Lipid Oxidation by Heart Mitochondria from Young Adult and Senescent Rats

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1. State-3 (i.e. ADP-stimulated) rates of O_2 uptake with palmitoylcarnitine, palmitoyl-CoA plus carnitine, pyruvate plus malonate plus carnitine and octanoate as respiratory substrate were all diminished in heart mitochondria isolated from senescent (24-month-old) rats compared with mitochondria from young adults (6 months old). By contrast, State-3 rates of O_2 uptake with pyruvate plus malate or glutamate plus malate were the same for mitochondria from each age group. 2. Measurements of enzyme activities in disrupted mitochondria showed a decline with senescence in the activity of acyl-CoA synthetase (EC 6.2.1.2 and 6.2.1.3), carnitine acetyltransferase (EC 2.3.1.7) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), but no change in the activity of carnitine palmitoyltransferase (EC 2.3.1.21) or acyl-CoA dehydrogenase (EC 1.3.99.3). 3. Measurement of DL-[3H]carnitine (in)/acetyl-L-carnitine (out) exchange in intact mitochondria showed decreased rates when the animals used were senescent. However, this followed from a decreased intramitochondrial pool of exchangeable carnitine, such that calculated first-order rate constants for exchange were identical in mitochondria from the two age groups. 4. The decline in acyl-CoA synthetase activity is thought to be the reason for the diminished rate of O_2 uptake with octanoate in senescence. The decline in carnitine acetyltransferase activity is considered to be the cause of the diminished rate of O_2 uptake with acetylcarnitine or with pyruvate plus malonate plus carnitine as substrate. The mechanism of the diminished rate of O_2 uptake with palmitoylcarnitine in senescence is discussed.

Fatty acids are a major substrate for oxidative metabolism in heart muscle, and indeed the oxidation of fatty acids tends to take precedence over the oxidation of glucose or pyruvate [see Neely *et al.* (1972) for a review]. In this light, the finding of Chen *et al.* (1972), that mitochondria isolated from the hearts of senescent rats show a 20% lower rate of palmitoylcarnitine oxidation than do mitochondria from young adult animals, may be of special significance. Conceivably the overall rate of energy transduction by the mitochondria might be decreased, placing a limitation on the intensity of performance of mechanical work, or alternatively carbohydrate oxidation might be responsible for a greater share of the total O_2 consumption, resulting in less sparing of carbohydrate for use by other tissues. There is some evidence that the latter may be true, as Hansford (1977) reported a lesser degree of pyruvate dehydrogenase inactivation in the presence of palmitoylcarnitine in mitochondrial preparations from the hearts of senescent rats. Chen *et al.* (1972) ruled out

the possibility of an age-linked decrement in the transfer of hydrogen from NADH to oxygen and suggested instead an age-linked lesion at the level of mitochondrial substrate transport or dehydrogenation, or at the level of ancillary enzymes, e.g. acyltransferases. They concluded that considerable scrutiny would be needed to decide between these various possibilities.

The present paper reports the results of some such scrutiny. The overall metabolic pathway of palmitoylcarnitine oxidation was dissected into portions by studying mitochondrial O_2 consumption with substrates that require the use of less than the whole pathway. Thus the oxidation of octanoate obviates the requirement for acylcarnitine translocation and acyl-group transfer, and the oxidation of acetylcarnitine involves acyl-group transfer but does not involve β -oxidation. In addition to these studies with intact mitochondria, the activities of five enzymes that the above work suggested might be diminished in senescence were measured directly in detergent-solubilized mitochondria. This allows comment to be made on the suggestion by Chen *et al.* (1972) that carnitine palmitoyltransferase (EC 2.3.1.21) might

Abbreviation used: Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid.

be diminished in activity. It also allows the statement that a rather general loss in activity of the enzymes associated with fatty acid oxidation occurs with senescence, with at least three enzymes being diminished in activity by 30–40%.

Finally, the fact that intact mitochondria from senescent animals showed a lowered rate of O_2 consumption with palmitoylcarnitine, acetylcarnitine and pyruvate plus malonate plus carnitine, all substrate combinations that involve acylcarnitine transport, suggested that a direct measurement be made of the activity of the carnitine/acylcarnitine antiporter described by Pande (1975), Ramsay & Tubbs (1975, 1976) and Pande & Parvin (1976) and responsible for carnitine/acylcarnitine exchange across the mitochondrial membrane. Results from such experiments are presented.

Experimental

Preparation of mitochondria

Mitochondria were prepared from the hearts of 6- or 24-month-old male Wistar-derived rats from the Gerontology Research Center colony. The method was that described by Chappell & Hansford (1972), with the modification that only one heart was used and that the amount of the proteolytic enzyme Nagarse was decreased to 2.5 mg. Care was taken not to exceed a total volume of 20 ml at any stage of the preparation, a more dilute homogenate or suspension resulting in an unstable preparation.

Measurement of rates of O_2 consumption

O_2 partial pressure was measured with a Clark-pattern oxygen electrode and the mitochondrial suspension was contained in a water-jacketed chamber (Gilson) maintained at the temperature indicated. The basal incubation medium was either 0.12 M-KCl, 20 mM-Tes (potassium salt) and 20 mM-potassium phosphate, pH 7.2 (studies at 25°C), or 0.13 M-KCl and 20 mM-potassium phosphate, pH 7.2 (studies at 37°C). In general, the rate of respiration tabulated in Tables 1 and 2 is that elicited by the addition of ADP (final concn. 1 mM) 1 min after the addition of the mitochondrial suspension. With acetylcarnitine as substrate, a higher rate of respiration was obtained if the reaction was instead begun by the addition of mitochondria. For the oxidation of octanoate and acetate, the medium contained 10 mM-glucose, 1 mM-ATP and 1 mM-MgCl₂ in addition to the oxidizable substrate. Dialysed hexokinase was then added in small increments to generate the respiratory rate tabulated. An excess hexokinase activity, or the addition of ADP, led to respiratory inhibition. The protein content of the mitochondrial suspensions was determined as described by Murphy & Kies (1960).

Measurement of enzyme activities

All spectrophotometric determinations of rate were made at 25°C by using a Gilford 250 recording spectrophotometer. Acyl-CoA synthetase activity (probably the sum of EC 6.2.1.2 and 6.2.1.3) was measured by hydroxamate formation, basically by the method of Beinert *et al.* (1953). A mitochondrial suspension (100 μ l, containing 3 mg of protein) was added to 1.9 ml of an incubation mixture comprising 50 mM-Tris/HCl, pH 8, 40 mM-KCl, 0.05% (w/v) Triton X-100, 3.8 mM-ATP, 7.5 mM-MgCl₂, 7.5 mM-potassium octanoate, pH 8, oligomycin (10 μ g/ml), 0.75 mM-CoA, 15 mM-phosphocreatine, adenylate kinase (13 μ g/ml), creatine kinase (0.5 mg/ml) and 0.2 M-hydroxylamine neutralized daily to pH 8 with KOH. After 10, 20 and 30 min intervals 0.5 ml portions of the incubation mixture were removed and added to 0.5 ml of 2.5% (w/v) FeCl₃ in 0.5 M-HCl, on ice. A_{540} was read after centrifugation (10000 g for 5 min), and a millimolar absorption coefficient of 0.975 litre·mmol⁻¹·cm⁻¹ was assumed (Berg, 1962). Control incubations were carried out minus octanoate or CoA, and this absorbance was subtracted.

Carnitine acetyltransferase (EC 2.3.1.7) was measured as described by Chase (1969). A portion (5 μ l) of a mitochondrial suspension (approx. 30 mg of protein/ml) was added to a cuvette containing 1 ml of a reaction mixture comprising 0.1 M-Tris/HCl, pH 8, 0.25 mM-EDTA, 20 mM-potassium malate, pH 8, 0.25 mM-NAD⁺, rotenone (2.5 μ g/ml), 1 mM-CoA, malate dehydrogenase (25 μ g/ml), citrate synthase (50 μ g/ml) and 0.04% (w/v) Triton X-100. After 2 min, the reaction was initiated by the addition of 5 μ mol of acetyl-DL-carnitine, and increase in A_{340} was recorded. Controls established that the detergent concentration was optimal and the 'coupling' enzyme activities were sufficient.

Carnitine palmitoyltransferase was measured by the release of CoA from palmitoyl-CoA. Mitochondria (5 μ l of suspension, containing approx. 30 mg of protein/ml) were added to a cuvette containing 0.85 ml of 0.1 M-Tris/HCl, pH 7.8, 0.25 mM-EDTA, 100 μ M-palmitoyl-CoA, 12 mM-DL-carnitine, 0.05% (w/v) Triton X-100 and 0.12 mM-5,5'-dithiobis-(2-nitrobenzoate). The increase in A_{412} was measured and the millimolar absorption coefficient taken to be 13.6 (Ellman, 1959). The experiment was repeated without carnitine and the non-specific rate thus measured was subtracted. Carnitine palmitoyltransferase activity was also measured by thioester disappearance, at 232 nm. A portion (5 μ l) of a mitochondrial suspension was added to a cuvette containing 0.85 ml of 0.1 M-Tris/HCl, pH 7.8, 0.25 mM-EDTA, 100 μ M-palmitoyl-CoA and 0.012% sodium dodecyl sulphate. The reaction was initiated, 6 min later, by the addition of 10 μ mol of DL-carnitine. The

reaction was monitored by decrease in A_{232} and it was assumed that a millimolar absorption coefficient of 4.5 given by Stadtman (1957) for C_4 - C_8 saturated acyl-thioesters was applicable. The concentrations of detergent and palmitoyl-CoA used were shown to give maximal activities in each type of assay.

Acyl-CoA dehydrogenase (EC 1.3.99.3) was measured by 2,6-dichlorophenol-indophenol reduction, basically as described by Hoskins (1969). A portion (10 μ l) of a mitochondrial suspension (approx. 30 mg of protein/ml) was added to a cuvette containing 0.8 ml of 0.12 M-potassium phosphate, pH 6.8, 0.05% (w/v) Triton X-100, 80 μ M-2,6-dichlorophenol-indophenol and 1 mM-KCN. The reaction was initiated, 3 min later, by the addition of 100 nmol of palmitoyl-CoA. The decrease in A_{600} was recorded and the millimolar absorption coefficient was taken to be 21 (Armstrong, 1964). The palmitoyl-CoA concentration used gave essentially maximal rates; higher concentrations of 2,6-dichlorophenol-indophenol gave slightly higher rates, but were considered impractical owing to the high total absorbance. The assay was modified to include phenazine methosulphate, and some data obtained in this way are also presented in Table 3. The protocol suggested by Hoskins (1969) of extrapolating to infinite phenazine methosulphate concentration proved to be impractical, as the plots of $1/v$ versus $1/s$ were markedly non-linear. Instead the concentration of phenazine methosulphate giving the highest rate (0.8 mM) was adopted and used in subsequent assays. The inclusion of FAD gave no additional enhancement in rate.

3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) was measured by the oxidation of NADH by acetoacetyl-CoA. A portion (5 μ l) of a mitochondrial suspension (approx. 30 mg of protein/ml) was added to a cuvette containing 0.75 ml of 12.5 mM-sodium pyrophosphate buffer, pH 7.3, containing 0.05% (w/v) Triton X-100, 2.7 mM-KCN, pH 7.7, 0.2 mM-NADH and rotenone (2.5 μ g/ml). After 2 min, the reaction was initiated by the addition of 100 μ M-acetoacetyl-CoA. Higher substrate concentrations led to slight inhibition.

Measurement of carnitine/acylcarnitine transport

After preparation, mitochondria were loaded with DL-[3 H]carnitine essentially as described by Pande (1975). Rotenone (5 μ g) and antimycin (5 μ g) were added to 0.3 ml of mitochondrial suspension containing approx. 30 mg of protein; then 10 μ Ci of DL-[3 H]carnitine and DL-carnitine to total 600 nmol were added, the suspension was mixed well and incubated at 25°C for 5 min. After this, the mitochondria were cooled to 0°C and washed twice by centrifugation as described by Pande (1975). The final suspension was made up to 0.8 ml with mito-

chondrial preparation medium and 20 μ l samples were used for experiments. All transport experiments were performed at 5°C, by the protocols described in the appropriate Table heading and Figure legend. Incubations were terminated by the addition of mersalyl to 1 mM (Pande & Parvin, 1976), followed by sedimentation of the mitochondria by 3 min centrifugation in a Beckman microcentrifuge (model 152) at approx. 10000g. Portions (200 μ l) of the supernatants were added to 10 ml portions of Aquasol (New England Nuclear, Boston, MA, U.S.A.) for liquid-scintillation counting. The basal incubation medium used in all of the transport studies reported contained 0.13 M-KCl, 20 mM-potassium phosphate, pH 7.2, antimycin (2 μ g/ml) and rotenone (2 μ g/ml).

Materials

DL-[3 H]Carnitine was from Amersham/Searle, Arlington Heights, IL, U.S.A. Palmitoyl-L-carnitine, CoA, acetyl-CoA and palmitoyl-CoA were from P-L Biochemicals, Milwaukee, WI, U.S.A. Acetyl-DL-carnitine and acetoacetyl-CoA were from Sigma Chemical Co., St. Louis, MO, U.S.A. DL-Carnitine was from Calbiochem, La Jolla, CA, U.S.A. Acetyl-L-carnitine was a much-appreciated gift from Otsuka Pharmaceutical Factory, Naruto, Tokushima, Japan. NAD $^+$, NADH and all enzymes were from Boehringer (Mannheim) Corp., Indianapolis, IN, U.S.A. Mersalyl (sodium salt) was from Sigma and was dissolved in water and protected from the light.

Results and Discussion

Rates of O₂ uptake by intact mitochondria from the hearts of young adult and senescent rats

Table 1 presents the results of experiments with heart mitochondria from young adult (6-month) or senescent (24-month) rats. Whereas there is no difference in the specific activity of pyruvate oxidation between these two age groups at either saturating (2.5 mM) or non-saturating (0.05 mM) pyruvate concentration, there is a significant decline in O₂ consumption with palmitoyl-L-carnitine. The age-linked decrement is approx. 30% under each of the two near-optimal experimental conditions presented in Table 1. This finding is entirely consistent with the results of Chen *et al.* (1972). When the respiratory substrate is glutamate plus malate, however, the same rates of O₂ consumption are obtained with mitochondria from rats of each age group. This is contrary to the results of Chen *et al.* (1972), who found a decline with age. Absolute rates with glutamate plus malate are lower in the present work, though rates with pyruvate and palmitoylcarnitine as substrates are similar, and it may be that the use of a lower glutamate

Table 1. *Rates of O₂ consumption by mitochondria isolated from the hearts of young adult and senescent rats, with fatty acids and other substrates (25°C)*

Mitochondria (1.2–2.4 mg of protein) were added to 2 ml of air-saturated incubation medium containing one of the substrate combinations given below. The composition of the medium and the experimental protocols are given in the Experimental section. Rates of O₂ uptake were measured with an oxygen electrode and are those at 25°C in the presence of 1 mM-ADP. Results are presented from independent experiments or are given as means $\pm$ S.E.M. of determinations carried out with the numbers of mitochondrial preparations given in parentheses. Statistical significance was evaluated by Student's *t* test: N.S., not significant (*P* > 0.05).

Substrate (mM)	Rate of O ₂ consumption (ng- atoms of O/min per mg of protein)		Significance of difference (6 month versus 24 month) <i>P</i>
	6-month	24-month	
Pyruvate (2.5)	325 $\pm$ 10 (10)	312 $\pm$ 16 (7)	N.S.
+L-malate (0.5)			
Pyruvate (0.05)	250 $\pm$ 10 (18)	233 $\pm$ 21 (4)	N.S.
+L-malate (0.5)			
Palmitoyl-L-carnitine (0.02)+L-malate (0.5)	305 $\pm$ 18 (7)	223 $\pm$ 12 (6)	0.001–0.005
Palmitoyl-L-carnitine (0.05)+L-malate (0.5)+albumin (0.04)	266 $\pm$ 18 (8)	187 $\pm$ 16 (7)	0.001–0.005
Palmitoyl-CoA (0.02) +L-malate (0.5) +albumin (0.04) +DL-carnitine (5)	178, 182	123, 178	
Octanoate (0.1) +L-malate (0.5)	198 $\pm$ 5 (7)	125 $\pm$ 10 (5)	<0.001
Acetyl DL-carnitine (5) +L-malate (0.5)	162 $\pm$ 7 (6)	119 $\pm$ 6 (5)	0.001–0.005
Acetate (5) +L-malate (0.5)	58 $\pm$ 9 (4)	52 $\pm$ 4 (4)	N.S.
Pyruvate (2.5) +malonate (2.5) +DL-carnitine (10)	63	57	
L-Glutamate (5) +L-malate (5)	182 $\pm$ 9 (5)	187 $\pm$ 8 (5)	N.S.
L-Malate (0.5)	9, 11	12	

concentration and a higher phosphate concentration in the present work has imposed a limitation of rate at the level of the entry of glutamate into the mitochondrion and may mask any age-linked decrement in activity of intramitochondrial enzymes. Such speculation notwithstanding, the identity of the rates of oxidation of glutamate plus malate and of pyruvate exhibited by

mitochondria from young adult and senescent animals means that the decline in the rate of oxidation of palmitoylcarnitine cannot be attributed to an artifact of preparation, such as contamination with myofibrils or other protein not active in substrate oxidation.

In an attempt to define more closely the enzymic step limiting the rate of O₂ consumption with palmitoylcarnitine, and presumably the locus of the age-linked decrement, octanoate was used as a substrate. This is thought to penetrate the mitochondrion as such, and does not require the participation of the carnitine acyltransferase system [see Bode & Klingenberg (1965) for the oxidation of decanoate]. There is a large decrement in the rate of O₂ consumption obtained with mitochondria from senescent animals (Table 1). Some care was taken to define optimal experimental conditions. Control experiments (results not shown) showed that 100 μ M-octanoate gave a maximal rate and also revealed the existence of an optimal ATP/ADP ratio. Simple addition of ADP resulted in a low rate of respiration, presumably because the mitochondrial-matrix ATP/ADP ratio was too low to support a high rate of fatty acid activation. Thus for every preparation a maximal respiratory rate was generated by careful addition of increments of hexokinase. The fact that rates of O₂ consumption are less with octanoate than with palmitoylcarnitine, in both young adult and senescent animals (Table 1), makes it unlikely that any of the enzymes of β -oxidation limits the rate of octanoate oxidation. For this reason, the age-linked decrement in the rate of octanoate oxidation was thought to reside at the level of the acyl-CoA synthetase, and this is directly examined below. In contrast with octanoate, acetate oxidation, which also involves acyl-CoA formation, did not decrease with senescence (Table 1). This is explicable if acetate and octanoate are activated by different acyl-CoA synthetases, as is believed (Jencks, 1962). Finally, Table 1 presents rates of O₂ consumption with acetyl-DL-carnitine as substrate: the activity is significantly diminished in mitochondria from the senescent animals. As the pathway of acetyl-group oxidation is the same, whether the donor is pyruvate or acetylcarnitine, the much lower rates of O₂ consumption with acetyl-DL-carnitine relative to pyruvate, especially in the senescent rats, would argue for rate limitation, and an age-linked decrement, at the level of acetyl-carnitine transport into the mitochondrion or at the level of carnitine acetyltransferase. These possibilities are directly examined below.

It became evident during direct experiments on [³H]carnitine/carnitine exchange across the mitochondrial membrane (results not shown) that Tes, used as a buffer in the studies reported in Table 1, is an inhibitor. In addition, Ramsay & Tubbs (1976) demonstrated a very high temperature-dependence

of carnitine/acetylcarnitine exchange, at least within the range 0–18°C. Thus it became untenable to study substrate oxidations under the conditions used above (Table 1), even though the great majority of experiments with mitochondrial preparations have been done at temperatures no higher than 30°C, and very often with Tris or Tes buffers (Hansford, 1976). Accordingly, the experiments were repeated, by using different mitochondrial preparations, at 37°C and in media buffered with phosphate in the hope that meaningful extrapolations could be made to the physiology of the animal. There is no age-linked difference in the rate of oxidation of pyruvate or of glutamate plus malate, but there is a decrement in the

rate of palmitoylcarnitine oxidation (Table 2) as seen at the lower temperature. In the presence of 40 μ M-bovine serum albumin, 30 μ M-palmitoyl-L-carnitine gave a maximal respiratory rate in mitochondria from both young and old rats. Under these conditions, the decrement in oxidative rate in mitochondria from the senescent rat was 19%. In addition a large decrement in the rate of octanoate oxidation was seen, as at 25°C. With the availability of the natural isomer of acetylcarnitine, this was used as a substrate, in addition to the DL mixture, which was used to allow comparison with Table 1. In each case, there is a decrement in rate of approx. 20% with mitochondria from the senescent animals. Absolute rates are higher with acetyl-L-carnitine than with the DL-mixture, although a large portion of the difference can be explained by lack of saturation by 5 mM-acetyl-DL-carnitine, rather than by inhibition by the unnatural isomer.

In addition, results with two other substrate combinations which involve carnitine/acetylcarnitine exchange are shown in Table 2. The oxidation of palmitoyl-CoA plus carnitine differs from that of palmitoylcarnitine only in that it requires the action of carnitine palmitoyltransferase external to the permeability barrier of the inner mitochondrial membrane (Fritz & Yue, 1963; Fritz & Marquis, 1965; Pande, 1971). The lower rate of O₂ consumption with palmitoyl-CoA plus carnitine, relative to palmitoylcarnitine, seen with the mitochondria from the young animal suggests that the external acyltransferase is rate-limiting for the whole oxidative pathway, under these experimental conditions. No such statement can be made for the mitochondria from the senescent animal, which give similar rates with the two substrates. Thus there is no evidence here to suggest that the external palmitoyltransferase is the site of the age-linked lesion. Finally, the rate of oxidation of pyruvate in the presence of malonate and carnitine is 22% less in mitochondria from senescent animals (Table 2). This oxidation involves pyruvate transport into the mitochondrion, pyruvate dehydrogenase, carnitine acetyltransferase and carnitine/acetylcarnitine exchange activities. Previous work (Hansford, 1977) has shown a stoichiometry of O₂ uptake/pyruvate utilization of 1 μ g-atom/0.3 μ mol, during State-3 pyruvate oxidation (terminology of Chance & Williams, 1955). From this ratio, pyruvate oxidation (Table 2, line 1) involves pyruvate transport and pyruvate dehydrogenase activities of approx. 180 nmol/min per mg of protein in mitochondria from animals of each age group. Thus it is likely that the rate of pyruvate oxidation in the presence of malonate and carnitine is limited by either carnitine acetyltransferase or carnitine/acetylcarnitine exchange activity, and that one or both of these activities is decreased in senescence. Direct measurements of these activities are reported below.

Table 2. Rates of O₂ consumption by mitochondria isolated from the hearts of young adult and senescent rats, measured at 37°C

Procedures were as described for Table 1 and in the Experimental section, except that the temperature was 37°C and the amount of mitochondrial protein used was 0.6–1.2 mg, depending on the substrate. None of the mitochondrial preparations used in these experiments was also used in the experiments described in Table 1.

Substrate (mM)	Rate of O ₂ consumption (ng- atoms of O/min per mg of protein)		Significance of difference (6-month versus 24- month) <i>P</i>
	6 month	24 month	
Pyruvate (2.5)	613 ± 26	593 ± 11	N.S.
+L-malate (0.5)	(6)	(5)	
Palmitoyl-L-carnitine (0.03)+L-malate (0.5)+albumin (0.04)	527 ± 24	428 ± 15	0.005–0.01
	(3)	(5)	
Palmitoyl-L-carnitine (0.05)+L-malate (0.5)+albumin (0.04)	534 ± 15	398 ± 23	<0.001
	(5)	(5)	
Palmitoyl-CoA (0.02) +L-malate (0.5) +albumin (0.04) +DL-carnitine (5)	483 ± 5	424 ± 15	0.005–0.01
	(4)	(4)	
Octanoate (0.1) +L-malate (0.5)	427 ± 18	239 ± 30	<0.001
	(5)	(4)	
Acetyl-DL-carnitine (5) +L-malate (0.5)	393 ± 12	314 ± 15	0.001–0.005
	(5)	(4)	
Acetyl-L-carnitine (5) +L-malate (0.5)	489 ± 33	397 ± 9	0.025–0.05
	(4)	(4)	
Pyruvate (2.5)+ malonate (2.5)+ DL-carnitine (10)	178 ± 2	139 ± 5	<0.001
	(4)	(5)	
L-Glutamate (5) +L-malate (5)	462 ± 18	444 ± 4	N.S.
	(5)	(5)	
L-Malate (0.5)	10	9	

Activities of enzymes involved in fatty acid oxidation

This section reports the results of experiments designed to give a further description of the changes in the mitochondria from senescent rats, at the level of isolated enzymes. Acyl-CoA synthetase (EC 6.2.1.2 plus 6.2.1.3) was measured (Table 3), being considered to be rate-limiting for octanoate oxidation for the reasons given above. There is a highly significant decrease in activity in mitochondria from senescent animals. However, the picture is not fully satisfactory, as the activity recovered is too low to account for the flux through this reaction in the mitochondrion, calculated from O₂-uptake data. It is very likely that the hydroxylamine in the assay is inhibitory. The hydroxamate-formation method (Table 3) was used nevertheless, being linear, reproducible and probably acceptable for comparative purposes. Methods based on CoA depletion were inaccurate and those based on ADP formation non-specific. Radioisotope experiments involving separation of acyl-CoA esters and non-esterified fatty acids might have been superior, but were not investigated.

The clear decrease in the rate of O₂ uptake with acetylcarnitine (Tables 1 and 2) or pyruvate plus malonate plus carnitine (Table 2) in the senescent rat, despite unchanged rates of pyruvate oxidation in the presence of malate, prompted measurement of carnitine acetyltransferase activity (EC 2.3.1.7). The activity is 35% less in the mitochondria from senes-

cent animals (Table 3). Although the carnitine acetyltransferases are often held to be sufficiently active in heart to maintain near-equilibrium (Pearson & Tubbs, 1967; Oram *et al.*, 1973), it is clear from rates of O₂ consumption given in Table 1 that there is less than a 3-fold excess of enzyme activity (measured under optimal conditions) over the flux through this step. The latter can be derived directly from the rate of pyruvate oxidation in the presence of malonate and carnitine (Table 1), or, approximately, from the rate of O₂ consumption with acetylcarnitine divided by 2 [the value of 3, measured for pyruvate oxidation (Hansford, 1977), being modified for one less oxidative step]. Thus there is a real possibility that the lowered rates of O₂ uptake with these substrates in mitochondria from senescent animals reflect the lowered activity of carnitine acetyltransferase. The other possible locus for the age effect, the carnitine/acetylcarnitine exchange, is discussed below.

Carnitine palmitoyltransferase activity was measured as being the first enzyme involved in the metabolism of added palmitoylcarnitine. The methods used involve the disruption of the mitochondria and therefore measure activities both internal and external to the permeability barrier (Brosnan *et al.*, 1973). Each of two methods shows that there is no change in the activity of this enzyme with senescence (Table 3).

Finally, two enzymes of the β -oxidation pathway

Table 3. Activities of some enzymes involved in fatty acid oxidation in mitochondria isolated from the hearts of young adult and senescent rats

Enzyme activities were measured at 25°C in the presence of Triton X-100 or sodium dodecyl sulphate to remove mitochondrial permeability barriers. Conditions of assay are described in detail in the Experimental section. They were as near optimal as possible and the activities generally approximate to $V_{\max}$, with the exceptions noted in the Experimental section. The number of mitochondrial preparations is given in parentheses; the result for any one preparation is the mean of duplicate determinations. Activities are expressed in nmol/min per mg of mitochondrial protein. In some cases, results from more than one assay technique are presented for the same enzyme (see the Experimental section).

Activity measured	Enzyme activity		Significance of difference (6-month versus 24-month) <i>P</i>
	6-month-old rat	24-month-old rat	
Acyl-CoA synthetase (EC 6.2.1.2 plus 6.2.1.3)	2.68 ± 0.18 (9)	1.61 ± 0.15 (8)	0.001–0.005
Carnitine acetyltransferase (EC 2.3.1.7)	150 ± 6 (5)	98 ± 6 (6)	<0.001
Carnitine palmitoyltransferase (EC 2.3.1.21), by CoA appearance	16.1 ± 0.8 (4)	15.8 ± 1.2 (5)	N.S.
Carnitine palmitoyltransferase (EC 2.3.1.21), ΔA_{232}	46.8 ± 2.7 (3)	50.0 ± 4.1 (5)	N.S.
Acyl-CoA dehydrogenase (EC 1.3.99.3)	7.73 ± 0.42 (19)	7.87 ± 0.48 (12)	N.S.
Acyl-CoA dehydrogenase (EC 1.3.99.3), with phenazine methosulphate	12.0 ± 1.8 (3)	14.2 ± 0.8 (3)	N.S.
3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)	687 ± 27 (8)	462 ± 31 (10)	<0.001

were measured. Acyl-CoA dehydrogenase was considered likely to be rate-limiting for the pathway, on the grounds that although long-chain acyl-CoA, the substrate, is found in considerable amounts in mitochondria, none of the acyl-CoA derivatives subsequent to this step is found in more than trace amounts (Garland *et al.*, 1965; Stanley & Tubbs, 1974). An exception is 3-hydroxyacyl-CoA, which is found under non-physiological conditions of highly reduced NAD (Stanley & Tubbs, 1974). However, measurement of acyl-CoA dehydrogenase activity (Table 3) revealed no difference with age, when two variants of the assay were used. The results can be criticized on the grounds that the measured activity may not be adequate to account for the computed flux through this reaction in the mitochondrion. Experiments in which limitingly small additions of palmitoylcarnitine generate cycles of O₂ uptake (results not shown) give values of 4–5 O atoms per turn of the β -oxidation spiral. Thus a rate of O₂ uptake of 300 ng-atoms/min per mg of protein (Table 1, young animal) requires acyl-CoA dehydrogenase activity of between 60 and 75 nmol/min per mg of protein. A difficulty in interpretation, however, is that it is not known what fraction of the total dehydrogenase activity, represented by the numbers 60–75, will be measured with palmitoyl-CoA as substrate. No experiments were conducted with short-chain acyl-CoA derivatives. It is concluded that the assay conditions are less than optimal, possibly reflecting the use of phenazine methosulphate rather than the natural electron acceptor, but that there is no obvious reason why this should have obscured an age-linked decrement, if there were one. Finally, the activity of 3-hydroxyacyl-CoA dehydrogenase was measured, as often being taken as indicative of the capacity of a tissue to carry out β -oxidation (see, e.g., Bass *et al.*, 1969). There is a 33% decrease with senescence (Table 3). The physiological significance of this is not clear, as the enzyme activity, admittedly measured under artificial and optimal conditions, is in large excess over the flux. Nevertheless, it makes it plain that there is not simply one age-linked lesion in fatty acid oxidation but what is probably a concerted decrease, by about the same extent, in the activities of several enzymes.

Effect of mersalyl on substrate oxidation involving carnitine/acylcarnitine exchange

The work by Pande & Parvin (1976) showing that mersalyl inhibits carnitine/acylcarnitine exchange at concentrations below those required to inhibit other mitochondrial processes suggested that a titration of acetylcarnitine oxidation with mersalyl might provide evidence on whether carrier activity is different in mitochondria from young and old animals. Fig. 1 presents the results of such an experiment with two preparations of mitochondria from each age group.

Clearly respiratory activity is less throughout the range of inhibitor concentration in the mitochondria from 24-month-old animals. The titration curves appear to indicate an excess exchange activity, in that approx. 1 nmol of mersalyl can be added per mg of protein with very little inhibition of O₂ uptake. However, Pande & Parvin (1976) found a similar result when inhibition of transport was studied directly. In addition, rates of acetylcarnitine oxidation are not maximal in the present study, as uncoupling agent was used to stimulate O₂ uptake, to avoid the complication of mersalyl inhibition of phosphate uptake. It has been shown (and confirmed in the present study) that uncoupling mitochondria inhibits acylcarnitine oxidation (Levitsky & Skulachev, 1972). Thus such an inference of excess exchange activity would be premature. Although no statement can be made about whether carnitine/acetylcarnitine exchange or carnitine acetyltransferase activity is rate-limiting (see above) in the absence of added inhibitor, when sufficient mersalyl has been added to inhibit the rate by, e.g., 50%, the carrier has been made rate-limiting. Under these circumstances, at any mersalyl concentration, the respiratory rate is invariably lower in the mitochondria from senescent animals. This suggests a lower carrier activity, or number of groups that react with mersalyl, in the

Fig. 1. Inhibition of acetyl-DL-carnitine oxidation by mersalyl at 25°C

Rates of O₂ consumption were measured by the general procedure described in the Experimental section. Mitochondria (2 mg of protein) were added to a medium comprising 0.12 M-KCl, 20 mM-Tes, 20 mM-potassium phosphate, 5 mM-acetyl-DL-carnitine, 0.5 mM-L-malate and 1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The volume was 2 ml and the final pH was 7.2. In addition mersalyl was present at the concentrations indicated. The low rate of O₂ consumption due to malate alone has been subtracted. ●, Mitochondria from 6-month-old rats (mean $\pm$ S.E.M. of three preparations); ■, mitochondria from 24-month-old rats (mean $\pm$ range of values of two preparations).

Fig. 2. Percentage inhibition by mersalyl of O_2 consumption with acetyl-L-carnitine or pyruvate plus malonate plus DL-carnitine as substrates

To study the oxidation of acetyl-L-carnitine, mitochondria (0.8 mg of protein) were added to a medium comprising 0.13 M-KCl, 20 mM-potassium phosphate, 5 mM-acetyl-L-carnitine, 0.5 mM-L-malate and 0.5 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The volume was 2 ml, the final pH was 7.2 and the temperature was 37°C. Mersalyl was present as indicated. The range of values obtained with two preparations from each age group is shown. The mean uninhibited velocity was 411 ng-atoms of O/min per mg of protein for the 6-month-old animals and 273 ng-atoms of O/min per mg for the 24-month-old animals. To study the oxidation of pyruvate plus malonate plus DL-carnitine, mitochondria (1.4 mg of protein) were added to a medium of the same composition as that above, except that 2.5 mM-pyruvate, 2.5 mM-malate and 10 mM-DL-carnitine were present instead of acetyl-L-carnitine and malate. Other conditions were identical. The rate of O_2 consumption obtained in the absence of carnitine was subtracted. The range of values obtained with two preparations from each age group is shown: neither preparation was also used for the acetyl-L-carnitine experiments. The mean uninhibited velocity was 154 ng-atoms of O/min per mg of protein for the 6-month-old animals and 127 ng-atoms of O/min per mg for the 24-month-old animals. ●, Acetyl-L-carnitine, 6-month-old animals; ■, acetyl-L-carnitine, 24-month-old animals; ○, pyruvate plus malonate plus DL-carnitine, 6-month-old animals; □, pyruvate plus malonate plus DL-carnitine, 24-month-old animals.

senescent rat. When these experiments were repeated at 37°C, with acetyl-L-carnitine as substrate and omitting nitrogen-containing bases that might inhibit carnitine transport (Fig. 2), a larger amount of mersalyl was required to give the same percentage inhibition of rate, when comparison is made with the experiments of Fig. 1. This may reflect a greater temperature-dependence of the exchange (Ramsay & Tubbs, 1976) than of the succeeding enzymic reactions, or, possibly, the lack of inhibition of transport by the unnatural isomer of acetylcarnitine in the experiments of Fig. 2. Under these experimental conditions there was a clear difference in the absolute activities of acetyl-L-carnitine oxidation given by preparations of mitochondria from young adult and senescent rats. Thus the uninhibited rate of oxidation was 32% lower for the latter group (legend to Fig. 2). However, when expressed as a percentage of the uninhibited rate (Fig. 2), velocities at different mersalyl concentrations are nearly superimposable for mitochondria from young and old rats, and may be so within the error of the experiment. Moreover, rates of pyruvate plus malonate plus carnitine oxidation derived from four different mitochondrial preparations also show a minimal age-difference when plotted on a percentage basis (Fig. 2), although absolute activities with this substrate differ by 22% in young and old rats (Table 2). It is concluded from these results that the activity of carnitine/acetyl-L-carnitine exchange (a requisite for the oxidation of each of these substrates) is diminished in the senescent rat in concert with the diminution in carnitine acetyltransferase activity shown in Table 3.

Measurement of DL-[3H]carnitine/acetyl-L-carnitine exchange

It was thought that the mersalyl titrations of O_2 uptake provided a sufficient indication of an altered activity of carnitine/acetyl-L-carnitine exchange in the preparations from senescent animals to justify direct measurement of exchange. Fig. 3 shows that mersalyl inhibits DL-[3H]carnitine (in)/acetyl-L-carnitine (out) exchange, in good agreement with the report of Pande & Parvin (1976), and that at a given mersalyl concentration exchange activity is lower in the mitochondria from senescent rats. This result is entirely consistent with the results of indirect assays based on O_2 uptake (Figs. 1 and 2). However, the statistical significance of the displacement between points at any one mersalyl concentration is not better than $P = 0.025-0.05$, and for some points there is no significant difference.

However, other studies, originally designed to measure K_m and V_{max} for the exchange process, provided evidence that the rate of exchange was indeed less in the preparations from senescent animals, and that the difference was highly significant

Fig. 3. Inhibition by mersalyl of carnitine/acetylcarnitine exchange in heart mitochondria from young adult and senescent rats

Mitochondria were loaded with [^3H]carnitine as described in the Experimental section. Mitochondria (0.8 mg of protein) were added to 0.4 ml of basal medium containing 2.5 mM-acetyl-L-carnitine and the amount of mersalyl indicated. The temperature was 5°C. The incubation was terminated at 30 s by adding mersalyl to 1 mM. Control incubations without acetyl-L-carnitine were alternated with test incubations, and the extent of exchange was obtained by comparing the extra [^3H]carnitine in the supernatant (after removal of the mitochondria by centrifugation) due to the presence of acetyl-L-carnitine with the total exchangeable [^3H]carnitine released on incubation for 10 min. Data are mean values derived from two mitochondrial preparations of each age group: ●, 6 months old; ■, 24 months old.

retained more radioactivity than did mitochondria from senescent animals (four preparations). However, the results were not quantitative, owing to an unknown degree of isotope dilution by the endogenous carnitine and acylcarnitine, and are not shown. For the experiments described in Table 4 the mitochondria were loaded with DL-[^3H]carnitine of known low specific radioactivity, such that dilution by the endogenous pool became negligible. The mitochondria from senescent animals contain less carnitine, after the loading procedure (Table 4). Moreover, if the rate achieved at a saturating external substrate concentration [$V_{\text{max. (apparent)}}$] is divided by the internal carnitine pool ([carnitine]_{in}), a first-order rate constant (K_t) is obtained, which is the same for young and old rats (Table 4). In some of these experiments multiple determinations of $V_{\text{max. (apparent)}}$ were made as [carnitine]_{in} declined, and for these preparations a mean value of K_t was used in the statistics. In other cases a single value of K_t was calculated for a single mitochondrial preparation by using values of [carnitine]_{in} and $V_{\text{max. (apparent)}}$ obtained over a short time period, and tabulated. Thus the lowered exchange activity of mitochondria isolated from the hearts of senescent rats can be explained in terms of a diminished mitochondrial content of carnitine, and there is no evidence for a change in the translocase molecule itself. The altered mersalyl-titration curve (Fig. 3) could perhaps be explained on the same grounds, as mersalyl inhibition of carnitine/carnitine exchange is competitive (Pande & Parvin, 1976). However, the fact that mersalyl is a charged species and probably a mitochondrial non-penetrant complicates this argument.

Interrelation and possible physiological significance of the findings

The present study has confirmed the diminished activity of palmitoylcarnitine oxidation reported by Chen *et al.* (1972) for mitochondria from the hearts of senescent rats and extended the findings to include the oxidation of octanoate, acetylcarnitine, palmitoyl-CoA plus carnitine and pyruvate in the presence of malonate and carnitine. The decrement in the rate of octanoate oxidation (Tables 1 and 2) is probably explained by the decreased acyl-CoA synthetase activity (Table 3). The oxidation of all the other substrates given above depends on the exchange of carnitine and acylcarnitine across the mitochondrial membrane, and so the activity of this process has been examined, and found to be diminished. However, this reflects only a decreased intramitochondrial pool of exchangeable carnitine in preparations made from senescent rats (Table 4). Nevertheless, Abu-Erreish *et al.* (1977) report a diminished total carnitine content in whole hearts

($P < 0.001$ for a comparison of apparent $V_{\text{max.}}$, Table 4). It was found by always alternating control incubations (lacking acetyl-L-carnitine) with those containing substrate that the DL-[^3H]carnitine with which the mitochondria were loaded leaked out at a rate sufficient materially to alter the internal pool size during a series of experiments. When the external substrate was 20 mM-acetyl-L-carnitine, which was found to be saturating, the rate of exchange was called $V_{\text{max.}}$ (Table 4), but was dependent on the internal pool size at that time. Eight preparations of mitochondria were loaded with carrier-free DL-[^3H]carnitine (1.7 Ci/mmol), and in each of four preparations mitochondria from young adult animals

Table 4. *Properties of carnitine/acylcarnitine exchange in heart mitochondria from young adult and senescent rats*

Mitochondria were preloaded with [^3H]carnitine and exchange was followed by the appearance of [^3H]carnitine external to the mitochondria on the addition of mitochondria to an incubation medium containing acetylcarnitine. Incubations were terminated by the addition of mersalyl, followed by centrifugation at approx. 10000g for 3 min, and controls were carried out in which acetylcarnitine was omitted from the medium. The protocol is given in detail in the Experimental section. Total exchangeable mitochondrial carnitine was derived from the radioactivity released on 10 min incubation with acetylcarnitine at 5°C; control studies confirmed that equilibrium was attained at this time. Molarity was calculated from the known specific radioactivity of the [^3H]carnitine added to the mitochondria during the preloading phase; dilution by the endogenous mitochondrial carnitine was minimal, as it comprised less than 10% of the [^3H]carnitine added on a molar basis. $V_{\text{max. (apparent)}}$ was derived approximately from the exchange occurring in 30 s at 5°C in the presence of 20 mM-acetyl-L-carnitine. For each preparation the result was the mean of at least three determinations. Mitochondrial carnitine at the time of the measurement of $V_{\text{max. (apparent)}}$ was obtained by subtracting the extramitochondrial [carnitine] given by control (minus acetylcarnitine) incubations from total exchangeable mitochondrial carnitine. A first-order rate constant, K_t , was obtained in one of two different experimental types. It was derived either from studies in which multiple determinations of $V_{\text{max. (apparent)}}$ and $[\text{carnitine}]_{\text{in}}$ were made over a short time interval, giving a single value of K_t , or from studies in which $[\text{carnitine}]_{\text{in}}$ was allowed to decrease over 4 h, allowing multiple determinations of $V_{\text{max. (apparent)}}$. In this latter case (four preparations) K_t was calculated at each of 10 values of $[\text{carnitine}]_{\text{in}}$, and the mean of these values is entered for that preparation. For each parameter, the error is the S.E.M. and the number of mitochondrial preparations is given in parentheses.

Parameter measured or calculated	6-month-old rat	24-month-old rat	Significance of difference (6-month versus 24-month) <i>P</i>
Total exchangeable mitochondrial carnitine pool (nmol/mg of protein)	1.05 ± 0.03 (5)	0.82 ± 0.02 (5)	<0.001
$V_{\text{max. (apparent)}}$ at 5°C, = $K_t \times [\text{carnitine}]_{\text{in}}$ (nmol/min per mg of protein)	0.71 ± 0.03 (7)	0.46 ± 0.04 (5)	<0.001
Mitochondrial carnitine at time of measurement of $V_{\text{max. (apparent)}}$ = $[\text{carnitine}]_{\text{in}}$ (nmol/mg of protein)	0.57 ± 0.03 (7)	0.37 ± 0.03 (5)	
First-order rate constant for exchange = K_t (min ⁻¹)	1.24 ± 0.03 (7)	1.24 ± 0.04 (5)	N.S.

from senescent rats, so that the diminished mitochondrial content reported here apparently reflects the situation *in vivo*. Moreover, the present paper shows an unchanged K_t in mitochondria from young and old animals, and therefore suggests that rates of transport would be less *in vivo* in the hearts of senescent animals, because of a decreased degree of saturation of the translocase. Certainly the values for K_m that have been reported are high (Ramsay & Tubbs, 1976) compared with whole-tissue carnitine content (Abu-Ereish *et al.*, 1977). Although a decreased activity of carnitine/acylcarnitine exchange across the mitochondrial membrane may lead to decreased rates of fatty acid metabolism in the hearts of senescent animals, it is not clear that it is a rate-limiting factor in any of the experiments *in vitro* reported here. In this regard an important difference in the whole organ is the presence of futile exchanges, e.g. carnitine (in)/carnitine (out), which are minimized in the present experiments. It is possible that decreased exchange activity is responsible for de-

creased rates of O_2 uptake with acetylcarnitine and pyruvate plus malonate plus carnitine (senescent compared with young adult, Tables 1 and 2). However, the decrease in carnitine acetyltransferase activity with senescence (Table 3) and bare excess of this activity over the flux through this reaction (see above) are consistent with carnitine acetyltransferase being the locus of the age-linked lesion. This is further supported by the fact that the decreased rate of O_2 uptake is still seen when the mitochondria from the senescent heart have been incubated for several minutes in 5 mM-acetylcarnitine at 25°C (Table 1) or 37°C (Table 2), or when they have been preincubated for 10 min with 20 mM-DL-carnitine (results not shown). Such treatments would be expected to increase the intramitochondrial pool of exchangeable carnitine, by means of a slow unidirectional movement (not exchange) described by Ramsay & Tubbs (1976), and thus abolish age differences, if they were due to differences in pool size alone. The factors responsible for limiting the rate of palmitoylcarnitine

oxidation, and for causing the decrement in senescence, are less clear. The decreased carnitine/acyl-carnitine exchange activity is not the answer, as a mersalyl titration of the oxidation of 20 μ M-palmitoyl-carnitine at 25°C (results not shown) revealed no inhibition whatsoever with less than 2 nmol of mersalyl/mg of protein. Thus only a portion of the exchange activity is needed to potentiate maximal rates of O₂ uptake, and this is presumably expected from the slightly lesser carrier activity with palmitoyl-carnitine than with acetylcarnitine (Pande & Parvin, 1976), but the 10–20-fold greater O₂ uptake per molecule with the former substrate. On the other hand acyl-CoA dehydrogenase, which is potentially rate-limiting for β -oxidation (see above), shows no decline with senescence (Table 3).

Finally, all of these studies with intact mitochondria involved either ADP-stimulated or uncoupled respiration, and thus allow comment on maximal potential rates of energy transduction and changes with senescence. Controlled rates of respiration (minus ADP) are not reported, as two excellent earlier studies (Gold *et al.*, 1968; Chen *et al.*, 1972) showed no change in these rates in heart mitochondria from senescent animals with several different substrates.

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