We measured production of reactive oxygen species by intact mitochondria from rat skeletal muscle, heart, and liver under various experimental conditions. By using different substrates and inhibitors, we determined the sites of production (which complexes in the electron transport chain produced superoxide). By measuring hydrogen peroxide production in the absence and presence of exogenous superoxide dismutase, we established the topology of superoxide production (on which side of the mitochondrial inner membrane superoxide was produced). Mitochondria did not release measurable amounts of superoxide or hydrogen peroxide when respiring on complex I or complex II substrates. Mitochondria from skeletal muscle or heart generated significant amounts of superoxide from complex I when respiring on palmitoyl carnitine. They produced superoxide at considerable rates in the presence of various inhibitors of the electron transport chain. Complex I (and perhaps the fatty acid oxidation electron transfer flavoprotein and its oxidoreductase) released superoxide on the matrix side of the inner membrane, whereas center o of complex III released superoxide on the cytoplasmic side. These results do not support the idea that mitochondria produce considerable amounts of reactive oxygen species under physiological conditions. Our upper estimate of the proportion of electron flow giving rise to hydrogen peroxide with palmitoyl carnitine as substrate (0.15%) is more than an order of magnitude lower than commonly cited values. We observed no difference in the rate of hydrogen peroxide production between rat and pigeon heart mitochondria respiring on complex I substrates. However, when complex I was fully reduced using rotenone, rat mitochondria released significantly more hydrogen peroxide than pigeon mitochondria. This difference was solely due to an elevated concentration of complex I in rat compared with pigeon heart mitochondria.

The free radical theory of aging states that it is the mitochondrial production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, and the resulting accumulation of damage to macromolecules that causes aging and determines maximum lifespan (MLSP). Comparative approaches have shed considerable light on the relationship between ROS and MLSP. Notably, the rate of superoxide production by submitochondrial particles (3) and the rate of \( \text{H}_2\text{O}_2 \) production by mitochondria (4) are inversely related to MLSP in different species. A complicating factor is the association of longer MLSP with lower metabolic rates within mammals or other groups, but this complication has been resolved by the observation that birds tend to have longer MLSP than mammals with the same metabolic rate. Thus pigeons (long MLSP) have a lower rate of mitochondrial \( \text{H}_2\text{O}_2 \) production than rats (shorter MLSP), even though these two species have similar standard metabolic rates (5–8). Similarly, canaries and parakeets (budgerigars) (long MLSP) have lower rates of mitochondrial \( \text{H}_2\text{O}_2 \) production than mice (shorter MLSP), although all three species have similar standard metabolic rates (9).

Despite numerous studies reporting that mitochondria release \( \text{H}_2\text{O}_2 \), there is some controversy as to whether mitochondria are an important source of ROS under physiological and pathological conditions (10). In agreement with these concerns, Staniek and Nohl (12) reported that mitochondria respiring on complex I and complex II substrates do not generate \( \text{H}_2\text{O}_2 \) except in the presence of the complex III inhibitor antimycin A. They proposed that unspecific interactions between the commonly used methods of \( \text{H}_2\text{O}_2 \) detection and mitochondria cause artificial rates of \( \text{H}_2\text{O}_2 \) production (11, 12).

Two principal sites of superoxide generation have been identified in mitochondria: complex I and complex III. The relative importance of these two sites seems to vary with experimental conditions and between tissues and species (13). There is no clear consensus in the literature about which side of the mitochondrial inner membrane superoxide is generated by complex I and complex III. In the traditional view, complex III generates superoxide on the matrix side of the mitochondrial inner membrane (14). The semiquinone at center o of complex III of heart mitochondria was shown to be the main producer of superoxide based on inhibitor studies (15). However, the x-ray structure of complex III reveals that center o is oriented toward the intermembrane space (16, 17), suggesting that superoxide production by complex III is directed toward the cytoplasm and not toward the matrix. In support of this view, a recent study has reported that antimycin A-supplemented mitoplasts (mitochondrial devoid of portions of outer membrane and cytochrome c) can release superoxide (18). In complex I, either the iron-sulfur centers (7, 19) or the active site flavin (20) are thought to be mainly responsible for superoxide production. There is no x-ray crystal structure of complex I, but all of these centers are likely to face the matrix side of the membrane.

30 years ago, it was shown that the oxidation of palmitoyl carnitine by mitochondria leads to the generation of \( \text{H}_2\text{O}_2 \) (21, 22). These results received little attention and, to our knowledge, no study has examined how lipid metabolism could cause ROS generation in mammalian mitochondria. The oxidation of
fatty acids involves the electron transfer flavoprotein (ETF) and the electron transfer flavoprotein quinone oxidoreductase (ETF-QOR) that could act as potential sources of ROS production. The role of lipid metabolism in the generation of ROS by mitochondria gained our attention recently when it was shown that the expression of mitochondrial uncoupling proteins (UCPs) correlates with the use of lipid as fuel substrates (29, 22). A recent study that UCPs are activated by superoxide (26). An interesting hypothesis is that the elevated expression of UCPs during increased reliance on lipid metabolism is a part of a mechanism to protect against high levels of ROS production during the oxidation of fatty acids.

In light of the controversy regarding the production of H2O2 by mitochondria and its relevance to aging, additional studies to characterize the generation of ROS by mitochondria are required. The aims of the present study were 1) to quantify the production of ROS by intact mitochondria from different tissues, 2) to identify the electron transport sites involved in ROS generation, 3) to determine the topology of ROS production, on which side of the inner membrane ROS are produced, and 4) to re-examine differences in the production of ROS by heart mitochondria from rat and pigeon, species with similar standard metabolic rate but markedly different MLSP.

EXPERIMENTAL PROCEDURES

Materials—The horseradish peroxidase, homovanillic acid, superoxide dismutase (SOD), and hydrogen peroxide solution, were purchased from Sigma. The substrates (malate, pyruvate, succinate, carnitine, and palmitoyl carnitine), inhibitors (rotenone, antimycin A, myxothiazol, and oligomycin), and bovine serum albumin (BSA) were also obtained from Sigma.

Isolation of Mitochondria—Female Wistar rats aged between 5 and 8 weeks were used. Pigeons were provided by Abbott Brothers, Norfolk, UK. Skeletal muscle, heart, and liver mitochondria were isolated as described (26), resuspended in standard assay medium containing 120 mM KCl, 5 mM KH2PO4, 3 mM Heps, 1 mM EGTA, and 0.3% BSA (pH 7.2 at 20 °C) and kept on ice. Mitochondria were of good quality (at least 3-fold increase in respiration rate in the presence of an uncoupler). Protein content was determined using the Buret method (27) with BSA as standard.

Measurement of Mitochondrial H2O2 Production—The rate of mitochondrial production of H2O2 was determined by following its reaction with homovanillic acid in the presence of horseradish peroxidase (28) using a Shimadzu RF5301 PC spectrofluorophotometer. Rat and pigeon mitochondria were incubated at 0.4 mg/ml at 37 °C and 41 °C, respectively, in standard assay medium. The following reactants were incorporated to the standard assay medium at the final concentrations indicated in parentheses. First, horseradish peroxidase (12 units/ml; one unit forms 18 μM per min of purpuragallin from pyrogallol at 25 °C) and homovanillic acid (0.1 mM) were added and a 3-min incubation period was allowed for temperature equilibration. Oligomycin (1 μg/ml mitochondrial protein), an inhibitor of the F1F0-ATP synthase, was added to prevent ATP synthesis. Production of H2O2 was started by adding substrate: malate (1 mM) and pyruvate (2.5 mM) for complex I; succinate (5 mM), in the presence of rotenone (10 μM), for complex II or palmitoyl carnitine (60 μM), in the presence of 1-carnitine (2 mM), as a lipid-derived substrate. Oxidation of palmitoyl carnitine generates equal amounts of NADH, which enters the electron transport chain at complex I, and FADH2, which enters via the ETF and ETF-QOR.

Calibrations and Corrections—Calibration curves were obtained by adding known amounts of H2O2 to assay medium in the presence of the reactants (homovanillic acid and horseradish peroxidase). They were performed in the absence and presence of mitochondria to establish whether any mitochondrial components interfered with the assay. Standard curves were linear up to 6 μM H2O2 (Fig. 1). Mitochondria quenched the fluorescence: the slopes of the standard curves obtained in the presence of liver, skeletal muscle, and heart mitochondria were 66, 81 and 86%, respectively, of the slope of the control curve without mitochondria (Fig. 1). Fig. 2 illustrates the effects of various corrections that we applied to the rates of H2O2 production using skeletal muscle as an example. Horseradish peroxidase and SOD were in excess in the experiments since doubling the final concentration of these enzymes did not affect the results (data not shown). Rates of mitochondrial H2O2 production were higher if calculated using the standard curve with mitochondria than using the control standard curve (Fig. 2; compare the black and white bars). There were significant rates of apparent H2O2 production in the absence of mitochondria (calibrated using the control standard curve). SOD, added at 50 units/ml (one unit inhibits the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25 °C in a 3-ml reaction volume), caused significant interference to the detection method, increasing the fluorescence signal under all conditions (Fig. 2, gray bars, and Fig. 3). The final rates of H2O2 production (fully corrected signals; dotted bars in Fig. 2) were obtained by subtracting the rates measured in the absence of mitochondria (calibrated without mitochondria: gray bars) from the rates measured in the presence of mitochondria (calibrated with mitochondria; white bars). All the following results represent fully corrected rates of H2O2 production.

Sites and Topology of Mitochondrial H2O2 Production—Various inhibitors of electron transport were used to define more precisely the sites of ROS production (28). Rotenone inhibits complex I, while myxothiazol (used at 0.625 nmol/ml protein) and antimycin A (used at 0.625 nmol/ml protein) inhibit center o and center i of complex III respectively.

To determine which side of the mitochondrial inner membrane superoxide (O2-) was generated, we measured the rate of H2O2 production in the presence and absence of exogenous SOD. If mitochondria produce O2- on the cytoplasmic face of the inner membrane, addition of exogenous SOD will increase the natural dismutation rate of O2- (and that catalyzed by any contaminating or endogenous SOD) and compete with other side reactions, leading to an elevated rate of H2O2 production. If mitochondria generate O2- on the matrix side of the inner membrane, much of it will be converted to H2O2 by the matrix SOD. H2O2 will diffuse out, and there will be no difference in the rates of extramitochondrial H2O2 appearance in the presence and absence of exogenous SOD. Complex I concentration was measured as described by Burch (29).

Statistical Analyses—Statistical analyses were performed using SigmaStat 2.0. The H2O2 production rate of mitochondria from a given tissue with a given substrate was compared between different experimental conditions using one-way analysis of variance and the a posteriori Tukey test. Comparisons between rat and pigeon heart mitochondria were carried out using a paired t test. The level of significance was p = 0.05.
RESULTS

Skeletal Muscle and Heart Mitochondria—The rate of \( \text{H}_2\text{O}_2 \) production by skeletal muscle or heart mitochondria incubated with malate and pyruvate was very low and was not increased by addition of SOD (Figs. 4A and 5A). However, the addition of rotenone to mitochondria respiring on malate and pyruvate induced a significant rate of \( \text{H}_2\text{O}_2 \) generation (Figs. 4A and 5A). This rotenone-stimulated \( \text{H}_2\text{O}_2 \) production came primarily from the matrix side of the inner membrane since the signal was insensitive to addition of exogenous SOD (Figs. 4A and 5A). These results indicate that complex I in skeletal muscle and heart mitochondria can generate superoxide on the matrix side of the inner membrane when it is fully reduced and inhibited by rotenone but that the rate measured when the complex is not inhibited by rotenone is extremely low.

There was no detectable rate of \( \text{H}_2\text{O}_2 \) production when skeletal muscle or heart mitochondria were fed succinate (in the presence of rotenone) in the absence or presence of SOD (Figs. 4B and 5B). There was little or no rate of \( \text{H}_2\text{O}_2 \) production in the presence of myxothiazol in the absence or presence of SOD (Figs. 4B and 5B), showing that neither complex II nor the ubiquinone pool can produce significant amounts of \( \text{O}_2^- \) on either side of the membrane when they are extensively reduced. Addition of antimycin A led to a low but measurable rate of \( \text{H}_2\text{O}_2 \) production (Figs. 4B and 5B) that was significantly increased by addition of exogenous SOD (Figs. 4B and 5B). These results indicate that center \( \text{o} \) of complex III can generate \( \text{O}_2^- \) on the cytoplasmic face of the inner membrane of skeletal muscle or heart mitochondria when it is reduced following inhibition of the complex at center \( \text{i} \) by antimycin A but that the rate measured when the complex is not inhibited by antimycin A is extremely low.

There was some \( \text{H}_2\text{O}_2 \) production in the presence of antimycin A even without exogenous SOD, which might suggest that center \( \text{o} \) can also produce \( \text{O}_2^- \) on the matrix side of the inner membrane. However, even if mitochondria only produce \( \text{O}_2^- \) on the cytoplasmic face of the membrane, there will be a detectable rate of \( \text{H}_2\text{O}_2 \) production without exogenous SOD because of contaminating or endogenous SOD and the natural dismutation of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \). In other words, if a rate of \( \text{H}_2\text{O}_2 \) production has a SOD-sensitive and a SOD-insensitive part, we can state that the SOD-sensitive part emanates from the cytoplasmic face of the membrane but we cannot be certain whether the SOD-insensitive part represents the natural dismutation rate of \( \text{O}_2^- \) coming from the cytoplasmic face of the membrane or \( \text{H}_2\text{O}_2 \) coming from the matrix side of the membrane.

Skeletal muscle or heart mitochondria respiring on palmitoyl carnitine had a significant rate of \( \text{H}_2\text{O}_2 \) production that was not significantly increased by addition of SOD (Figs. 4C and 5C). This result indicates that oxidation of palmitoyl carnitine (unlike oxidation of malate and pyruvate or succinate plus rotenone) leads to significant ROS production and that this ROS is produced mostly on the matrix side of the inner membrane. The addition of rotenone led to a slight increase in the rate of \( \text{H}_2\text{O}_2 \) production that did not reach statistical signifi-
cance and was SOD-insensitive (Figs. 4C and 5C). The rates of 
H$_2$O$_2$ production in the presence of palmitoyl carnitine and 
rotenone were very similar to those in the presence of malate, 
pyruvate, and rotenone (Figs. 4A and 5A), suggesting that 
complex I was the source of this ROS with either substrate 
when complex I was fully reduced in the presence of rotenone. 
In the absence of rotenone, perhaps complex I is more reduced 
with palmitoyl carnitine as substrate (due to reversed electron 
transport and competition with ETF-QOR for oxidized Q) than 
it is with malate and pyruvate, leading to greater endogenous 
ROS production from complex I with palmitoyl carnitine.

In the presence of palmitoyl carnitine, the addition of myx-
thiazol led to an increase in the rate of H$_2$O$_2$ production 
that did not reach statistical significance in skeletal muscle mito-
chondria and was SOD-insensitive (Figs. 4C and 5C). Statistical significance (as in Fig. 4) is 
indicated by different letters.

Addition of antimycin A to skeletal muscle or heart mito-
chondria supplemented with palmitoyl carnitine greatly in-
creased the rate of H$_2$O$_2$ production (Figs. 4C and 5C). This 
rate was increased by addition of SOD (in skeletal muscle 
mitochondria), showing that much of it was due to production 
of O$_2^-$ on the cytoplasmic face of the inner membrane. Under 
these conditions complex I, complex II, ETF-QOR, the ubiqui-
none pool, and center o of complex III may all be strongly 
reduced. In the presence of SOD, H$_2$O$_2$ production with palmi-
toyl carnitine plus antimycin A appeared to be higher than the 
sum of the individual contributions from complex I, complex II, 
ETF-QOR, the ubiquinone pool, and center o of complex III. 
The reason for this discrepancy is unclear.
Liver—In general, the rates of H$_2$O$_2$ production of liver mitochondria were lower than those of skeletal muscle and heart mitochondria, particularly with succinate or with palmitoyl carnitine as substrate (compare Figs. 4, 5, and 6), making reliable interpretations difficult. Liver mitochondria respiring on malate and pyruvate, produced less than 0.2 nmol H$_2$O$_2$/min/mg mitochondrial protein in the absence of SOD (Fig. 6A). The addition of rotenone did not increase H$_2$O$_2$ production, but SOD appeared to increase the rate of H$_2$O$_2$ production in the presence of rotenone (Fig. 6A). These results appear to suggest that complex I from liver mitochondria generates ROS on the cytoplasmic face of the inner membrane as well as on the matrix side, but because of the small signal we consider them to be unreliable.

Liver mitochondria respiring on succinate did not generate H$_2$O$_2$ (Fig. 6B), except perhaps for a small signal in the presence of SOD and antimycin A or myxothiazol. Liver mitochondria respiring on palmitoyl carnitine did not produce H$_2$O$_2$ (Fig. 6C), except perhaps for a small signal in the presence of rotenone, antimycin A, or myxothiazol. However, the rates of H$_2$O$_2$ production were minimal, making it difficult to interpret their significance.

Comparisons of the Rate of H$_2$O$_2$ Production between Rat and Pigeon Heart Mitochondria—In a separate series of experiments, neither rat nor pigeon heart mitochondria generated measurable amounts of H$_2$O$_2$ when supplemented with malate and pyruvate (Fig. 7A). The addition of rotenone increased the rate of H$_2$O$_2$ production in both species (Fig. 7A). In the presence of rotenone, the H$_2$O$_2$ production rate per mg of mitochondrial protein was higher with rat mitochondria than with pigeon mitochondria (Fig. 7A). The amount of complex I present in the two types of mitochondria was measured (Fig. 7B) to see whether the greater capacity of rat heart mitochondria to produce ROS in the presence of rotenone was caused by a greater concentration of complex I. Complex I was significantly greater in rat mitochondria than in pigeon mitochondria. Fig. 7C shows that the different capacities for ROS production between rat and pigeon heart mitochondria were lost when H$_2$O$_2$ production rate was expressed per mole of complex I.

**DISCUSSION**

Mitochondria from rat skeletal muscle, heart, and liver respiring on substrates feeding exclusively to complex I (malate plus pyruvate) or complex II (rotenone plus succinate) in the absence of other inhibitors generated little or no measurable H$_2$O$_2$ (Figs. 4, 5, and 6). The absence of significant generation of H$_2$O$_2$ from mitochondria respiring on complex I and complex II substrates supports results from Staniek and Nohl (12) showing a lack of H$_2$O$_2$ production from rat heart mitochondria respiring on glutamate/malate, malate/pyruvate, and succinate. Hansford et al. (36) and Liu et al. (20) also reported minimal rates of H$_2$O$_2$ production from rat heart, brain, or liver mitochondria with glutamate/malate or rotenone/succinate as substrates. In the absence of rotenone, succinate supports considerable ROS production from complex I by reverse electron transport (e.g., see Ref. 20); this aspect was not pursued in the present paper. However, many other studies have reported significant rates of H$_2$O$_2$ production in isolated mitochondria (reviewed in Ref. 13). Most of these studies used SOD in the assay (6–8, 30). We obtained considerable rates of H$_2$O$_2$ generation in the presence of SOD if we did not correct for background effects (Fig. 2). Therefore, some of the discrepancies in production of H$_2$O$_2$ by mitochondria respiring on complex I and II substrates between various studies might be due to the presence or absence of inhibitors of reversed electron transport or to methodological artifacts as suggested by Forman and Azzi (10) and Staniek and Nohl (12).

In contrast, rat skeletal muscle and heart mitochondria produced H$_2$O$_2$ at measurable rates when respiring on palmitoyl carnitine with no added inhibitors (Figs. 4 and 5). This H$_2$O$_2$ was produced primarily on the matrix side of the membrane, since it was not significantly enhanced by addition of exogenous SOD. The greater H$_2$O$_2$ production with palmitoyl carnitine than with complex I substrates could be because complex I is more reduced with palmitoyl carnitine, due to reversed electron transport and competition with ETF-QOR for oxidized Q. Greater steady-state reduction of complex I would lead to greater matrix ROS production with palmitoyl carnitine as substrate. Additionally, it could be that ETF-QOR and ETF can also produce O$_2^-$ on the matrix side of the membrane when palmitoyl carnitine is added.

ETF accepts electrons from eight primary dehydrogenases in the mitochondrial matrix including those of the $\beta$-oxidation cycle. ETF is rapidly reduced to the semiquinone form (ETF$_{SQ}$) and more slowly to the fully reduced form, suggesting that ETF$_{SQ}$ is the electron donor to ETF-QOR (31). ETF-QOR can be fully reduced by accepting three electrons, but it accepts only...
two electrons when ETF is the electron donor (32). It was proposed that the FAD prosthetic group of ETF-QOR cycles between the oxidized and semiquinone forms (31). These data suggest that ETF and ETF-QOR could act as generators of superoxide owing to their presence in partially reduced states during lipid metabolism.

Early papers on the production of \( \text{H}_2\text{O}_2 \) by mitochondria showed that rat liver and pigeon heart mitochondria release \( \text{H}_2\text{O}_2 \) when respiring on 10 \( \mu \text{M} \) palmitoyl carnitine (21, 22). These results gained little attention since succinate gave higher rates of \( \text{H}_2\text{O}_2 \) production. However, the lower rates of \( \text{H}_2\text{O}_2 \) production with palmitoyl carnitine in these early reports might reflect the absence of catalytic carnitine and the use of subsaturating concentrations of palmitoyl carnitine. It is tempting to speculate that increased production of ROS by mitochondria during lipid metabolism might lead to an increase in the expression and activity of UCPS to counteract the deleterious effects of ROS on mitochondria. In fact, UCP2 and UCP3 expression is increased when fatty acids are high (23, 35). Fatty acids and superoxide activate uncoupling by UCPs, and it was recently suggested that the primary role of UCP2 and UCP3 might be for protection against ROS (25).

It is commonly repeated that 2% of electron flow during mitochondrial respiration gives rise to \( \text{H}_2\text{O}_2 \) (33). More recently, Hansford et al. (36) reported values of free radical leak in the range of 0.4–0.8% for heart mitochondria respiring on physiological concentrations of succinate (less than 0.5 \( \mu \text{M} \)). Even if we consider our results with saturating palmitoyl carnitine (0.15 \( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) per minute of mitochondrial protein) for skeletal muscle mitochondria (Fig. 4), only 0.15% of electron flow gives rise to \( \text{H}_2\text{O}_2 \) under resting conditions with a respiration rate of 200 \( \mu \text{M} \) of electrons/minute of mitochondrial protein. This estimate of free radical leak would be lower at physiological partial pressure of oxygen, since the rate of \( \text{H}_2\text{O}_2 \) production by mitochondria decreases steadily with oxygen tension (22). It would be even lower in more physiologically realistic conditions of subsaturating palmitoyl carnitine and lower mitochondrial membrane potential due to background ATP synthesis. Therefore, our upper estimate of free radical leak is one to two orders of magnitude lower than the frequently cited values.

The results presented in this paper show that rat heart and skeletal muscle mitochondria generally display considerably higher rates of \( \text{H}_2\text{O}_2 \) production than liver mitochondria either in the absence or presence of inhibitors, consistent with the idea that postmitotic tissues generate more ROS through accumulation of oxidative damage (34). A simpler explanation for these results is that liver mitochondria have a reduced content of mitochondrial electron transport chain components compared with heart and skeletal muscle (35). Specifically, rat liver mitochondria have approximately 10 times less complex I and six times less complex III activity than heart or skeletal muscle mitochondria (35).

To identify the major potential sites of mitochondrial ROS production and the topology of ROS production from them, we used inhibitors of complex I and III of the electron transport chain. The inhibitors should strongly reduce different respiratory complexes and cause them to generate sufficient ROS to override the natural antioxidant systems of mitochondria. This allowed us to determine which complex has the largest capacity to generate ROS. The presence and absence of SOD permitted the identification of the topology of ROS production. We found that center \( o \) of complex III (rotenone plus succinate plus antimycin A) can generate more ROS than complex I (malate plus pyruvate plus rotenone) (Figs. 4 and 5). Center \( o \) of complex III generates superoxide (at least in part, and probably exclusively) on the cytoplasmic face of the mitochondrial inner membrane, whereas complex I produces ROS solely on the matrix side. Experiments using mitoplasts indicated that complex III can release superoxide on the cytoplasmic face of the inner membrane (18). Many studies using intact mitochondria have reported increases in \( \text{H}_2\text{O}_2 \) production in the presence of antimycin A and rotenone (12, 13, 22, 36, 37). However, none of these studies examined the topology of ROS production. Mitochondria respiring on complex I substrates increase their \( \text{H}_2\text{O}_2 \) production in the presence of myxothiazol (13, 36). Since the rates in the presence of rotenone in these studies are similar or higher than those in the presence of myxothiazol, it is probable that the ROS generated in the presence of myxothiazol actually originated from complex I. Finally, the possible SOD-insensitive increase in the rate of \( \text{H}_2\text{O}_2 \) production by mitochondria supplemented with palmitoyl carnitine and myxothiazol leads us to suggest that ETF and ETF-QOR may also produce ROS on the matrix side of the inner membrane (Figs. 4 and 5).

At a physiological level, our results showing very low \( \text{H}_2\text{O}_2 \) production by mitochondria respiring on complex I and II substrates in the absence of inhibitors fail to provide support for the conclusions that there is an inverse relationship between MLSP and \( \text{H}_2\text{O}_2 \) production by mitochondria from various species (4) or that pigeon mitochondria respiring on complex I or II substrates generate less \( \text{H}_2\text{O}_2 \) than rat mitochondria (5–8, 30). Indeed, we found that heart mitochondria from pigeons and rats respiring on malate/pyruvate did not generate measurable amounts of \( \text{H}_2\text{O}_2 \). In the presence of rotenone, however, rat mitochondria produced more \( \text{H}_2\text{O}_2 \) per mg of mitochondrial protein than did pigeon mitochondria (Fig. 7A), showing that the capacity of rat mitochondria to generate ROS is indeed higher. This was caused by a higher content of complex I in rat heart mitochondria (Fig. 7B). The rates of ROS production per mole of complex I were not higher in rat mitochondria, suggest-
ing that complex I from pigeon heart mitochondria does not have a decreased intrinsic capacity for ROS production. These data confirm that the maximum capacity of pigeon heart mitochondria to generate ROS from complex I is less than in rat, but fail to provide support for the theory that the elevated MLSP of pigeons compared with rats is partially due to their lower endogenous mitochondrial production of H$_2$O$_2$. It would be enlightening to compare mitochondrial H$_2$O$_2$ production between animals of different MLSP using palmitoyl carnitine.

Finally, our results should not be interpreted as meaning that mitochondria produce no ROS under physiological conditions, where the major substrates will usually be pyruvate from glycolysis or long chain fatty acyl carnitines from lipid oxidation. Our results suggest that if complexes I and III of mitochondria do produce ROS physiologically during oxidation of pyruvate through the Krebs cycle, these ROS are mostly scavenged by antioxidant defense systems and little or no H$_2$O$_2$ escapes into the cytosol. On the other hand, fatty acid β-oxidation does lead to release of ROS, primarily from complex I on the matrix side of the inner membrane, which can lead to H$_2$O$_2$ spilling out into the cytosol. However, it is important to emphasize that whatever the substrate, complex I, complex III, and perhaps ETF and ETF-QOR do have the capacity to generate superoxide. A consistent small leak of electrons to oxygen that escapes the antioxidant defense systems during normal metabolism might still be sufficient to cause accumulation of oxidative damage, ultimately resulting in aging.

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