Uncoupling activity of endogenous free fatty acids in rat liver mitochondria

SAMUEL H. P. CHAN AND EDWARD HIGGINS, JR.
Department of Biology, Syracuse University, Syracuse, NY, U.S.A. 13210

Received June 20, 1977
Accepted October 25, 1977

Chan, S. H. P. & Higgins, E. Jr. (1978) Uncoupling activity of endogenous free fatty acids in rat liver mitochondria. *Can. J. Biochem. 56*, 111–116

Changes in the respiratory control index (RCI) and ADP:O ratio were found to be related to alterations in the free fatty acids levels of rat liver mitochondria aging in 0.25 *M* sucrose–Tris buffer at 0°C. Free fatty acid levels increased with time after isolation of mitochondria while a concomitant decrease in the RCI and ADP:O ratio occurred. The changes in free fatty acid levels corresponded with the reported increasing levels of phospholipase A activity in aged mitochondrial preparations. Washing these mitochondria with sucrose buffer containing 1% defatted bovine serum albumin (BSA) counteracted the aging effect on the RCI (e.g., 2.5 to 3.5) and reduced the free fatty acid levels (e.g., 50 to 16 nmol/mg protein). This reversible phenomenon could be repeated several times during the *in vitro* aging at 0°C. Use of ^{125}I-iodinated BSA showed that approximately 5 μg BSA/mg mitochondria was adsorbed by the mitochondrial membranes during washing. These results indicate a direct correlation between the level of endogenous fatty acids and the uncoupling of mitochondrial oxidative phosphorylation. The mechanism of counteracting the aging effect by BSA involves the removal of some of the free fatty acids.

Chan, S. H. P. & Higgins, E. Jr. (1978) Uncoupling activity of endogenous free fatty acids in rat liver mitochondria. *Can. J. Biochem. 56*, 111–116

Nous avons trouvé que les changements de l'index du contrôle respiratoire (RCI) et du rapport ADP:O sont reliés aux variations des taux d'acides gras libres dans les mitochondries de rat qui vieillissent dans un tampon saccharose–Tris 0.25 *M* à 0°C. Après l'isolation des mitochondries, les taux d'acides gras libres augmentent avec le temps alors que le RCI et le rapport ADP:O diminuent de façon concomitante. Ces changements des taux d'acides gras libres correspondent à l'augmentation connue de l'activité de phospholipase A dans des préparations mitochondriales âgées. Le lavage de ces mitochondries dans un tampon saccharose contenant 1% d'albumine sérique bovine délipidée neutralise l'effet du vieillissement sur le RCI (e.g., 2.5 à 3.5) et réduit les taux d'acides gras libres (e.g., 50 nmol à 16 nmol/mg de protéine). Ce phénomène réversible peut être répété plusieurs fois durant le vieillissement *in vitro* à 0°C. L'emploi d'albumine sérique bovine marquée à ^{125}I montre qu'environ 5 μg d'albumine sérique bovine par mg de mitochondries sont adsorbés par les membranes mitochondriales durant le lavage. Ces résultats montrent une corrélation directe entre le taux d'acides gras endogènes et le découplage de la phosphorylation oxydative mitochondriale. Le mécanisme de neutralisation de l'effet du vieillissement par l'albumine sérique bovine implique l'enlèvement de quelques-uns des acides gras libres.
[Traduit par le journal]

Introduction

The interest in the uncoupling activity of long-chain fatty acids began in 1955 when Pressman and Lardy (1, 2) identified a mixture of long-chain fatty acids to be the active substance from microsomes which stimulated the respiration and ATPase of mitochondria. Unsaturated long-chain fatty acids such as oleic acid have been shown to be more potent uncoupling agents than saturated ones (3, 4), and their mode of action was similar to that exhibited by a classical artificial uncoupler, 2,4-dinitrophenol.

Pullman and Racker (5) in 1956 described the formation of an uncoupler in aged mouse liver mitochondria whose action on fresh mitochondria could be counteracted by BSA. Since then, BSA has been routinely employed to isolate tightly coupled mitochondria, particularly from adipose (6) and tumor tissues (7).

Recent studies of phospholipase A in rat liver mitochondria suggest that the endogenous enzyme assists in disrupting the structural integrity of the aging mitochondrial membranes (8, 9). Scarpa and co-workers (10, 11) demonstrated a correlation between phospholipase A_2 activity in mitochondria and respiratory activity, and a protection against the effects of aging by the local anesthetic nupercaine, a powerful phospholipase inhibitor. These authors also observed some protection of respiratory activity when mitochondria were aged in the presence of BSA although they did not measure the fatty acid contents of the aged mitochondria. In contrast, Boime et al. (12) demonstrated that free fatty acids accumulate in mitochondria during ischemia in concentrations which if added to normal mitochondria significantly lower the RCI, primarily by increasing state 4 respiration.

ABBREVIATIONS: RCI, respiratory control index; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

However, no studies have been done to link the diminishing RCI and ADP:O ratio during isolation and aging directly with an accumulation of free fatty acids. The present study offers information directly relating this aging process of diminishing RCI and ADP:O ratio to the accumulation of free fatty acids, and the mechanism of the counteraction by BSA on the partially uncoupled mitochondrial preparations. A preliminary report of some of these studies has appeared (13).

Materials and Methods

Liver mitochondria were isolated from male Sprague–Dawley rats according to the method of Johnson and Lardy (14) using 0.25 *M* sucrose, 10 m*M* Tris–Cl, and 1 m*M* EDTA pH 7.4 as the isolation medium. The mitochondrial preparation was washed three times and finally suspended for immediate use in 0.25 *M* sucrose at a protein concentration of 20 mg/ml. The procedure of washing mitochondria with BSA was as follows: Aliquots of the mitochondrial preparation were suspended at 1 mg/ml protein concentration with the isolation medium containing 10 mg/ml of the charcoal defatted BSA (15) (BSA was from Armour Laboratory, lot No. P13504). After complete mixing for 5 min at 0°C, the mitochondrial pellet was obtained by centrifugation at 26 000 × *g* for 5 min in a Sorval centrifuge. The mitochondria were again suspended at 1 mg/ml protein concentration with plain 0.25 *M* sucrose and spun down as before to remove any residual BSA in the mitochondrial pellet. This washing process was repeated one more time. The mitochondria were finally suspended in 0.25 *M* sucrose at 20 mg/ml protein concentration. All isolation procedures and storage of the mitochondria were at 0°C, unless otherwise indicated.

Oxygen uptake was measured polarographically with a Clark electrode coupled to a Perkin–Elmer recorder and the RCI and ADP:O ratio were calculated according to the method of Estabrook (16). The RCI is the ratio of state 3 respiration measured in the presence of ADP and substrate to state 4 respiration measured in the absence of ADP but in the presence of substrate, and represents a measure of the 'tightness' of the coupling of oxidation to phosphorylation (17). ADP:O is the ratio of ADP being phosphorylated to oxygen in solution being consumed. The ADP:O ratio is equivalent to a P:O ratio (16).

The assay medium was based on one used by Sordahl and Schwartz (7), i.e., 1 m*M* EDTA, 5 m*M* $MgCl_2$, 17.5 m*M* K_2HPO_4, 50 m*M* Tris–HCl, and 75 m*M* KCl at pH 7.3. The total volume of the oxygen cuvette was 2.5 ml, and the temperature was 30°C. The concentration of ADP in the solutions was measured in a coupled enzymatic assay employing pyruvate kinase and lactate dehydrogenase (18).

Free fatty acids were extracted both directly from mitochondrial suspensions and indirectly via a BSA wash using a chloroform and methanol mixture according to the procedure of Bligh and Dyer (19) at pH 3.5. Following phase separation by centrifugation, the methanol–aqueous layer was removed by aspiration. The chloroform layer was washed with 0.5 volumes aqueous 0.05% NaCl. The washed chloroform extracts were either used immediately or stored at −20°C and used within 2 wk. The free fatty acids were determined colorimetrically according to the procedure of Duncombe (20) and (or) Falholt et al. (21) using both palmitic and oleic acids separately as standards. The free fatty acids were also determined both quantitatively and qualitatively using a Searle analytic 4720 H dual flame gas–liquid chromatograph after they were converted into methyl esters using boron trifluoride as a catalyst according to the procedure of Morrison and Smith (22). Aliquots of 0.1 to 1 μl of the fatty acid methyl esters in *n*-hexane were injected into the gas chromatograph column. The stationary phase in the column was 10% diethylene glycol succinate polyester on 80–100 mesh Chromosorb Q (Applied Science, College Station, PA). The chromatographic column was run either isothermally at 180°C or with temperature programming from 120 to 180°C at increments of 2°/min, with purified nitrogen as the carrier gas. The retention times for the fatty acid methyl esters were determined by comparison with standards and the concentrations were calculated using the peak area of the internal standard.

^{125}I-Labelled BSA was prepared according to the method of McFarlane (23) utilizing ICl. Only one to two atoms of iodine per molecule of BSA were calculated to be incorporated. The partially iodinated BSA was observed to have the same fatty acid binding capacity as that of native BSA, as judged by its ability to counteract the aging effect. After iodination in 0.2 *M* glycine buffer pH 8.6, the BSA was passed through a G50 Sephadex column (50 × 1 cm) equilibrated with the same buffer to remove unreacted iodine. The BSA was then extensively dialyzed (four changes) against 100 times the sample volume of 0.25 *M* sucrose, 10 m*M* Tris–Cl buffer. The radioactivity was measured with a Nuclear Chicago automatic crystal well scintillation detector.

Mitochondrial protein was determined by the biuret method in the presence of 0.4% dioxycholate (24) using BSA as standard. Fatty acids and their methyl ester derivatives were purchased from Applied Science, College Station, PA. All other chemicals were of enzyme grade or analytical reagent grade, mostly from Schwarz-Mann and Fisher Scientific Co.

Results

The RCI and ADP:O ratio was measured in mitochondria in the presence of succinate (3.2 m*M*) and ADP (100–150 μ*M*). Both the ADP:O ratio and RCI decreased when the mitochondrial preparations were aged at 0°C. For example, as indicated by the experimental tracings in Fig. 1, in 24 h, there was a 36% decrease in the RCI and 22% in the ADP:O ratio (tracing C compared with tracing A). This indicates that the coupling of respiration to oxidative phosphorylation in the mitochondria is gradually being inhibited with time. Furthermore, the decreases in the RCI and ADP:O ratio are primarily due to an increase in the state 4 respiration rate. For example, the state 4 respiration rate was increased from 32.6 nmol O_2/min (tracing A) to 49.2 nmol O_2/min (tracing C). When BSA was added in the assay cuvette, in freshly isolated mitochondria, there was a slight decrease in state 4 respiration rate from 32.6 to 27 nmol O_2/min (tracing B). In contrast, in aged mitochondria, the presence of BSA greatly reduced the state 4 respiration rate from 49.2 to 30.2 nmol O_2/min (tracing D), a value almost equal to that of freshly isolated mitochondria. The increase in the state 4 respiration rate by aging is very similar to the respiration rate stimulated by the addition of a potent uncoupler, 3 μ*M* CCCP, as indicated in Fig. 1.

In order to understand the nature of the uncoupling, the effect of washing the mitochondria in BSA on the RCI and ADP:O ratio was determined. At various time intervals during aging of the mitochondria, two washes were performed. The first, using a 1% BSA buffer solution; and the second, with the normal sucrose buffer to remove the residual BSA in the mitochondrial pellet as described in the Materials and Methods section. As shown in Fig. 2 following these washes, the RCI and ADP:O ratio were found to increase. Washing the mitochondria after agin

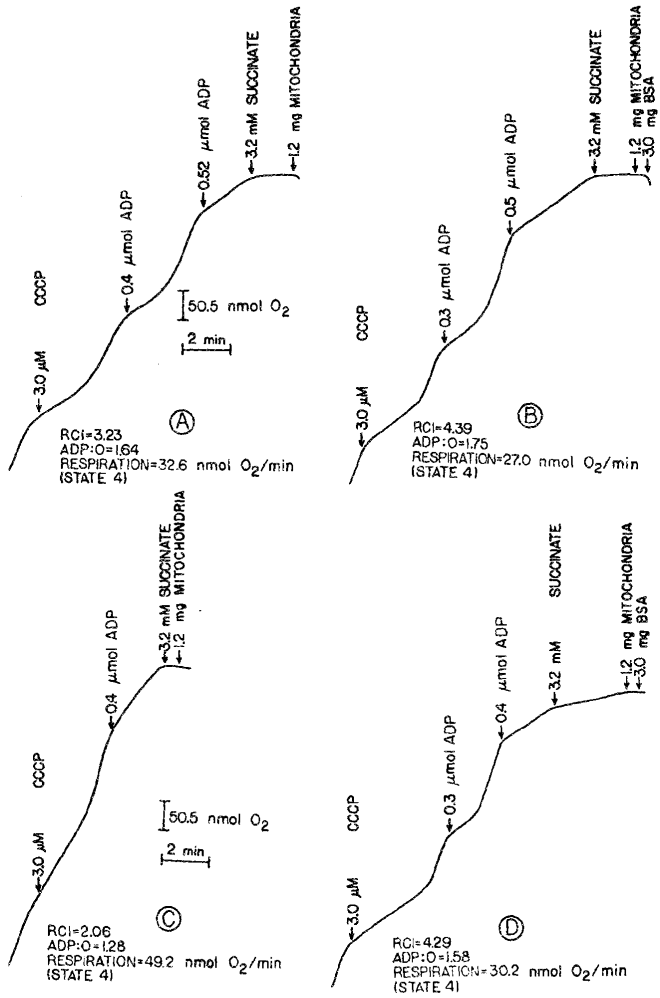

FIG. 1. The effect of BSA on the RCI, ADP:O ratio, and state 4 respiration rate in freshly isolated and aged rat liver mitochondria. Assay medium contained the following: (final volume 2.5 ml) 17.5 m*M* K_2HPO_4, 50 m*M* Tris–HCl, 75 m*M* KCl, 1 m*M* EDTA, 5 m*M* $MgCl_2$, and 4 μM rotenone. Additions are indicated in the figure. Tracings B and A are fresh mitochondria (3 h after sacrificing the animal) with and without 3 mg BSA respectively. Tracings C and D are mitochondria after 24 h of incubation in isotonic sucrose at 0°C.

at 6 h, for example, the RCI increased from 3.4 to 4.0 and the ADP:O ratio from 1.68 to 1.85. At 30 h, the RCI rose from 2.0 to 2.8 while the ADP:O ratio rose from 1.35 to 1.57. This counteraction of the aging effect with the BSA wash was possible while the mitochondria remained viable (RCI > 1) during *in vitro* aging. Possibly, free fatty acids might be involved as BSA is known to bind fatty acids tightly (15). In addition, experiments were undertaken to determine the mechanism of the effect of BSA on the mitochondria; that is, whether it prevented the aging process or simply improved the activity and did not influence the aging process, since the presence of BSA in the assaying cuvette also improved the RCI and ADP:O ratio. Data were obtained showing that after the initial BSA wash at 4 h, which increased the RCI (from 3.4 to 3.9) and the ADP:O ratio (from 1.70 to 1.83), the aging process proceeded along a time course similar to that in the unwashed mitochondria. Therefore, although BSA is capable of counteracting the aging process, the effect is temporary. Apparently, BSA does not act by repairing the mitochondrial membranes. Furthermore, when BSA-prewashed mitochondria were subsequently washed with BSA again, the uncoupling of the aging mitochondria preparation was again counteracted. This suggests that BSA acts by removing some endogenous compounds that are inhibiting mitochondrial coupling activity.

Previous studies have indicated that the aging phenomena were probably a result of membrane phospholipid degradation to long-chain free fatty acids (8). In an attempt to confirm the observation that added free fatty acids influenced the RCI and ADP:O ratio, experiments were performed in which endogenous free fatty acids were extracted from mitochondria after incubation

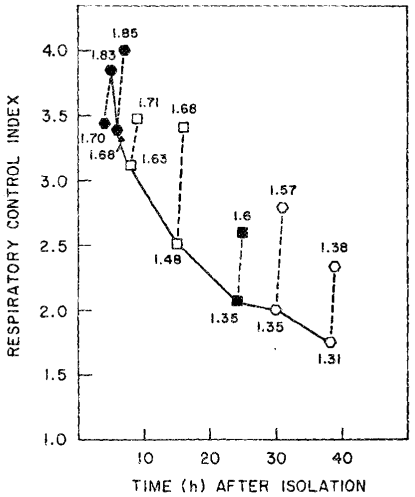

FIG. 2. Counteraction of the aging effect by 1% BSA wash in isolated mitochondria. See text for the BSA washing procedures. Solid lines represent the normal aging effect and broken lines represent the reversal of the RCI and ADP:O ratio by periodically washing the aging mitochondrial preparations with BSA. The ADP:O ratio is indicated by the numbers in the figure.

medium for various times. The results in Fig. 3 show that, at 0°C, the level of long-chain free fatty acids increased from 25 nmol/mg mitochondrial protein initially to 80 nmol/mg protein at 30 h, and appeared to approach a steady state beginning at 30–40 h. Since the decrease in the RCI associated with aging is concomitant with an increase in free fatty acid level, the mechanism of action of BSA in counteracting aging is presumed to involve the binding of the free fatty acids.

Figure 3 also shows the results of an experiment in which the changes in the RCI correlated with the levels of free fatty acids in the mitochondrial preparation during incubation. At various times, aliquots of mitochondria were taken and the immediate effects of washing with BSA on the RCI and free fatty acid levels was simultaneously determined. Associated with the reversal in the decline of the RCI caused by BSA was a significant decrease in the levels of free fatty acids. The magnitude of the effects of BSA on both the RCI and free fatty acid levels was about the same at 4 and at 24 h.

It was shown that the effect of BSA on the aging process is temporary: Washing with BSA at 4 h increased the RCI, but subsequently the RCI declined with a time course similar to that of an unwashed preparation. The result obtained on the decrease in free fatty acids caused by BSA is also temporary, and subsequent to the wash, the increase in free fatty acids corresponds with that observed in the control preparation. In spite of the temporary removal of free fatty acids by BSA, the long-term free fatty acid production is not prevented. Further exposures to BSA were still able to remove the free fatty acids and increase the RCI. Therefore, it is evident that the fatty acids in aged mitochondria are being continuously released from membrane phospholipids (8, 10) and acting as endogenous uncouplers.

Results obtained by gas–liquid chromatography on the free fatty acids extracted from mitochondria aged for 6 h are shown in Table 1. The total amount of free fatty acids of the mitochondria was 41 nmol/mg mitochondrial protein. Approximately 56% of the free fatty acids were saturated long-chain fatty acids, while 43% were unsaturated. Washing the mitochondria with BSA removed approximately half of the total free fatty acids. Of the free fatty acids remaining in the mitochondrial pellet after the wash, only 24% were unsaturated, whereas in unwashed mitochondria, 43% were unsaturated. Therefore, unsaturated free fatty acids, particularly oleic acid, were preferentially removed by BSA. This observation is sig-

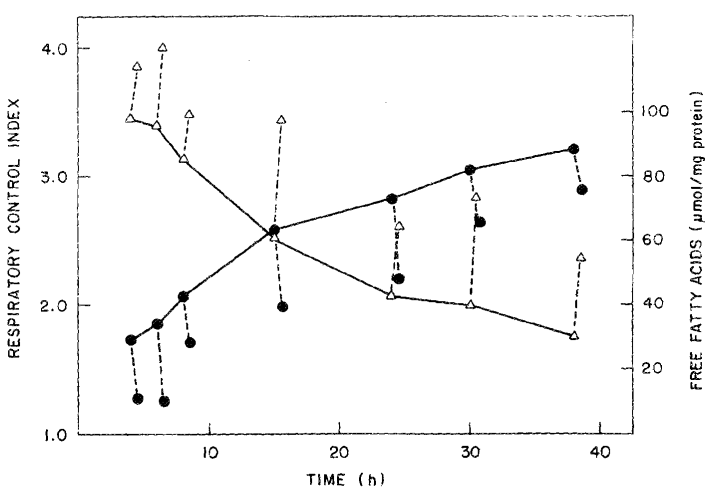

FIG. 3. The relationship between the fatty acid levels and oxidative phosphorylation in rat liver mitochondria during aging. Experimental conditions were the same as those for Figs. 1 and 2. One half of each mitochondrial sample after the BSA wash was used for fatty acid determination and the other half for the RCI determination. ●, Fatty acids; △, RCI. Broken lines represent results obtained after washing the aging mitochondria with BSA (see text).

TABLE 1. Free fatty acids extracted from rat liver mitochondria aged 6 h before (control) and after a BSA wash

Fatty acids	Analysis of methyl esters, %[a]	
	Control (RCI = 2.8)	After BSA wash (RCI = 3.8)
Myristic, $C_{14:0}$	Trace	Trace
Palmitic, $C_{16:0}$	24.4	29.8
Stearic, $C_{18:0}$	31.5	41.9
Palmitoleic, $C_{16:1}$	2.0	Trace
Oleic, $C_{18:1}$	18.9	9.7
Linoleic, $C_{18:2}$	13.2	8.2
Linolenic, $C_{18:3}$	5.0	3.3
Arachidonic, $C_{20:4}$	3.9	3.5
Total free fatty acids, nmol/mg mitochondrial protein[b]	41	21

[a]Determined by gas–liquid chromatography.
[b]Determined colorimetrically according to the procedure of Falholt et al. (21). Total fatty acid content after complete hydrolysis of membrane phospholipids (22) was determined to be 320 nmol/mg mitochondrial protein.

nificant since unsaturated long-chain free fatty acids, particularly oleic acid, have been shown to be more potent uncouplers than saturated ones (3). For comparative purposes, the total fatty acids formed by hydrolysis from membrane phospholipids was 320–340 nmol/mg mitochondrial protein. Thus, when approximately one-quarter of the total phospholipids were hydrolysed, the respiratory control became almost completely lost.

To determine whether large quantities of BSA were incorporated into the mitochondria during the wash process, the appropriate experiments were carried out with ^{125}I-labelled BSA. Mitochondria were exposed to various concentrations of BSA (the ratio of BSA (in milligrams) to mitochondrial protein (in milligrams) was 0.5 to 10) for times up to 5 h at 0°C. In one experiment, the incubation was at 30°C for 30 min. Although 30–50 μg BSA/mg protein (depending on the amounts used for washing) were nonspecifically adsorbed by the mitochondrial pellet, after additional washing with plain 0.25 *M* sucrose to remove residual BSA in the mitochondrial pellet, there were only small amounts (0.6–4.7 μg) of BSA adsorbed by the mitochondria. In fact, results indicate that less BSA was adsorbed when incubated at 30°C (0.4 μg) than at 0°C (2.4 μg). It also seems that the longer the incubation time, the less was adsorbed by the mitochondria. Finally, both fresh and aged mitochondria were incubated with BSA with no apparent difference in their uptake of BSA. Thus, BSA is not taken up to any significant extent by mitochondrial membranes even after lengthy exposures or at BSA concentrations 10-fold higher than those normally employed in the washing procedures of the experiments described above.

Discussion

Free fatty acids have been suggested to be intrinsic uncouplers of oxidative phosphorylation in mitochondria (25). Studies by Chefurka (26, 27) and others (28) indicated that the respiratory rate of aged mitochondria *in vitro* failed to respond to ADP, and that added BSA protected the activity from decay in aged mitochondria. Chefurka (27) also measured the fatty acid contents of fresh and aged mitochondria and concluded that the accumulation of long-chain fatty acids during aging prevented stimulation of latent ATPase activity by dinitrophenol in aged mitochondria. Thus, uncoupling of oxidative phosphorylation by free fatty acids has been well documented. More recently, Scarpa and Lindsay (10) and Aleksandrowicz et al. (29) concluded from their studies with nupercaine that endogenous phospholipase activity in mitochondria was largely responsible for the damage to aged mitochondria. The present study confirms these observations and directly correlates the stoichiometric relation between the oxidative phosphorylation capacity and the fatty acid contents of mitochondria during aging *in vitro*. Boime et al. (12) reported that exposure of rat liver to periods of ischemia resulted in a progressive increase in mitochondrial free fatty acids and when comparable amounts of free fatty acids were added to normal mitochondria, a significant depression of the RCI was observed. The present study identifies the source of free fatty acids as being due to the lipolysis of lipids of mitochondrial origin. This is in agreement with reports that endogenous mitochondrial phospholipase A activity is related to alterations in respiratory control in aged mitochondria (8, 10). The role of BSA, however, in preventing the uncoupling phenomenon has never been determined with certainty. BSA has been used during mitochondrial isolation by many investigators, particularly in tumor mitochondria, to preserve mitochondrial function and improve the activity. Its intrinsic nature of binding to free fatty acids caused many to speculate that this BSA effect could account for the improved activity in mitochondria. Of particular interest in the present study is the observation that in normal liver mitochondria, BSA counteracts the aging effect by binding the accumulated free fatty acids but not by preventing the aging process. Indeed, using radioactive BSA, it was observed that under various conditions, only small quantities of BSA are incorporated into the liver mitochondria and result in the reversal of the RCI and ADP:O ratio. This is in contrast with the proposal of Sordahl and Schwartz (7) that in tumor mitochondria, BSA acts by repairing the mitochondrial membrane and maintaining its structural integrity so that restoration of activity comparable with normal mitochondrial oxidative phosphorylation capacity can be observed. We are presently testing this hypothesis by washing mitochondria isolated from Ehrlich ascites tumor and L1210 cells with radioactive BSA. Preliminary results indicate that higher levels of BSA are indeed incorporated into the tumor mitochondrial pellets which cannot be removed by additional washing with plain 0.25 *M* sucrose. This observation may indicate an important difference between the normal and neoplastic mitochondria *in vivo* and may reflect the state of the altered energy metabolism in some tumor tissues (30).

Although BSA may preserve mitochondrial activity by

counteracting the aging process, there is eventually an irreversible change signified by a rapid decline of activity with no reversible activity possible when approximately one-fourth of the membrane phospholipids are hydrolysed to fatty acids and lysophospholipids. It may be the result of a critical loss of phospholipids by the action of the phospholipase in the mitochondrial membrane (10, 29).

Spector and Brenneman (31) compared the binding of different long-chain free fatty acids to rat liver mitochondria and reported that saturated fatty acids were taken up to a greater extent than unsaturated fatty acids. The present study confirms their observations, i.e., proportionally more unsaturated fatty acids are washed out by BSA with a consequent higher percentage of saturated fatty acids retained by the mitochondria. It is interesting to note that mitochondrial fatty acid uptake experiments by Spector and Brenneman (31) were performed in the presence of BSA in the incubation medium; most of the fatty acid uptake was due to the incorporation after transfer from albumin to the mitochondria, and not to the direct incorporation of the free fatty acids into mitochondria, nor to the direct incorporation of the intact fatty acid – albumin complex. This observation, together with our present results that washing with 10-fold excess BSA did not result in removal of all free fatty acids from mitochondria, indicates that either some fraction of the free fatty acids liberated from lipid hydrolysis in mitochondria are not accessible, or some intrinsic components within the mitochondria are specific for binding some fatty acids as we observed earlier (32). We are presently identifying this fatty acid binding component and studying its regulatory role in normal and tumor mitochondria.

Acknowledgement

This investigation was supported by grant CA 20454 awarded by the National Institutes of Health, U.S. Department of Health, Education, and Welfare, by a grant from American Heart Association, Upstate New York Chapter, and by a Syracuse University Research and Facilities grant. We thank Dr. P. Dunham for his criticism and reading of the manuscript.

1. Pressman, B. C. & Lardy, H. A. (1955) *Biochim. Biophys. Acta 18*, 482–487
2. Pressman, B. C. & Lardy, H. A. (1956) *Biochim. Biophys. Acta 21*, 458–466
3. Borst, P., Loos, J. A., Christ, E. J. & Slater, E. C. (1962) *Biochim. Biophys. Acta 62*, 509–518
4. Bos, C. J. & Emmelot, P. (1972) *Biochim. Biophys. Acta 64*, 21–29
5. Pullman, M. E. & Racker, E. (1956) *Science 123*, 1105–1107
6. Pedersen, J. I. & Grav, H. J. (1972) *Eur. J. Biochem. 25*, 75–83
7. Sordahl, L. A. & Schwartz, A. (1972) in *Methods in Cancer Research VI* (Busch, H., ed.) pp. 159–186, Academic Press, New York
8. Parce, J. W. & Cunningham, C. C. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 33*, 1284
9. Scherphof, G. L. & van Deenen, L. L. M. (1965) *Biochim. Biophys. Acta 98*, 204–206
10. Scarpa, A. & Lindsay, J. G. (1972) *Eur. J. Biochem. 27*, 401–407
11. Scherphof, G. L., Scarpa, A. & van Toorenenberger, A. (1972) *Biochim. Biophys. Acta 270*, 226–240
12. Boime, I., Smith, E. E. & Hunter, F. E. (1970) *Arch. Biochem. Biophys. 139*, 425–443
13. Higgins, E. & Chan, S. H. P. (1975) *Biophys. J. 15*, 281
14. Johnson, D. & Lardy, H. (1967) in *Methods in Enzymology* (Estabrook, R. W. & Pullman, M. E., eds.), Vol. X, pp. 94–96, Academic Press, New York
15. Chen, R. F. (1967) *J. Biol. Chem. 242*, 173–181
16. Eastabrook, R. W. (1967) in *Methods in Enzymology* (Estabrook, R. W. & Pullman, M. E., eds.), vol. X, pp. 41–47, Academic Press, New York
17. Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. 17*, 65–134
18. Adams, H. (1962) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 573, Verlag Chemie, Weisheim
19. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917
20. Duncombe, W. G. (1963) *Biochem. J. 88*, 7–10
21. Falholt, K., Lund, B. & Falholt, W. (1973) *Clin. Chim. Acta 46*, 105–111
22. Morrison, W. E. & Smith, L. M. (1964) *J. Lipid Res. 5*, 600–608
23. McFarlane, A. S. (1958) *Nature (London) 182*, 53
24. Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766
25. Hülsmann, W. C., Elliott, W. B. & Slater, E. C. (1960) *Biochim. Biophys. Acta 39*, 267–276
26. Chefurka, W. (1966) *Biochemistry 12*, 3887–3903
27. Chefurka, W. & Dumas, T. (1966) *Biochemistry 12*, 3904–3911
28. Bjorntorp, P., Ells, H. A. & Bradford, R. H. (1964) *J. Biol. Chem. 239*, 339–344
29. Aleksandrowicz, Z., Świerczyński, J. & Wrzołkowa, T. (1973) *Biochim. Biophys. Acta 305*, 59–66
30. Barbour, R. L. & Chan, S. H. P. (1977) *J. Biol. Chem.*, in press
31. Spector, A. A. & Brenneman, D. E. (1972) *Biochim. Biophys. Acta 260*, 433–438
32. Chan, S. H. P. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 30*, 1246