE SIMULTANEOUS PRESENCE OF HIGH LONGEVITY AND HIGH AEROBIC METABOLIC RATE IN BIRDS

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Birds are unique since they can combine a high rate of oxygen consumption at rest with a high maximum life span (MLSP). The reasons for this capacity are unknown. A similar situation is present in primates including humans which show MLSPs higher than predicted from their rates of O_2 consumption. In this work rates of oxygen radical production and O_2 consumption by mitochondria were compared between adult male rats (MLSP = 4 years) and adult pigeons (MLSP = 35 years), animals of similar body size. Both the O_2 consumption of the whole animal at rest and the O_2 consumption of brain, lung and liver mitochondria were higher in the pigeon than in the rat. Nevertheless, mitochondrial free radical production was 2–4 times lower in pigeon than in rat tissues. This is possible because pigeon mitochondria show a rate of free radical production per unit O_2 consumed one order of magnitude lower than rat mitochondria: bird mitochondria show a lower free radical leak at the respiratory chain. This result, described here for the first time, can possibly explain the capacity of birds to simultaneously increase maximum longevity and basal metabolic rate. It also suggests that the main factor relating oxidative stress to aging and longevity is not the rate of oxygen consumption but the rate of oxygen radical production. Previous inconsistencies of the rate of living theory of aging can be explained by a free radical theory of aging which focuses on the rate of oxygen radical production and on local damage to targets relevant for aging situated near the places where free radicals are continuously generated.

KEY WORDS: Mitochondria; free radicals; hydrogen peroxide; maximum life span; longevity; aging.

INTRODUCTION

It is well established that between the majority of mammals the maximum life span (MLSP) of a given species increases as the aerobic metabolic rate at rest (VO_2; O_2 consumed/time × g) decreases and body size increases.[1-3] The same occurs between bird species.[4-5] This is the basis of the rate of living theory of aging[2] which is generally followed between mammals or between birds. This is nowadays based in studies on 77 mammalian[3] and 131 bird[5] species. A possible present explanation of this phenomenon is that species with higher aerobic metabolic rates produce oxygen radicals at mitochondria at a higher rate and then they age faster. It has been recently shown that mitochondrial free radical production is inversely correlated with MLSP in five species of mammals which follow the rate of living theory.[6-7] Nevertheless

This work is dedicated by Dr. G. Barja to Eduardo Rosell. In memoriam.

this theory of aging is largely discredited because there are large groups of animal species in which the MLSP is much higher than predicted from VO_2 values. A well known example are primates which show MLSPs 2–4 times greater (4 times in the human case) than predicted by the rate of living theory. The other only known example of large groups of animals in which VO_2 can not fully explain MLSP are birds. As stated above the rate of living theory holds true between bird species, but bird species show MLSPs around 3–4 times higher than those of mammals of similar size or VO_2^4. Birds are unique since they are the only vertebrates which were able to increase simultaneously MLSP and VO_2 during evolution.[8-10] Due to this the metabolic potential (O_2 consumed/g during the whole life span = MLSP × VO_2) of birds is 3–4 times higher than that of mammals[4] and is most probably the highest among vertebrate groups.

If free radicals are responsible for the rate of living phenomenon and constitute an important cause of aging, it must be explained why species such as birds and primates show MLSPs substantially higher that predicted from their rates of O_2 consumption. We have previously performed comparative studies of the main seven endogenous antioxidants in the liver,[8] brain,[9] and lung[10] of 6–8 vertebrate species showing different MLSPs, including mammals and birds. The results obtained in those studies led us to propose the hypothesis that the exceptionally high longevity of birds and primates is due in part to the presence of a low rate of free radical production in their tissues.[8-10] In order to test this hypothesis we decided to study mitochondrial free radical production in the same tissues of the two species showing maximum and minimum MLSPs used in our previous studies with antioxidants, the rat (MLSP = 4 years) and the pigeon (MLSP = 35 years). This difference in MLSP is striking since these two animals show a similar body weight and the VO_2 is even higher in the pigeon than in the rat.[8] Young but true adult animals were used in the present study. The results confirm our hypothesis since they show that mitochondrial free radical production in brain, lung and liver is 2–4 times lower in the pigeon than in the rat and that free radical production per unit O_2 consumption is one order of magnitude smaller in pigeon than in rat mitochondria.

MATERIALS AND METHODS

Animals

Adult male Wistar rats of 7 months of age were obtained from Iffa-Creddo (Lyon, France). Adult male pigeons (*Columba livia*) of 2–4 years of age were obtained from a Spanish breeder. Young adult ages were selected to be within 9–14% of the MLSP of the species.

Preparation of Mitochondria

Both rats and birds were acclimated in the laboratory during two weeks at 25°C and 12:12 L:D illumination conditions. Animals were sacrificed by decapitation and the organs were processed directly to obtain and assay mitochondria from fresh tissues. The whole period of isolation plus measurement of production of reactive oxygen species (ROS) and oxygen consumption (VO_2) by mitochondria was smaller than 2–3 hr in both species. One rat and one pigeon was sacrificed every day and their mitochondria were processed and assayed simultaneously. Measurement of mitochondrial VO_2 and ROS production was performed simultaneously in the same

samples at the same temperature and using the same buffer and substrate concentrations in order to be able to relate ROS production to mitochondrial VO_2. Liver (2–3 g) and the whole brain and lungs were briefly and gently homogenized with a loose fitting pestle hand-operated glass-glass homogenizer in 10 ml of MSE buffer (225 mM manitol, 75 mM sucrose and 1 mM EGTA, pH 7.4) containing 5 mg of nagarse and 25 mg of albumin. After standing for 1 min 25 ml of additional MSE buffer containing 25 mg of albumin were added and homogenization was gently performed again with a tighter fitting pestle. The homogenates were centrifuged 3 min at 1,500 × g (5°C) in a RC5C Sorvall centrifuge. The supernatants were centrifuged 10 min at 9,800 × g and the pellets were resuspended in 2 ml of MSE buffer using a plastic Pasteur pipette, protected from light at 0–5°C and immediately used for assays of mitochondrial ROS production and VO_2. This procedure minimizes functional damage to mitochondria since it shortens isolation time and decreases the manipulation of mitochondrial samples (Del Maestro *et al.* personal communication). All the procedures were performed over ice.

Mitochondrial ROS Production

Mitochondrial production of ROS was assayed by the fluorimetric determination of H_2O_2 production[11] adapted to tissue mitochondria (Del Maestro *et al.* personal communication). The linear increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to H_2O_2 production was measured in Hepes buffer (145 mM KCl, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4 at 25°C) in the presence of 0.25–0.5 mg of mitochondrial protein, 0.1 mM homovanilic acid, 6U/ml of horseradish peroxidase, 50U/ml of SOD, and 10 mM succinate in a LS50B Perkin-Elmer fluorimeter. Production of H_2O_2 was followed kinetically in order to measure the slope of the linear increase in fluorescence with time without taking into account the lag phase after addition of succinate nor the flat plateau which appeared at the end of the run (10–15 min) in a few very active samples. Addition of excess SOD avoided limitation of $O_2^{\cdot-}$ conversion to H_2O_2 by endogenous mitochondrial SOD. The results are then representative of total mitochondrial ROS ($O_2^{\cdot-}$ plus H_2O_2) production and are expressed as nmoles of H_2O_2 produced per min per mg protein. No increase in fluorescence was detected when succinate or mitochondria were omitted from the reaction system and the linear increase in fluorescence was totally abolished by catalase or GSH plus GSH-peroxidase addition. No inhibitors of the mitochondrial respiratory chain were added in order to obtain a rate of mitochondrial ROS production more representative of the physiological *in vivo* situation. Protein content was measured by the method of Lowry.[12]

Mitochondrial VO_2 Consumption

Stage 4 VO_2 was measured simultaneously with ROS production in fresh mitochondrial preparations using a Clark-type O_2 electrode in the same conditions in which mitochondrial ROS production was assayed: Hepes buffer pH 7.4 at 25°C with 0.25–0.50 mg of mitochondrial protein per ml and 10 mM succinate. Respiratory control ratios of 3.5 to 4 (lung), 2 to 4.8 (brain) and 2 (liver) were obtained after addition of ADP (0.37 mM final concentration) to succinate supplemented mitochondria.

RESULTS AND DISCUSSION

Previous studies from this laboratory in 6–8 vertebrate species from four of the five main vertebrate groups showed that the principal endogenous antioxidants, superoxide dismutase, catalase, Se-dependent and total GSH-peroxidases, GSH-reductase, GSH and ascorbate in liver,[8] brain,[9] and lung[10] negatively correlate with the species-specific MLSP. Among the 21 correlations studied in these works, 17 were significantly negative, four did not show correlation with MLSP and positive correlation was not obtained in any case. Furthermore, the species with the longest MLSP included in the study, the pigeon, showed the lowest level of many tissue antioxidants, whereas the rat showed very high levels of antioxidants in many comparisons. When we reviewed all previous works of this kind known to us in mammals in which primates were included we observed that strong negative correlations with MLSP were obtained for catalase,[3] GSH-peroxidases,[13-14] GSH[15] and ascorbate[3,15] in liver, brain or kidney and no correlation was found for superoxide dismutase.[16] Interestingly, in all these works the species with the highest MLSP (humans in the majority of works) showed the minimum levels of the antioxidant studied.

Taking into account all that information we hypothesized,[8-10] that species with an extraordinarily long MLSP not explainable by their basal metabolic rates (like birds and primates; see the introduction) show very low levels of antioxidants because their rates of free radical production are low, whereas the high rates of free radical production of short lived species (like the rat) would be compensated with high levels of endogenous antioxidants. The scheme of Figure 1 shows the application of our hypothesis to mitochondria. Compensation between rates of production and elimination of H_2O_2 would lead to gradients of H_2O_2 concentration away from mitochondria and to similar levels of H_2O_2 in many parts of the cell in species with short or long MLSP. This is consistent with data from this laboratory showing lack of correlation between MLSP and the GSH/GSSG ratio or lipid peroxidation in liver[8] and brain.[9] But near the places of free radical production at the inner mitochondrial membrane the *local* concentration of reactive oxygen species (ROS) would be much higher in species with high free radical production (and short MLSP) than in species with low free radical production (and long MLSP).[9,17] This would lead to much higher levels of oxidative damage to relevant targets (like mitochondrial DNA) situated near the places of free radical production in species with short than in species with long MLSP. It has been shown that mit-DNA shows much higher levels of 8-OH-deoxyguanosine (HPLC-EC) than nuclear DNA[18] and exhibits an increase in mutation rate[19] and in the proportion of various deletions[20,21] during aging in various human tissues. An exponential increase in 8-OH-deoxyguanosine (measured by HPLC/MS) and in deletions in mitDNA with age in the heart of normal humans from 30 to 100 years of age has been recently reported.[22] Our hypothesis would also be compatible with many "aging experiments" showing that MLSP is not changed by pharmacological, dietary or genetic antioxidant suplementation even though the mean life span is usually prolonged.[17] The determinant factor for aging would be the local ROS concentration near places of production, not at other cellular sites.

An inverse relationship between the rate of mitochondrial ROS production and MLSP has been observed in liver,[6] heart and kidney[7] of six mammalian species following the rate of living theory. In the last of these works[7] it was shown that mitochondrial ROS production positively correlated with mitochondrial VO_2 in kidney and heart and with the VO_2 of the whole animal. Nevertheless, the six species used in these works (mouse, rat, guinea pig, rabbit, pig and cow) followed

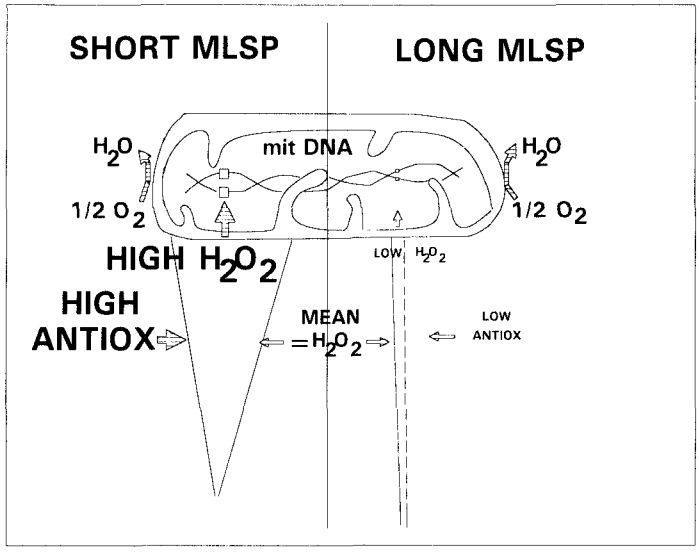


FIGURE 1 We hypothesized that animals with long MLSP show low levels of ROS production in their tissues because they had low levels of tissue antioxidants[8–10] whereas the contrary occurs in short-lived species. Our model predicts the existence of gradients of H_2O_2 concentration diminishing away from the places of ROS production at mitochondria. Similar levels of *mean* cellular H_2O_2 in short- and long-lived species are expected from the model. But the local concentration of ROS and H_2O_2 near places of ROS production would be much lower in long- than in short-lived animals. This would decrease oxidative damage at targets critical for aging situated near the places of ROS production (e.g., mitochondrial DNA). Modified from Barja *et al.*, 1994.[9]

the rate of living theory. Thus, we must critically consider the possibility that the inverse correlation observed between mitochondrial ROS production and MLSP in these works is due to other unknown factors (different from free radicals) causing the aging rate and also correlating with metabolic rate and then, necessarily with ROS production (since ROS production correlates with VO_2 in these species). An approach to solve this uncertainty is to study species with MLSPs much higher than predicted by their metabolic rates (e.g. birds and primates). If a high ROS production were found in these specially longevous species, this would discredit the free radical theory of aging similarly to the previous discredit of the rate of living theory of aging after finding relatively high metabolic rates (in relation to MLSP values) in these species.

If our hypothesis is generally applicable it must also be true in species in which longevity can not be explained by the rate of living theory: primates or birds when compared to the majority of mammals. In other words, the validity of our hypothesis needed the demonstration that primates and birds show low mitochondrial ROS production values and lower ROS production per unit O_2 consumption at mitochondria than the majority of mammals. This would explain their high MLSP in spite of their high (birds) or relatively high (primates) aerobic metabolic rate.

In order to test our hypothesis we selected the two species showing minimum and maximum MLSPs and antioxidant levels (Table 1) used in our previous studies involving antioxidants, the rat and the pigeon. These two species seemed to us a good

TABLE 1
Ratio (rat/pigeon) of levels of endogenous antioxidants in three different tissues

Tissue	SOD	CAT	TOT.-GPx	Se-GPx	GR	GSH	ASC
Brain	4	2	3	3	4	0.9	1.5
Lung	1.7	6	17	14	6	2	9
Liver	0.6	3	30	68	1.6	2.4	0.9

Values are levels of each antioxidant in the rat divided by levels of the same antioxidant in the pigeon in the same tissue. Superoxide dismutase (SOD); Catalase (CAT); Total (TOT.-) and Selenium-dependent (Se-) GSH-peroxidase (GPx); GSH-reductase (GR); Ascorbate (ASC). Values were obtained in our laboratory as described in recent publications.[8-10]

choice taking into account their similarity of body size, the strong difference in MLSP (4 years in the rat and 35 years in the pigeon,[23] Figure 2) and the much lower levels of the majority of tissue antioxidants shown by the pigeon (Table 1). Furthermore, the VO_2 of the whole animal at rest is around two times higher in the pigeon than in the rat (Figure 2). As a result, the metabolic potential of the pigeon (465 10_2/g) is 17 times higher than that of the rat (28 10_2/9).

Mitochondrial VO_2 stimulated by succinate and in the absence of ADP (stage 4) was measured in the same three tissues of rats and pigeons in which we had previously studied the antioxidant levels: liver, brain and lung. The results show that mitochon-

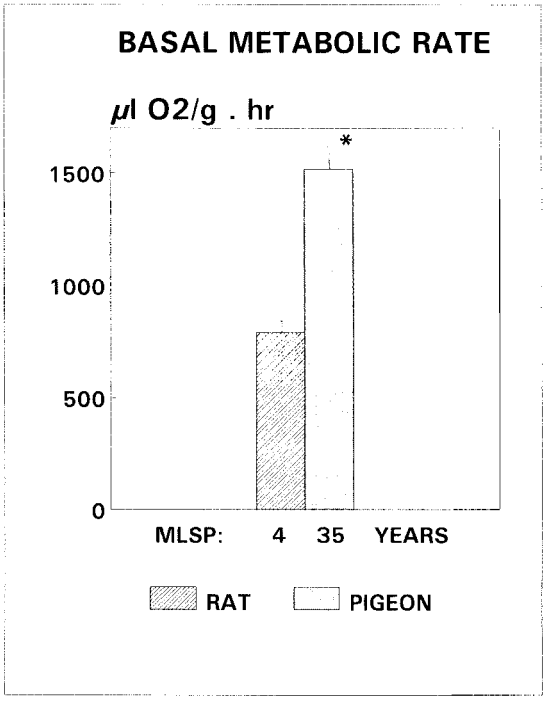


FIGURE 2 Basal metabolic rate of the whole animal at rest in adult male rats (MLSP = 4 years) and pigeons (MLSP = 35 years). VO_2 data from López-Torres *et al.*, 1993.[8]

drial VO_2 is higher (2–4 times) in the pigeon than in the rat in the three tissues studied (Figure 3). This *in vitro* finding in three tissues relevantly contributing to the total metabolic rate is in agreement with the higher *in vivo* VO_2 of the whole animal observed in the pigeon in relation to rats (Figure 2). Mitochondrial ROS production (in the absence of any respiratory inhibitor) was measured in the same samples from the same animals in which mitochondrial VO_2 was being measured. In order to be able to relate the rates of ROS production to those of VO_2 in mitochondria both values were measured simultaneously and under the same conditions (equal buffer, succinate concentration and temperature). As expected from our hypothesis, the rate of mitochondrial ROS production was lower in pigeon than in rat mitochondria in the three tissues (two times lower in liver an lung and four times lower in the brain, Figure 4) in spite of the higher rate of VO_2 of pigeon mitochondria (Figure 3). The difference is maximum in the brain, an organ of special importance in the aging process. Figure 5 shows representative tracings directly obtained at the fluorimeter screen showing the lower slopes of the lines of ROS production in pigeon than in rat mitochondria. During the performance of this work a paper from another research group was published showing a lower ROS production in pigeon than in rat mitochondria in heart, kidney and brain.[24] Thus, the lower ROS production of pigeon mitochondria in relation to that of the rat has been confirmed by two independent groups and seems to be a general characteristic since it helds true in at least five vital tissues. Some minor discrepancies were apparent between both works, specially for mitochondrial VO_2 which was reported to be lower in pigeon than in rat mitochondria in brain, heart and kidney.[24] A possible explanation of this discrepancy with our work is that these authors used 3 months old rats and 6 months old pigeons whereas we used animals which had attained full growth and had an age 9–14% of their MLSP (7 months in the rat and 2–4 years in the pigeon).

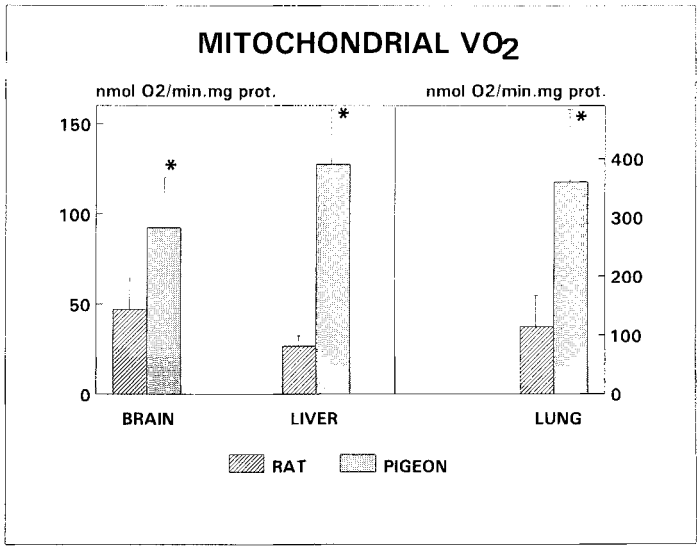


FIGURE 3 Mitochondrial O_2 consumption in three tissues from adult rats and pigeons in the presence of 10 mM succinate (Hepes buffer pH 7.4, 25°C); $n = 5$ animals per species.

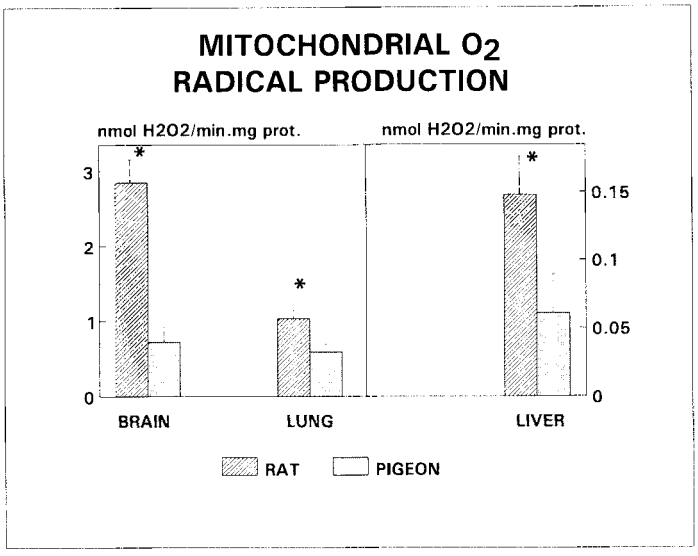
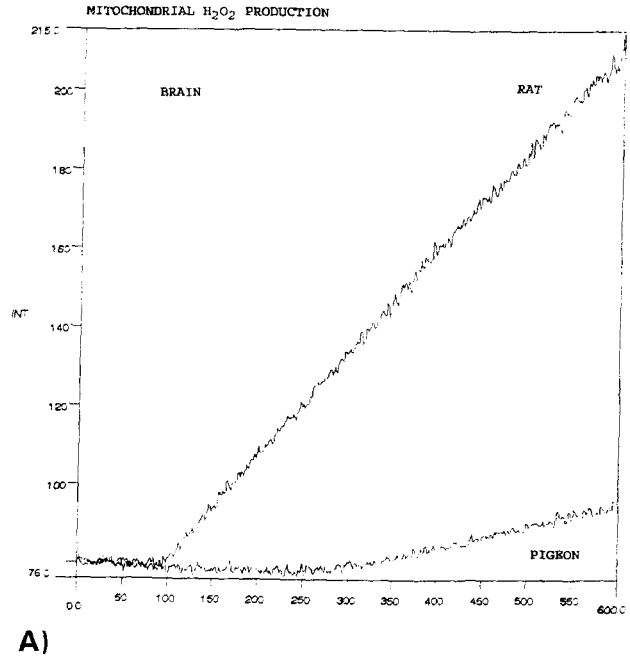


FIGURE 4 Mitochondrial O_2 radical production in three tissues from adult rats and pigeons in the presence of 10 mM succinate (Hepes buffer pH 7.4, 25°C); $n = 5$ animals per species.

When we related mitochondrial ROS production to mitochondrial O_2 consumption we obtained a value one order of magnitude lower in the pigeon than in the rat in the three tissues studied (Figure 6). This is the first time that the capacity to reduce ROS production per unit O_2 consumption at mitochondria is described in a specially longevous animal. The large quantitative difference for ROS/VO_2 values between rat and pigeon suggests that this was a physiologically relevant change which ocurred at some stage during bird evolution. This result confirms our hypothesis and suggests that the high longevity of birds in relation to mammals can be due in part to the capacity of bird mitochondria to decrease free radical leakage at the respiratory chain. If this result turns out to be a general characteristic of bird species it will explain the unique capability of these animals to increase simultaneously MLSP and VO_2. If the same increase in "efficiency" of mitochondrial electron transport is also found in primates and humans it will be possible to reformulate the rate of living theory in terms of free radical production. A low free radical production would be a longevity determinant in all animal species. In those following the rate of living theory a low free radical production would be a consequence of a low rate of oxygen consumption: ROS production/VO_2 would be constant across these species (it has been shown that ROS production positively correlates with VO_2 in at least six of these species[7]) and this will explain the strong association between MLSP and metabolic rate if a high ROS production accelerates aging rate. Species like birds and primates (when compared to the majority of mammals) would not follow the rate of living theory because they would show a lower free radical production per unit oxygen consumption. This would explain in part their extraordinarily high MLSP in relation to their metabolic rate if ROS production is a determinant of aging rate. Thus, species considered as "exceptions" to the rate of living theory would be so due to the possibility that the appropriate parameter to relate to MLSP is not oxygen con-



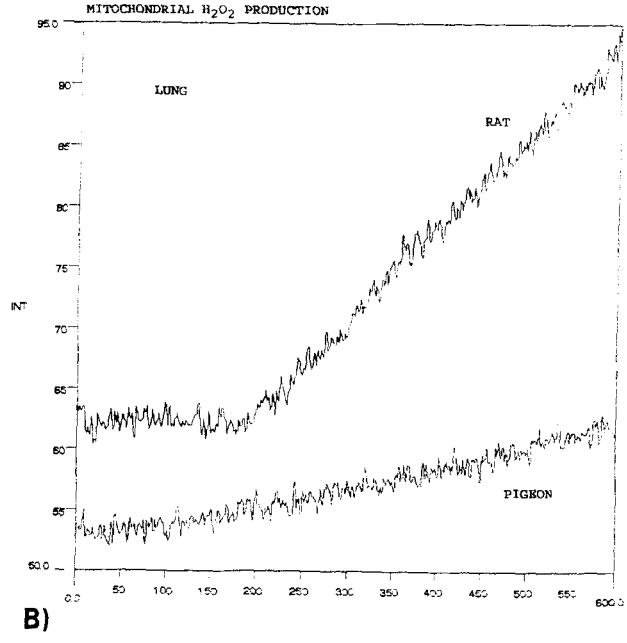


FIGURE 5 Representative tracings directly obtained from the fluorimeter screen simultaneously showing the linear rates of H_2O_2 production by pigeon and rat mitochondria as a function of time (sec). Conditions as in Figure 4 and Materials and Methods. A: brain mitochondria; B: lung mitochondria.

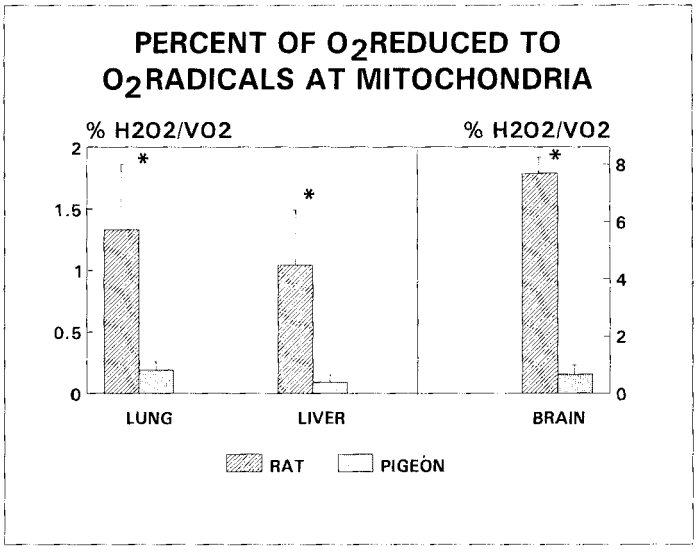


FIGURE 6 Rate of production of O_2 radicals at mitochondria as percentage of mitochondrial O_2 consumption; $n = 5$ animals per species.

sumption but free radical production. The strong association between ROS production (not VO_2) and MLSP in all studied species suggests that this is an important parameter determining aging rate and maximum longevity. We think that the results obtained here also strengthen the available evidence suggesting the involvement of mitochondria in the aging process[25-26] as well as the free radical theory of aging[27] if it is viewed as follows: the factor relating oxidative stress to aging and longevity is the rate of free radical production.

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