Age-related increase in mitochondrial proton leak and decrease in ATP turnover reactions in mouse hepatocytes

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1Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5; 2Wisconsin Regional Primate Research Center, University of Wisconsin, Madison 53715; and 3Department of Medicine, University of Wisconsin, and Veterans Administration Geriatric Research, Education and Clinical Center, Madison, Wisconsin 53705

Harper, Mary-Ellen, Shadi Monemdjou, J on J. Ramsey, and Richard Weindruch. Age-related increase in mitochondrial proton leak and decrease in ATP turnover reactions in mouse hepatocytes. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E197–E206, 1998.—Age-related changes in mitochondria, including decreased respiratory control ratios and altered mitochondrial inner membrane lipid composition, led us to study oxidative phosphorylation in hepatocytes from old (30 mo) and young (3 mo) male C57BL/J mice. Top-down metabolic control analysis and its extension, elasticity analysis, were used to identify changes in the control and regulation of the three blocks of reactions constituting the oxidative phosphorylation system: substrate oxidation, mitochondrial proton leak, and the ATP turnover reactions. Resting oxygen consumption of cells from old mice was 15% lower (P < 0.05) than in young cells. This is explained entirely by a decrease in oxygen consumption supporting ATP turnover reactions. At all values of mitochondrial membrane potential assessed, the proportion of total oxygen consumption used to balance the leak was greater in the old cells than in the young cells. Metabolic control coefficients indicate a shift in control over respiration and phosphorylation away from substrate oxidation toward increased control by leak and by ATP turnover reactions. Control of the actual number of ATP molecules synthesized by mitochondria for each oxygen atom consumed by the ATP turnover and leak reactions was greater in old than in young cells, showing that efficiency in older cells is more sensitive to changes in these two blocks of reactions than in young cells.

oxidative phosphorylation; uncoupling; oxidative stress; free radicals; aging

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Although isolated mitochondria have been extensively investigated in aging studies, it is known that populations of mitochondria from tissues of old and young organisms can be differentially damaged by mitochondrial isolation procedures. Histological studies have shown that, with increasing age, mitochondria tend to be larger, there is increased matrix vaculization, cristae are shorter, and there is a decrease in dense granules (47). Importantly, it has been estimated that only about one-half of these enlarged mitochondria are retained in mitochondrial isolations from tissues of older animals (47). Thus the quality of the mitochondrial preparation from old organisms may, in fact, be improved by the loss of unhealthy mitochondria during the isolation procedures (47), leading potentially to artifactual conclusions about changes in mitochondrial function with age. Moreover, oxidative damage occurs throughout the cell, emphasizing the importance of metabolic studies conducted using intact cells and tissues.

The experimental approach that we have used is one that has been useful in identifying the sites of action of hormones and other external effectors in metabolic pathways in intact cells as well as in mitochondrial preparations. The approach is referred to as top-down elasticity analysis (7), and it is an extension of metabolic control analysis. Whereas metabolic control analysis allows the identification of the important sites of control within metabolic pathways, top-down elasticity analysis is used in the comparison of varying metabolic conditions to identify differences in pathway regulation. The latter is extremely useful, for example, in the identification of the "sites of action" of hormones, drugs, and metabolic defects. Some of the useful measurements to emerge from an elasticity analysis include "elasticity coefficients," "flux control coefficients," and "concentration control coefficients." In very general terms, an elasticity coefficient (often referred to simply as an "elasticity") describes the responsiveness of a branch of a metabolic pathway to changes in the amount of an intermediate in that pathway. If the elasticity for a branch differs between the drug-treated and the control pathways being compared, then one site of action of the drug is located within the reactions encompassed by that branch. Other measurements include flux control and concentration control coefficients. Values of the latter describe, respectively, the relative proportion of control by branches of the pathway over the rate of the pathway and over the amounts of intermediates. Several useful reviews on this approach have been published recently (see Refs. 8, 26).

In intact cells it has been successfully used to investigate the sites of action of glucagon (9), thyroid hormones (19, 20, 24, 25), and butylated hydroxyanisole (16) in mitochondria, and of fatty acids in isolated hepatocytes of rats (37). Here we use the approach to quantitatively identify in mouse hepatocytes the effects of aging on reactions that are central to oxidative phosphorylation.

On the basis of the above documented age-related changes in mitochondria, particularly the decreases in the State 3/State 4 and changes in M1M lipid composition, we hypothesized that the mitochondrial proton leak might be greater in cells from old compared with young mice. Thus we aimed, in the following set of experiments, to compare the overall kinetics of the mitochondrial proton leak in hepatocytes from old (30 mo) and young (3 mo) mice. We also conducted the first complete metabolic control analysis on oxidative phosphorylation in intact cells from old mice and report herein that the overall kinetics of the mitochondrial proton leak are altered by aging. Moreover, we show that there is a significant decrease in the amount of oxygen used to support the synthesis and use of ATP in old compared with young cells. Top-down metabolic control analysis showed that there is a shift in control away from substrate oxidation reactions toward increased control by the leak and by ATP turnover reactions in hepatocytes from old mice.

**EXPERIMENTAL PROCEDURES**

Treatment of animals. Twenty-seven male 30-mo-old C57BL/6J mice were received from the Veterans Administration Geriatric Research, Education, and Clinical Center at the University of Wisconsin (Madison, WI). These mice were part of a cohort purchased at 1 mo of age from Charles River Laboratories (Wilmington, DE), group housed (three per cage), and given free access to Purina 5001 chow diet and water. Twenty-seven young (3 mo) control C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). On receipt in Ottawa, mice were caged individually at 23°C with light from 0700 to 1900 and were given free access to a Purina 5001 chow diet and water. For the isolation of hepatocytes, nonfasted mice were anesthetized with 1 mg of pentobarbitone/100 g body weight and were killed between 0730 and 1100. Mice used in this study were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care, the Institute of Laboratory Animal Resources (National Research Council, USA), and with the Guiding Principles for Research Involving Animals and Human Beings.

Isolation and incubation of hepatocytes. Hepatocytes were isolated and incubated as earlier described (19). The viability of cells was >92% as determined by the exclusion of 0.3% (wt/vol) trypan blue. Before incubations, the cells were stored on ice in the isolation medium containing (in mM) 148 NaCl, 5 KCl, 0.81 MgSO4 ·7H2O, 0.83 Na2HPO4, 0.14 KH2PO4, 1 CaCl2, 25 NaHCO3, and 15 glucose.

For incubations, the cells were diluted approximately sevenfold in an incubation medium containing (in mM) 106 NaCl, 5 KCl, 25 NaHCO3, 0.41 MgSO4, 10 Na2HPO4, 2.5 CaCl2, 10 glucose, 1 lactate, 1 pyruvate, and 2.25% (wt/vol) defatted BSA. Stock 9% BSA was defatted by the method of Chen (12) and dialyzed against 153 mM NaCl and 11 mM KCl. Cell suspensions (3–5 ml of 6–9 mg dry wt cells/ml) were incubated in 20-ml stoppered glass vials at 37°C in a shaking water bath (100 cycles/min). To allow equilibration of the medium to a pH of 7.4, the gas phase above each suspension during incubations was 95% air-5% CO2. The cells were preincubated at 37°C in the shaking water bath for 10 min to allow the hepatocytes to reestablish ion gradients after being stored on ice. Cells were then incubated a further 20 min in the presence of the various inhibitors, uncouplers, and isotopes before aliquots were taken for the measurements of oxygen consumption and mitochondrial membrane potential (ΔΨm). (Refer to Application of top-down elasticity analysis...
and top-down control analysis and to legends of Figs. 1–4 for the specific concentrations of inhibitors and uncouplers used in each experiment.)

Measurement of oxygen consumption. The respiration rate of hepatocytes was measured using a Hansatech (Norfolk, UK) Clark-type oxygen electrode, the incubation chamber of which was maintained at 37°C and magnetically stirred. Each rate was assessed in duplicate using 1.0 ml of cell suspension. Respiration rates are reported here as per 106 cells rather than per milligram dry weight of cells, as is more normal for rat hepatocyte preparations. This is simply because the yield of hepatocytes from a mouse is about one-tenth of that from a rat, and an inordinate amount of the final cell preparation would be needed to accurately assess dry cell weight. Cell counts were determined with a hemocytometer.

All respiration rates were determined simultaneously and in parallel with the measurements of ΔΨm. The resting respiration rate was defined as the oxygen consumption rate in the absence of inhibitors and uncouplers. Nonmitochondrial oxygen consumption was determined after the incubation of cells with maximal concentrations of oligomycin (1 µg/ml) and antimycin (5 µM) and with valinomycin (0.1 µM) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (20 µM).

Measurement of ΔΨm. Total aqueous volume, extracellular volume, and ΔΨm were estimated from the distributions of [3H]2O, [14C]methoxyinulin, and [3H]methyltriphenylphosphonium cation (TPMP+) as described previously (25, 36). ΔΨm can be calculated by knowing the proportion of cytoplasmic volume that is occupied by the mitochondrial matrix [mitochondrial volume/cell volume (Vc/V)], the apparent activity coefficient of TPMP+ in each compartment (aα, α = a, and aα, where subscripts e, c, and m represent extracellular, cytoplasmic plus nuclear, and mitochondrial), and the extent of the accumulation of TPMP+ into the whole cell [(TPMP+[e]m)/[TPMP+[e]] and into the cytoplasm in relation to the external medium ([Cl−][TPMP+]e/[Cl−]e). The relationship between ΔΨm and TPMP+ distribution at 37°C is shown below

$$ΔΨ_m = -61.5 \log \left( \frac{V_c \cdot a_m}{V_m \cdot a_c} \frac{[\text{Cl}^-][\text{TPMP}^+]_{\text{e}} (V_c + V_m)}{[\text{Cl}^-][\text{TPMP}^+]_{\text{e}} a_c V_c} - 1 \right)$$

The determination and the actual values of these correction factors are outlined briefly in the following paragraphs. Vc and Vm values were determined using quantitative morphometric cytology (45) as described previously (25). Briefly, isolated cells were fixed with glutaraldehyde, and electron micrographs of osmium-stained cells were prepared at a final magnification of 4,950-fold. Mitochondrial volume was determined from the number of intersections of a 1-cm grid overlaying the micrographs. Then the volume was calculated as the total number of intersections in mitochondria divided by the total number of intersections in cells (less the total number of intersections in lipid droplets). The cellular volume was corrected for the volume of lipid droplets, because TPMP+ is not taken up into fat (13). Mitochondrial matrix volume was calculated as 56.5% of total mitochondrial volume on the basis of the work of Loud (32), who calculated this weighted mean from the percentage matrix volume of midzonal, peripheral, and central liver cells and the percentage of these cell types in the whole liver.

The accumulation ratio, ([TPMP+]e/[Cl−]e), and plasma membrane potential (ΔΨp) were determined from the distribution of ³⁵Cl (35). ΔΨp was 36.0 mV ± 6.2 (n = 3) and 39.3 mV ± 0.7 (n = 3) in cells from old and young mice, respectively.

The proportion of TPMP+ that is free (i.e., not bound) in mitochondria (aα) and the cytoplasm (aα) was determined as described by Nobes et al. (36). Values for aα were 0.247 (± 0.039; n = 2) and 0.171 (± 0.073; n = 3) for old and young, respectively. The aα values were 0.356 (± 0.019; n = 2) and 0.322 (± 0.015; n = 2) for old and young, respectively.

At the end of each incubation, triplicate aliquots (0.70 ml) were removed and pipetted into 1.5-ml minitubes and immediately centrifuged in a minicentrifuge for 2 min. Then 200-µl aliquots of the supernatant were removed and pipetted into scintillation vials and immediately mixed with scintillant. The residual supernatant was aspirated; the sides of each tube were wiped dry, and 40 µl of 20% (vol/vol) Triton X-100 were added. After the suspension of the pellet by vortex mixing, the bottom of the tube was cut off into a scintillation vial and the pellet was resuspended in 3.0 ml of scintillant. The radioactivities of the supernatant and pellet were determined by dual-channel scintillation counting for ³H and ¹⁴C by use of the appropriate quench and crossover corrections.

The apparent volume of pellet available to each isotope (its space in µl) was calculated as disintegrations per minute in total pellet divided by disintegrations per minute of supernatant sample. The [³H]TPMP+ accumulation ratio, ([TPMP+]e/[TPMP+]m), was calculated as ([³H]TPMP+ space – [¹⁴C]methoxyinulin space)/(³H2O space – [¹⁴C]methoxyinulin space).

Application of top-down elasticity analysis and top-down control analysis. To quantitatively determine the important sites of effects of aging on oxidative phosphorylation processes, we used the top-down elasticity analytic approach described by Brand (7). We defined the oxidative phosphorylation system as the tripartite system shown in Fig. 1 and then determined the overall elasticities to changes in ΔΨm of the reactions that produce ΔΨm (cellular catabolic reactions, the tricarboxylic acid cycle, and the electron transport chain) and those that consume it (ATP synthesis and consumption and the proton leak). The kinetic response (or elasticity) of the ΔΨm to changes in ΔΨm was measured by titrating the ΔΨm consumers with oligomycin (0.01–0.05 µg/ml). The kinetic response of the leak to ΔΨm was assessed by titrating with antimycin (0.05–0.25 µM), an inhibitor of complex III of the respiratory chain.
chain, in the presence of saturating amounts of oligomycin (1.0 µg/ml). The elasticity of the phosphorylating subsystem to ΔΨm was measured from titrations with antimycin alone (0.10–0.20 µM). However, because the later titrations provide the kinetics of both ΔΨm-consuming subsystems (i.e., the phosphorylating and leak subsystems), corrections were made for the amount of oxygen required to balance the rate of the proton leak at each ΔΨm measured. This was done using the proton leak titration curve.

To determine the distribution of control over respiration rate and over ΔΨm in cells from old and young mice, we used top-down control analysis and the published equations for elasticities, flux control coefficients, and concentration control coefficients (11, 18).

Statistical analysis. Data were analyzed using unpaired Student's t-tests or ANOVA, which was followed by Tukey's post hoc tests. Linear regression lines were compared by analysis of covariance with use of Prism 2 for Windows. A P value of <0.05 was considered statistically significant. Unless otherwise stated, results are presented as means ± SE.

Materials. Oligomycin, antimycin, valinomycin, BSA (fraction V), collagenase (type IV), inulin, and trypan blue were from Sigma Chemical. FCCP and TPMP bromide were from Aldrich. 3H2O, Na[36Cl], [86Rb]Cl, [14C]methoxyinulin, and [3H]TPMP bromide were from Du Pont NEN. Water-insoluble compounds were dissolved in dimethyl sulfoxide.

RESULTS

Resting respiration rates and ΔΨm values in hepatocytes from old and young mice. The resting respiration rate of hepatocytes from old mice was significantly less than that of hepatocytes from young controls (P < 0.04). Values were 70.9 ± 3.4 (n = 10) and 83.8 ± 5.1 (n = 7) nmol O2·min⁻¹·10⁶ cells⁻¹, respectively. Resting state ΔΨm was not significantly different between cells from old and young mice. Values were 149 ± 4.4 mV (n = 8) and 147 ± 3.4 mV (n = 7) in cells from old and young mice, respectively. State 4 ΔΨm values were 155 ± 5.0 mV (n = 8) and 154 ± 3.0 mV (n = 7), respectively. Respiration rates and ΔΨm values are indicated in Fig. 2. The ΔΨm results indicate that any age-induced changes in the ΔΨm consumers (i.e., leak and ATP turnover in the resting state, and leak alone in State 4) are matched by any changes in the ΔΨm producers (i.e., substrate oxidation reactions).

Fig. 2. Kinetic responses of the mitochondrial proton leak (A), substrate oxidation (B), and phosphorylating (C) subsystems to ΔΨm in hepatocytes from old and young rats. Oxygen consumption rates are corrected for nonmitochondrial oxygen consumption. Open symbols, old cells; filled symbols, young cells. Each symbol marked with an asterisk in B and C represents a resting point [i.e., in the absence of carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) and oligomycin]. A: kinetic response of mitochondrial proton leak to ΔΨm (antimycin titration of oligomycin-inhibited respiration). B: kinetic response of substrate oxidation subsystem to ΔΨm. Lines were fitted by linear regression to oligomycin titration points; showing no difference in kinetics (P > 0.05 by analysis of covariance) of substrate oxidation subsystem. C: kinetic response of the phosphorylating subsystem to ΔΨm (antimycin titration of resting respiration rate from which rate of oxygen consumption used to balance the proton leak has been subtracted). Each point represents mean ± SE. Results are from cell preparations from the following nos. of old and young mice, respectively: 8 and 6 (A), 8 and 7 (B), and 5 and 5 (C). Each ΔΨm determination was carried out in triplicate; each oxygen consumption determination was carried out simultaneously in duplicate.
Comparison of the kinetic responses of the mitochondrial proton leak, substrate oxidation, and phosphorylation subsystems to Δψₘ in hepatocytes from old and young mice. The kinetic responses of the mitochondrial proton leak to Δψₘ in hepatocytes are shown in Fig. 2A. The oxygen consumption rates are corrected for nonmitochondrial oxygen consumption, as described in EXPERIMENTAL PROCEDURES. These results show that, over a wide range of Δψₘ values, the amount of oxygen used to support the mitochondrial proton leak is greater in cells from old mice. Because the kinetics of the proton leak subsystem are nonlinear, an analysis of covariance could not be used to test for statistically significant differences. However, taken together, the results show that the overall kinetics of the mitochondrial proton leak are altered in hepatocytes from old mice in relation to the results from young mice.

The kinetic responses of the substrate oxidation subsystem to Δψₘ in old and young hepatocytes are compared in Fig. 2B. The results indicate that, at any value of Δψₘ, there is no difference in the rate of the Δψₘ-producing reactions between old and young hepatocytes. Thus there are no age-related differences in the overall kinetics of the substrate oxidation reactions.

A comparison of the kinetic responses of the phosphorylating subsystem to Δψₘ in old and young hepatocytes revealed marked differences (P < 0.05; analysis of covariance) (Fig. 2C). At the resting state, the rate of the phosphorylating subsystem was ~30% lower in the old cells than in the young cells at identical values of Δψₘ (147 mV).

Quantitative analysis of the effects of the altered kinetics of the mitochondrial proton leak and phosphorylating subsystems to Δψₘ on respiration rate in old hepatocytes. The titrations of cellular respiration rate in old and young hepatocytes that were used to determine the kinetics of the subsystems described in Fig. 2 can be used to quantify the oxygen consumption that is used to sustain blocks of energy-dissipating reactions: nonmitochondrial oxygen consumption, proton leak reactions, and ATP turnover reactions as described by Brand (7). The proportion of resting cellular oxygen consumption that is nonmitochondrial was identified as that which was insensitive to saturating amounts of antimycin, oligomycin, valinomycin, and FCCP. There was no significant difference in the amounts of nonmitochondrial oxygen consumption; values for old and young cells were 22.1 ± 3.7 (n = 8) and 21.5 ± 1.2 (n = 7) nmol O₂·min⁻¹·10⁶ cells⁻¹, respectively. The total mitochondrial oxygen consumption and the amounts used to balance the proton leak and ATP turnover reactions at the resting value of Δψₘ are shown in Fig. 3. Despite the fact that the proportion of resting mitochondrial respiration used to balance the mitochondrial proton leak is doubled in the cells from old mice compared with young, there is a small but significant decrease in respiration (P < 0.04). This decrease can thus be accounted for entirely by a decrease (P < 0.03) in the rate of the only other block of reactions responsible for the dissipation of Δψₘ, i.e., ATP synthesis and consumption reactions.

Application of top-down control analysis. As well as being useful in the identification of the sites of action of an external effector and in the quantitative determination of the importance of changes induced within the system by an external effector, top-down elasticity analyses provide all the data needed for a top-down control analysis of the system (11, 18). A top-down control analysis was completed using the data from the present elasticity analyses around Δψₘ in old and young hepatocytes. The results for cells in the resting state and in State 4 are shown in Tables 1-3. The results were calculated using mitochondrial respiration rates; similar elasticities and control coefficients were obtained when calculations were based on total cellular respiration rates. The elasticities to Δψₘ of the substrate oxidation, phosphorylating, and proton leak subsystems are given in Table 1. All of the data needed for the calculation of elasticity and control coefficients can be obtained from Δψₘ and oxygen consumption values in Fig. 2, A, B, and C. Values of each, and the inverse slopes of the respective elasticity lines, are then used in the published series of equations (18) for the calculation of control coefficients.

The flux control coefficients of the three subsystems over the rate of each of the subsystems are shown in Table 2, A–C. The flux control coefficients describing the distribution of control over mitochondrial oxygen consumption (Jₛ) are shown in Table 2A. The results from the young control hepatocytes in the resting state indicate that the substrate oxidation reactions (0.51; i.e., 51% of the control) and phosphorylation reactions (0.45) exert most of the control over Jₛ, whereas the remaining of the control is through the proton leak...
AGE-RELATED CHANGES IN OXIDATIVE PHOSPHORYLATION

Table 1. Overall elasticities to $\Delta \Psi_m$ for substrate oxidation, phosphorylating, and proton leak subsystems in hepatocytes from old and young control mice

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<th>Young</th>
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<tr>
<td>$\varepsilon_m^S$</td>
<td>-10.2</td>
<td>-13.2</td>
<td>-449.5</td>
<td>-243.3</td>
</tr>
<tr>
<td>$\varepsilon_m^L$</td>
<td>5.2</td>
<td>10.3</td>
<td>20.4</td>
<td>14.5</td>
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<td>$\varepsilon_{am}$</td>
<td>3.3</td>
<td>8.3</td>
<td>20.4</td>
<td>14.5</td>
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$\Delta \Psi_m$, mitochondrial membrane potential; $S$, $P$, and $L$, substrate oxidation, phosphorylating, and proton leak subsystems; $\varepsilon$, elasticity; resting state, intermediate state between States 3 and 4 in cells incubated in a basal medium in the absence of inhibitors and uncouplers; State 4, respiratory state in which there is little or no ATP synthesis.

Results shown in Table 2 similarly show that in old cells compared with young, there is a shift in control over the phosphorylation reactions away from substrate oxidation reactions toward phosphorylation reactions and leak reactions. Control by the substrate oxidation reactions and phosphorylation reactions over the flux through the mitochondrial proton leak reactions (Table 2C) in old cells compared with young shows that there are decreases to roughly equal but opposite extents for these two blocks of reactions. Control over leak flux by the leak reactions themselves remains high in both old and young.

The concentration control coefficients of the three subsystems over the intermediate in the system, $\Delta \Psi_m$, are shown in Table 2D. Control coefficients describe the distribution of control by blocks of reactions in a system over the amount of the intermediate in the system, and, unlike flux control coefficients that sum to unity, concentration control coefficients sum to zero. The values are also similar to those determined for hypothyroid cells (25) and for euthyroid cells (11, 25). The results for old and young cells are roughly similar and show that most of the control over the amount of the intermediate, $\Delta \Psi_m$, is exerted by the substrate oxidation subsystem, and that the remainder of the control is through the activity of the $\Delta \Psi_m$ consumers: the proton leak and phosphorylating subsystems.

As described by Brand et al. (6), it is possible to estimate the actual number of ATP molecules synthesized by mitochondria for each oxygen atom consumed (effective P/O ratio) and the distribution of control (i.e., control coefficients) over the effective P/O by use of data such as those described above. Because phosphorylation flux is measured as the oxygen consumed to drive the phosphorylation reactions, the ratio of $J_p$ divided by $J_s$ provides the fraction of oxygen consumption that is used to support phosphorylation, irrespective of the true value of the maximum P/O. The remaining fraction of the oxygen consumption ($J_l/J_s$) is used to support mitochondrial proton leak reactions. Thus the effective P/O at any rate between State 3 and State 4 is this ratio multiplied by the maximum P/O, P/O$_{\text{max}}$. The effective P/O values, based on mitochondrial oxygen consumption data, are shown in Table 3. In theory, the oxidation of glucose by cells produces a maximum of 31 molecules of ATP per molecule of glucose (27); this corresponds to a P/O$_{\text{max}}$ of 2.58. The values shown in Table 3 for mitochondrial respiration are higher than the previously published values (6) and again support the postulate that cells are metabolically positioned close to State 3 respiration.

Brand et al. (6) reported the derivation of flux control coefficients, which quantitatively describe the control by the three blocks of reactions over the effective P/O

$$C^{PD}_s = C^{P}_s - C^{S}_s$$

$$C^{PD}_p = C^{P}_p - C^{S}_p$$

$$C^{PD}_l = C^{P}_l - C^{S}_l$$

These equations were used with the flux control coeffi-
Table 3. Control coefficients over effective P/O ratio in hepatocytes from old and young control mice

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<th>Resting</th>
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<tr>
<td></td>
<td>Old</td>
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<tr>
<td>Effective P/O ratio (J/P)</td>
<td>0.82</td>
</tr>
<tr>
<td>C_{P/O}</td>
<td>0.02</td>
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<tr>
<td>C_{P/O}</td>
<td>0.16</td>
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<tr>
<td>C_{P/O}</td>
<td>-0.18</td>
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In intact hepatocytes there is a substantial rate of nonmitochondrial oxygen consumption, so actual number of ATP molecules synthesized by mitochondria for each oxygen atom consumed (effective P/O ratio) differs depending on whether this is subtracted or not. When total cellular oxygen consumption is used as $J_S$, instead of mitochondrial oxygen consumption as above, the resulting effective P/O ratios are 0.56 and 0.67 for old and young, respectively.

The very recent findings of Brookes et al. (10) also support our findings. In their study on the effects of peroxynitrite on brain mitochondria, they found that three sequential additions of 200 µM peroxynitrite (initial concentration) to rat brain mitochondria (0.2 mg protein/ml) significantly stimulated mitochondrial proton leak. Cyclosporin A did not affect the stimulation, suggesting no involvement of the mitochondrial permeability transition pore. However, the stimulation was prevented by the vitamin E analog Trolox, supporting the involvement of lipid peroxidation, a proposed mechanism of peroxynitrite cytotoxicity. The authors link lipid peroxidation-mediated increases in mitochondrial proton leak to certain neurodegenerative disorders thought to proceed via mechanisms of mitochondrial oxidative damage.
The mechanism of proton leakage warrants discussion, particularly in light of recent developments in this area. Potential mechanisms underlying the mitochondrial proton leak are depicted in Fig. 4. Within the past year, two uncoupling proteins (UCP2 and UCP3) were identified and found to be distinct from the brown adipose tissue-specific uncoupling protein (UCP1) (4, 15, 44). These proteins explain at least some of the proton leak that has been assessed in mitochondria from a variety of tissues. Thus far, none of the three currently known uncoupling proteins have been found in parenchymal cells, which constitute 95–97% of the cells isolated using the techniques employed (2). Fleury et al. (15) report low levels of UCP2 mRNA in liver; however, recent findings localize UCP2 to the Kupffer cells of the liver (31). Nevertheless, it is possible, and likely, that there is an as-yet-unidentified uncoupling protein in the parenchymal cells. Furthermore, it is possible that there are changes in the amount and/or activity of such an uncoupling protein in these cells. However, again, our underlying hypothesis, founded on a substantial supportive literature, implicates free radical damage to lipids, thus affecting the integrity of the bilayer at the lipid-lipid or lipid-protein interfaces.

On the basis of previous reports of changes in the amounts and activities of a wide range of enzymes involved in substrate oxidation and specifically in components of the electron transport chain (3, 22, 43), we expected to observe changes in the kinetics of the substrate oxidation reactions. We hypothesized that we might observe results reflecting a decreased activity of this block of reactions. Alternatively, it was reasoned that, as a result of the increased proton leak, there may be a compensatory increase in the rate of the substrate oxidation reactions in an effort to restore protonmotive force to normal values; this would then accelerate the production of reactive oxygen metabolites, which in turn would induce further oxidative damage to cellular components. Our data (Fig. 2B) show clearly that any such changes as measured under our conditions are quantitatively insignificant. This does not indicate that free radical damage has not occurred but shows only that this group of reactions responds normally to imposed changes in the rate of its activity and may indicate also that the functioning of this block of reactions is at some level protected.

Our results describing decreases in the proportion of resting oxygen consumption of hepatocytes and altered kinetics of the phosphorylation reactions were not anticipated. Beyond the extensive analyses of respiratory control ratios in the literature (see for example Ref. 22), data on age-related alterations in ATP synthesis and turnover reactions are lacking. Thus it is difficult to speculate about the specific mechanisms responsible for the decreased amount of oxygen used by cells to support these reactions. As described in Fig. 1, ATP synthesis and turnover mechanisms include ATP synthetic reactions, such as the adenine nucleotide carrier and the phosphate transporter. This block of reactions also includes all cellular ATP-consuming processes, such as those involved in maintaining ion gradients across membranes (e.g., Ca\(^{2+}\)-ATPase, Na\(^+-\)K\(^{+}\)-ATPase) and in protein, DNA, and RNA synthetic reactions. Consistent with the oxidative stress theory, these findings may be related to known age-related increases in oxidative damage to mitochondrial proteins and DNA and should be examined further.

Importantly, the findings herein provide the first metabolic control analysis of oxidative phosphorylation in relation to the metabolic effects of aging. The data provide quantitative information about the control over resting oxygen consumption, over other blocks of reactions, and over \(\Delta W_m\). In old compared with young cells, there is a shift in control over resting oxygen consumption away from the substrate oxidation reactions toward phosphorylation and leak reactions. Thus oxygen consumption of old cells is more sensitive to changes in the rate of ATP turnover and in mitochondrial proton leak rate. Similarly, there is a shift in control over phosphorylation reactions away from substrate oxidation reactions toward phosphorylation reactions and the leak in old cells.

In addition, the control coefficients describing the control over the effective P/O denote that a greater amount of control is possessed by the ATP turnover reactions and the leak in old hepatocytes compared with young. This suggests an augmented capability of the latter blocks of reactions to affect changes in the efficiency of oxidative phosphorylation.
Overall, our findings, gathered from a relatively novel experimental perspective, extend our understanding of the effects of aging on oxidative phosphorylation in hepatocytes. The findings confer additional support for the oxidative stress theory of aging. They provide new quantitative data on the altered kinetics of the mitochondrial proton leak and of ATP turnover reactions and show shifts in metabolic control with aging.

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