Oxidative Phosphorylation in *in Vitro* Aged Mitochondria. I. Factors Controlling the Loss of the Dinitrophenol-Stimulated Adenosine Triphosphatase Activity and Respiratory Control in Mouse Liver Mitochondria*

W. Chefurka

ABSTRACT: The latent adenosine triphosphatase (ATPase) system and the respiratory rate of *in vitro* aged and phospholipase-treated mitochondria fail to respond to 2,4-dinitrophenol (DNP) and adenosine 5′-diphosphate (ADP), respectively. Bovine serum albumin and other proteins protect these activities from decay in aged mitochondria. During aging, phospholipids decrease and free long-chain fatty acids accumulate stoichiometrically suggesting the involvement of phospholipase A. Albumin does not inhibit the loss of phospholipids but it does "trap" the fatty acids suggesting that loss of function of aged mitochondria is related primarily to the accumulation of fatty acids. These results also suggest that inhibition by phospholipase does not constitute sufficient evidence for the requirement of phospholipids in oxidative phosphorylation.

The loss of the DNP-stimulated ATPase activity in aged mitochondria is also counteracted by EDTA but not Ca–EDTA. This protective effect is also associated primarily with the inhibition of production of free fatty acids presumably by chelation of calcium with the consequent inactivation of the mitochondrial phospholipase. An investigation of other aging lesions such as swelling, loss of nucleotides, and magnesium in relation to aging shows that these are unrelated to the inactivation of the DNP-stimulated ATPase activity in aged mitochondria.

The loss of capacity for oxidative phosphorylation by *in vitro* aged mitochondria and submitochondrial particles has been observed on numerous occasions (Hunter and Hixon, 1949; Kielley and Kielley, 1951; Kielley, 1952; Slater and Cleland, 1952, 1953; Cross *et al.*, 1949; Ernster, 1956; Boyer *et al.*, 1956; Lester and Hatefi, 1958; Azzone *et al.*, 1960; Helinski and Cooper, 1960; Rossi *et al.*, 1964). The mechanisms underlying this loss of function have not been clearly delineated. However, it has been generally assumed that this instability is due to the simultaneous production of several lesions by the process of aging. First, aged mitochondria often swell extensively (see Chappell and Greville, 1963) with concomitant morphological alterations (Harman, 1952a,b, 1953). Second, they lose to the surrounding medium such essential cofactors as adenine nucleotides (Kielley and Kielley, 1951; Raaflaub, 1953, 1955; Siekevitz and Potter, 1955; Weinbach, 1959), pyridine nucleotides (Kielley, 1952; Ernster and Löw, 1955a; Ernster, 1956; Lester and Hatefi, 1958), as well as other unidentified thermostable factors (Ernster and Löw, 1955b; Ernster *et al.*, 1955), which seem to be essential for the restoration of oxidative phosphorylation. Third, aged mitochondria may accumulate free or protein-bound lipids containing unesterified long-chain fatty acids (Lehninger and Remmert, 1959; Hülsmann *et al.*, 1960; Lehninger, 1962) which have uncoupling properties (Pressman and Lardy, 1956; Scholefield, 1956; Borst *et al.*, 1962). Fourth, a close relationship has been observed between the degree of breakdown of mitochondrial phospholipids and the extent of uncoupling of phosphorylation from oxidation (Rossi *et al.*, 1964). A sufficient awareness of these changing parameters has now been achieved to warrant a reexamination of the precise role played by these factors in the loss of oxidative phosphorylation and its partial reactions during aging. This becomes particularly urgent in the light of many recent studies which have employed aged mitochondria as experimental material (see Ernster and Lindberg, 1958; Chappell and Greville, 1963).

Although several reports have appeared on the loss of the dinitrophenol (DNP)-stimulated ATPase[1] activity of *in vitro* aged mitochondria and submitochondrial particles (Chappell and Perry, 1953; Potter *et al.*, 1953; Lardy and Wellman, 1953; Cooper and Lehninger, 1957), the mechanism of loss of this activity remains obscure. In a series of preliminary communications (Chefurka, 1961, 1963a,b, 1964) we have confirmed the high sensitivity of this ATPase to aging and showed that it might be related to the accumula-

* From the Research Institute, Research Branch, Canada Department of Agriculture, University Sub Post Office, London, Ontario. Contribution No. 296. *Received July 26, 1966.*

[1] Abbreviations used: ATP, adenosine triphosphate; TCA, trichloroacetic acid; PCA, perchloric acid; P:O ratio, ratio of micromoles of Pi esterified:microatoms of oxygen utilized; ADP, adenosine 5′-diphosphate.

tion of lipids containing free long-chain fatty acids. Cognizant, however, of the multiplicity of lesions which occur in aged mitochondria, we report further attempts at the elucidation of the relationship between several of these lesions and the loss of this mitochondrial activity in the hope of delineating the proximate factor responsible for the loss of DNP-stimulated ATPase activity, a partial reaction of oxidative phosphorylation (Lehninger, 1961; Pullman *et al.*, 1961).

The basis of the approach to the resolution of this problem was the finding that bovine serum albumin and EDTA were effective agents in stabilizing this enzyme system when added to mitochondria before aging (Chefurka, 1961). The theoretical position taken on this question was then a simple one. Those changes during aging which were not affected by these stabilizing agents were probably unrelated to the failure of the ATPase of aged mitochondria to respond to DNP.

While confirming a multiplicity of lesions in aged mitochondria, these were not affected by albumin or EDTA and hence were probably of no consequence in the loss of this partial reaction. Only the accumulation of free fatty acids was found to be closely associated with the loss of activation of the latent ATPase by DNP.

Experimental Section

Preparation of Mitochondria from: A. LIVER. The mice were albino females (20–35 g) from Rolfsmeyer Farms, Madison, Wis. They were maintained on a diet of Gaines Krunchons dog pellets and allowed free access to food and water. The animals were killed by cervical dislocation and the liver (usually 1–2 g) was excised, finely minced, and placed in ice-cold 0.25 M sucrose solution. After several washings with ice-cold 0.25 M sucrose the chopped liver was homogenized in 10 ml of 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. After an initial centrifugation at 700*g* for 5 min at about 1° in a refrigerated Servall Model RC-2 centrifuge, about three-quarters of the supernatant fluid was decanted and the mitochondria were sedimented at 8500*g* for 10 min. The mitochondrial pellet was resuspended in ice-cold sucrose by the Potter–Elvehjem homogenizer and centrifuged at 9500*g* for 10 min. The supernatant was decanted and the surface of the tan pellet was rinsed three times with ice-cold 0.25 M sucrose to remove the "fluffy" layer. The removal of this "fluffy" layer could be discerned by the occurrence of a sharply defined edge of the mitochondrial pellet. The mitochondrial pellet was resuspended in ice-cold 0.25 M sucrose with the aid of the homogenizer to give a suspension containing approximately 6–8 mg of protein/ml. The pH of the final suspension in 0.25 M sucrose was 6.6–7.0 as measured with the glass electrode.

B. HEART. Mouse heart mitochondria were prepared as described above. The final mitochondrial suspension contained about 1 mg of protein/ml.

C. HOUSEFLY THORACIC MUSCLES. The mitochondria from thoracic muscles of houseflies were isolated in 0.25 M sucrose as described previously (Chefurka, 1963b).

Aging of Mitochondria. Freshly prepared mitochondria were diluted with an equal volume of ice-cold 0.25 M sucrose solution with or without bovine serum albumin or EDTA at pH 7.0–7.1. This suspension, subsequently referred to as "aging medium," was then incubated without shaking at 23 or 35° in a water bath, at 1° in a cold room or at 0° in a mixture of ice and water in centrifuge tubes under air. Appropriate aliquots were withdrawn at specified periods of time and assayed for ATPase activity. Since the EDTA and ATP (W. Chefurka, 1966, unpublished data) prevent the decay of the DNP-stimulated ATPase activity during aging, their presence in the reaction mixture prevented any further inactivation. Thus the inactivation measured was due wholly to the preliminary *in vitro* aging.

Assay of ATPase Activity. The assay of the ATPase activity was carried out in a reaction medium that contained 75 mM KCl, 1 mM di-Na–EDTA, pH 7.2; 50 mM Tris–acetate buffer, pH 7.2; 50 mM sucrose and 2 mM Na–ATP, pH 7.2; and 0.33 mM DNP as indicated in the text. The total volume was 1.5 ml. The incubation was for 15 min in a thermostated water bath at 23 ± 0.5°. The reaction was stopped by the addition of an equal volume of 10% TCA. After centrifugation of the coagulated protein, the orthophosphate in aliquots of the TCA supernatant was measured.

Determination of Oxidative Phosphorylation. Oxygen uptake, ADP:O ratio and respiratory-control index (defined as the ratio between the respiratory rate during the active state of respiration and the respiratory rate after the ADP is phosphorylated; Chance and Baltscheffsky, 1958) were determined at 23.0 ± 0.05° in a closed Perspex vessel equipped with polyethylene-coated Clark oxygen electrode. Additions were made with microsyringes through a small opening in the vessel. The reaction mixture was stirred by a "flea" activated by a magnetic stirrer. The composition of the reaction mixture is given in the legend of the appropriate figure.

Extraction of Fatty Acids. The fatty acids were extracted from fresh and aged mitochondrial pellets (see legends of Tables II and III) with acidic isooctane (5% glacial acetic acid in isooctane, 2,3,4-trimethylpentane; Goodman, 1957) overnight at 1° or from mitochondrial suspensions with acidic heptane–isopropyl alcohol (Dole and Meinertz, 1960) using the double-extraction procedure to eliminate interfering substances (see Tables IV and V). The isooctane extract was reduced to dryness at 30–35° in a rotary evaporator and the residue was washed twice with distilled water to remove the residual acetic acid as well as possible water-soluble inhibitors.

The occurrence of free fatty acids in the sucrose aging medium with or without albumin (see Tables II and III) was determined by sedimentation of the mitochondria at about 80,000*g* for 10 min in a Spinco Model L centrifuge. The supernatant was then extracted three times with approximately equal volumes of

acidic isooctane. Occasionally the albumin solution was lyophilized and extracted with acidic isooctane at 1° overnight. The isooctane extracts were then reduced to dryness as described above.

Extraction of Phospholipids. The extraction of mitochondrial phospholipids was performed according to Fleischer *et al.* (1961). The washed organic phase was reduced to dryness at 35° under a stream of nitrogen in 20-ml Kjeldahl flasks, and the residue was digested by 0.5 ml of a perchloric–nitric–sulfuric acid mixture (Harris and Gambal, 1963) for about 2 hr. This was cooled, diluted to 10 ml, and assayed for inorganic phosphorus.

Mitochondrial Swelling. Mitochondrial swelling was followed by changes in optical density at 520 mμ in a Beckman spectrophotometer Model DU employing quartz cuvets with 5-mm path length for concentrated suspensions and 10 mm for dilute suspensions. The composition of the medium varied and is given in the appropriate figure.

Release of E_{260} Material. After aging for a specified period, 2-ml aliquots of mitochondrial suspension (equivalent to 300 mg of liver) were spun at 17,000*g* for 5 min in a refrigerated Servall RC-2 centrifuge. The soluble proteins in the supernatant were precipitated with 0.6 ml of 1.5 N PCA and centrifuged out in a clinical centrifuge. The coagulated protein was washed once with 0.5 ml of 0.5 N PCA, centrifuged, and this washing was added to the bulk supernatant. The absorbancy of the supernatant was read at 260 mμ in a Beckman Model DU spectrophotometer.

Release of Intramitochondrial Magnesium. The loss of intramitochondrial magnesium to the surrounding medium during aging was determined by the method of Schachter (1959) using the Farrand spectrofluorimeter. An aliquot of aged mitochondrial suspension was centrifuged to separate the mitochondria from the suspending medium. The pellet was then resuspended in an equivalent volume of fresh isotonic sucrose medium. To 0.1-ml aliquots of either the supernatant or resuspended mitochondria was added 3.9 ml of appropriate reagents (Schachter, 1959). The preparation was then actively shaken for 2 min, the coagulated protein was removed by centrifugation, and the supernatant was used for assays. Only that fluorescence was ascribed to magnesium which was quenched upon addition of EDTA (Schachter, 1959). The fluorescence of the supernatant was quenched completely by EDTA to the level of the water blanks suggesting no interference from other substances. However considerable fluorescence was still evident after the EDTA treatment in the case of mitochondria. The total fluorescence of mitochondrial suspensions was therefore corrected for this EDTA nonquenched fluorescence.

Treatment of Proteins. The proteins were defatted by extraction with 5% glacial acetic acid in isooctane (Goodman, 1957) overnight at about 1°. The acidic isooctane was decanted and the protein was washed several times with isooctane. The final traces of isooctane were removed under vacuum in a rotary evaporator. The protein was then dissolved in 0.25 M sucrose and the pH was adjusted to 7.0–7.1 with dilute NaOH.

Chemical Analyses. The orthophosphate in aliquots of the TCA supernatants was measured by the method of Sumner (1944). Mitochondrial protein was determined by the method of Lowry *et al.* (1951) (using bovine serum albumin as standard) or of McKenzie and Wallace (1954). Free unesterified fatty acids were determined by the titration method of Dole and Meinertz (1960). The microtitrations were performed with the aid of a Gilmont microburet (Monostat Corp., N. Y.). The alkali strength was 0.01 N NaOH unless stated otherwise. In preliminary studies cresol red was used as the indicator but it was discarded in favor of bromothymol blue at the recommended concentration. Palmitic acid was used as a standard with recoveries of added palmitic acid ranging from 98 to 101%.

Chemicals. Bovine serum albumin (fraction V) was obtained from California Corp. for Biochemical Research, Nutritional Biochemicals, and Mann Research Laboratories; crystalline bovine serum albumin, bovine β-lactoglobulin, bovine γ-globulin, ovalbumin, and lactalbumin were obtained from Nutritional Biochemicals Corp.

Sodium oleate was obtained from Fisher Scientific Co. Disodium hydrogen phosphate, magnesium chloride, sucrose, 2-4-dinitrophenol, and EDTA (disodium and magnesium salts) were reagents of analytical grade obtained from Fisher Scientific Co. The calcium salt of EDTA was obtained from Light and Co., Ltd., England. Isooctane (2,3,4-trimethylpentane) and heptane were products of Eastman Organic Chemicals Department. The venom from *Ancistrodon piscivorus* was used as the source of phospholipase. The venom was obtained as a lyophilized powder from the Ross Allen Reptile Institute. An appropriate amount of the powder was dissolved in 0.25 M sucrose or distilled water at pH 5.7, boiled for 15 min (Hughes, 1935), the coagulated protein was removed by centrifugation, and the pH of the clear supernatant was adjusted to pH 7.15 or as specified in the text.

Results

The Aging Phenomenon

Effect of Time. The data in Figure 1 represent a typical experiment showing the loss of the DNP-stimulated ATPase activity with aging time in the presence and absence of 3 mM Mg^{2+} in the reaction medium. Aged mitochondria fail to respond to DNP and the limiting value of the DNP-stimulated ATPase activity was approached within 20–30 min of aging; 10 min being sufficient for 50% inactivation. In most instances the inactivation proceeds without any lag period suggesting that traces of endogenous substrates and cofactors provide no protection. A similar time-course loss has been reported for the ATP–P_i exchange activity and for phosphorylation (Cross *et al.*, 1949; Boyer *et al.*, 1956; Polis and Shmukler, 1957; Helinski and Cooper, 1960). Paralleling this loss, was a small but definite activation of the endogenous ATPase

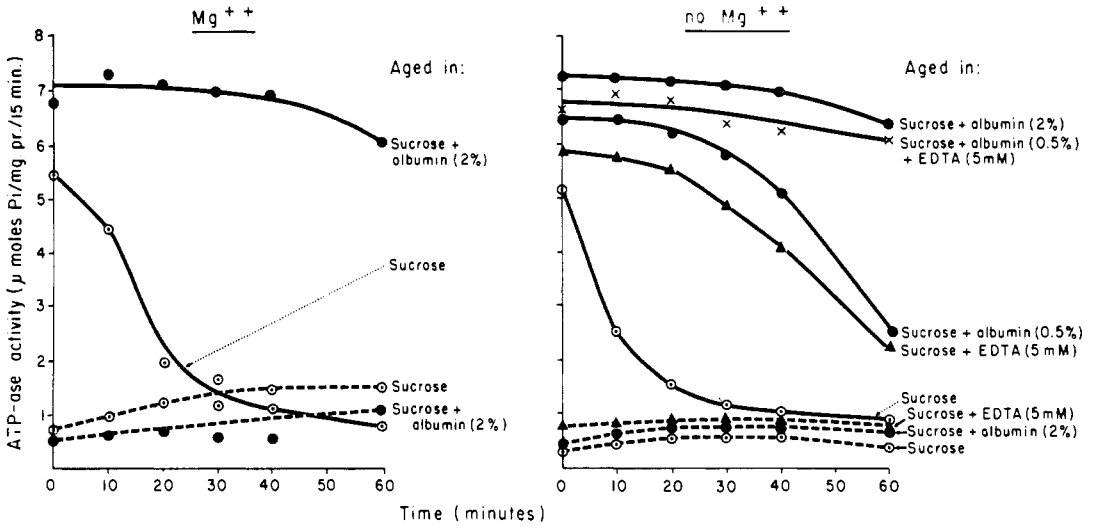

FIGURE 1: Effect of aging of mitochondria on the ATPase activity. Mitochondria were aged in 0.25 M sucrose solution with bovine serum albumin and EDTA (pH 7.1) either singly or in combination at final concentrations as indicated. The solid lines represent ATPase activity with DNP in the reaction mixture; and broken lines in absence of DNP. Concentration of $MgCl_2$ was 3 mM in the reaction mixture; temperature, 23°.

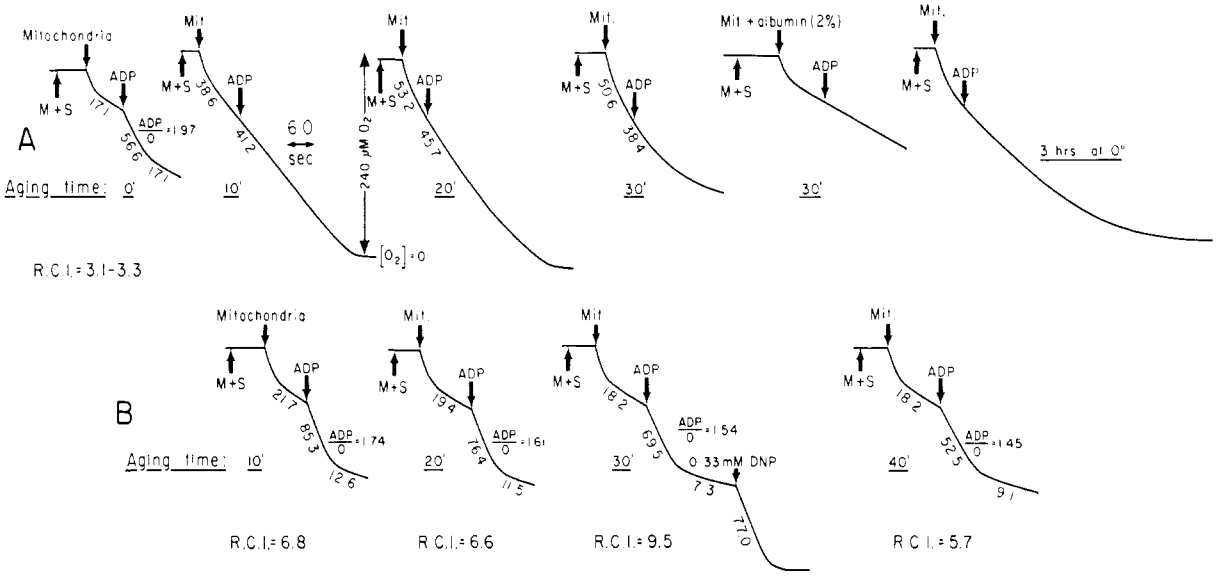

FIGURE 2: The effect of albumin on oxidative phosphorylation of aging mouse liver mitochondria. The upper set of tracings (A) are of mitochondrial activity aged in 0.25 M sucrose; the lower set (B) of mitochondria aged in 0.25 M sucrose + 2% albumin at 23° unless stated otherwise. The figures under the tracings are respiratory rates in micromoles of O_2 per minute which were calculated from the slopes of the traces. The air-saturated medium (M) at pH 7.5 contained sucrose 0.25 M, NaF 0.01 M, sodium phosphate 0.02 M, and KCl 0.01 M. Other additions as indicated in the figure were: succinate (S), 0.01 M, pH 7.5; 0.2 ml of mitochondria containing 4.52 mg of protein, and 10 μl of ADP (212.5 μM) at pH 7.5; total volume, 2.0 ml. Reaction vessel temperature was 23.0 ± 0.05°.

and a more substantial activation of the Mg^{2+}-stimulated ATPase. However, the latter activity in our preparation was never as high as in aged rat liver mitochondria (Kielley and Kielley, 1951). Possible complications of interpretation due to the Mg-stimulated ATPase were therefore avoided by withholding Mg^{2+} from the experiments to be reported.

The upper tracings (A) in Figure 2 show the loss of respiratory control with aging time. Mitochondria aged for 10 min virtually failed to respond to additions

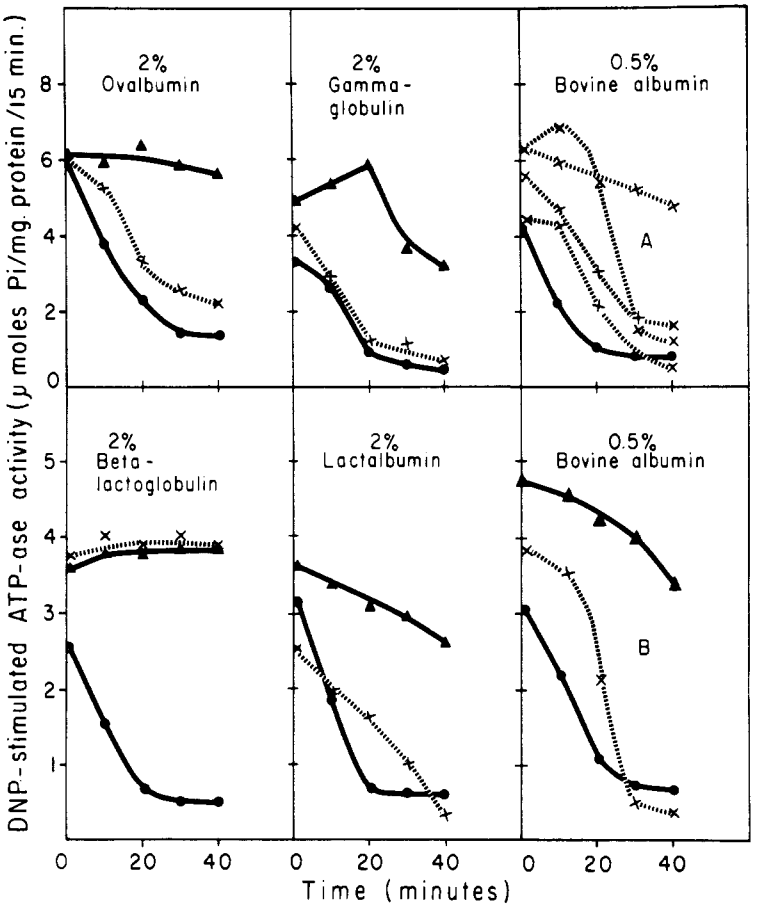

FIGURE 3: Effect of proteins on the DNP-stimulated ATPase activity of aging mitochondria. ●—●, no additions to aging medium. ×- - - -×, untreated proteins in aging medium. ▲—▲, defatted proteins in aging medium. Bovine serum albumin fraction V (A) from four commercial sources.

of ADP. In other preparations some evidence of respiratory control was evident after 10 min but not after 20 min of aging. Albumin (2%) added to mitochondria after they were aged for 30 min failed to reactivate the respiratory control. Prolonged aging (3 hr) at 0° also produced a loss of respiratory control.

Effect of Bovine Serum Albumin and Other Proteins. The data in Figure 1 show that bovine albumin in the aging medium exerts a twofold effect: (a) an activation of the zero-time DNP-stimulated ATPase activity, and (b) a striking attenuation of the loss of this activity during aging. The latter effect was markedly dependent on the concentration of albumin. Studies not reported here, show that inclusion of albumin in the preparative medium only was insufficient to maintain a high level of DNP-stimulated ATPase activity in mitochondria aged in sucrose.

The lower tracings (B) in Figure 2 show that the loss of respiratory control in aged mitochondria was also prevented by inclusion of albumin (final concentration of 2%) in the aging medium. Furthermore the ADP:O ratio of mitochondria aged for 40 min was still 1.45 as compared with 1.97 for the controls. Significantly, in the presence of albumin, the respiratory-control index was doubled and was negligibly decreased by aging.

The data in Figure 3 show that commercially available ovalbumin, lactalbumin, and γ-globulin as well as certain lots of bovine serum albumin failed to appreciably attenuate the decay of the DNP-stimulated ATPase in aged mitochondria. This is in keeping with the failure of ovalbumin and lactalbumin to activate (a) oxidative phosphorylation of aged mouse liver mitochondria (Pullman and Racker, 1956) and of fresh insect mitochondria (Sacktor *et al.*, 1958; Wojtczak and Wojtcazk, 1960) and (b) the ATP–P_i exchange reaction of fresh insect mitochondria (Wojtczak and Wojtczak, 1960). However, after extraction with acidic isooctane, the proteins readily counteracted the aging effects, implying that this property of proteins is dependent on the level of endogenously bound fatty acids and/or other lipids. On the other hand, some commercial lots of β-lactoglobulin and bovine serum albumin protected the mitochondria from aging without prior defatting.

Effect of EDTA. The data in Figure 1 demonstrate

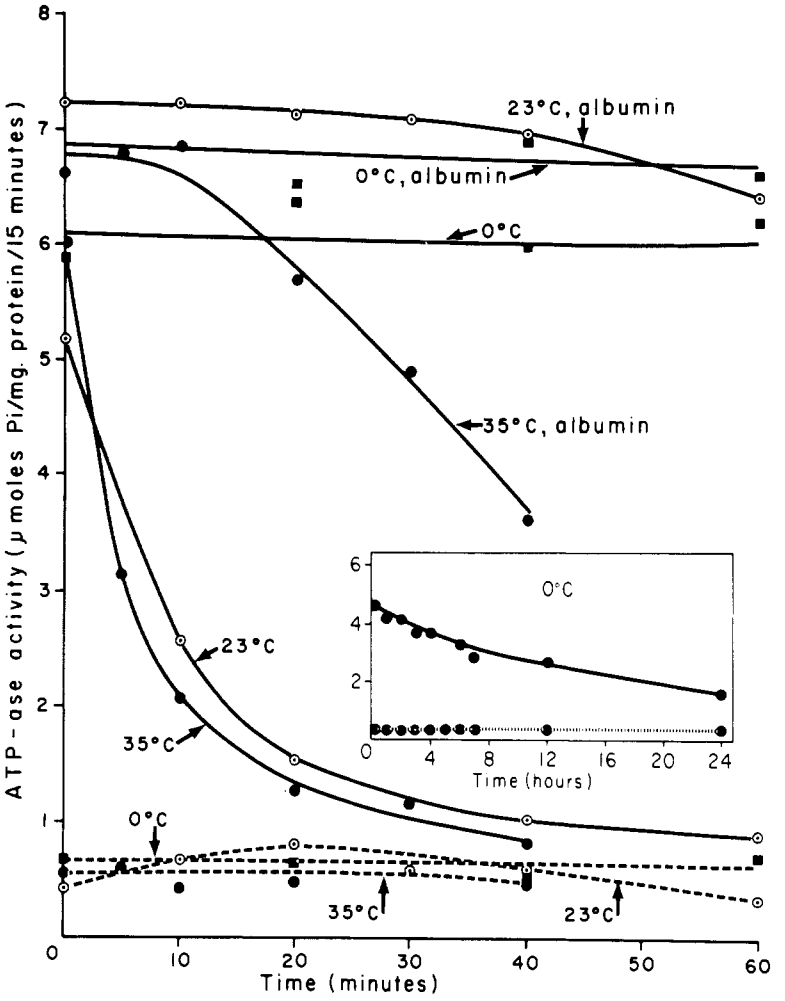

FIGURE 4: The effect of temperature on the loss of ATPase activity during aging. The solid lines represent ATPase activity with DNP in the reaction mixture and broken lines in absence of DNP. The concentration of bovine serum albumin in the aging medium was 2%. The mitochondria were aged in an ice bath at 0° or a thermostated water bath at 23.0 and 35.0 ± 0.5°.

the stabilizing effect of EDTA when added initially to the aging medium. The protective effect was concentration dependent being maximum at about 1–5 mM. Data not reported here indicate that the rate of decay of the DNP-stimulated ATPase of mitochondria suspended and aged in only sucrose did not depend on the presence of EDTA in the preparative medium. Only when EDTA was included in the aging medium did it counteract the aging effects. Furthermore, the data in Figure 1 show a more striking protection when both EDTA and albumin were included in the aging medium. These additive effects are seen most clearly in the later phases of aging suggesting that each agent counteracts the aging effects by different mechanisms.

Effect of Temperature. Several reports indicate that the stability of the energy-coupling mechanism and of the partial reactions is a function of temperature. As a general rule, these reactions are very nearly completely abolished by aging at 25–38° for short periods of time (Hunter and Hixon, 1949; Cross *et al.*, 1949; Boyer *et al.*, 1956; Helinski and Cooper, 1960) but are relatively stable at 0° (Hunter and Hixon, 1949; Weinbach, 1959). The data in Figure 4 show that short-term aging at 0° (*e.g.*, 1 hr) has little effect on the response of the ATPase system to DNP. However, only about 46% of this activity remained after storage of the mitochondria for 24 hr at 0° (see insert of Figure 4). As the temperature was increased so was the rate of loss of the DNP-induced ATPase activity. This loss was prevented to a greater or lesser extent by albumin depending on the temperature of the aging medium.

Effect of Source. The data in Figure 5 demonstrate a marked source difference in the rate of inactivation of the DNP-stimulated ATPase. The mitochondria

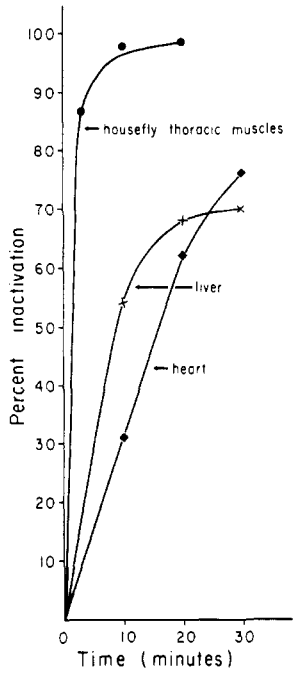

FIGURE 5: Effect of aging on the DNP-stimulated ATPase activity of mitochondria from several sources; temperature, 23.0°. Liver mitochondria contained 340 μg of protein; heart mitochondria, 76 μg of protein; and housefly mitochondria, 120 μg of protein.

from the housefly thoracic muscles were considerably less stable than those of either mouse liver or mouse heart.

Mitochondrial Lesions during Aging

Swelling. The relative insensitivity of the mitochondrial ATPase to DNP after a 20–30-min aging period does not appear to be due to swelling or disruption of mitochondria even though morphological alteration of mitochondria is known to abolish the DNP-stimulated ATPase activity (Kaltenbach and Harman, 1955; Hunter, 1956). The data in Figure 6 show negligible swelling of mouse liver mitochondria during the 60-min aging period in 0.25 M sucrose. This refractory property of mouse liver mitochondria to swelling confirms the observation of Emmelot and Bos (1957) and is in contrast to that of rat liver mitochondria (Tapley, 1956). Both phosphate and serum albumin induced swelling but it is significant to note that in this swollen state, the mitochondria were still capable of a high level of DNP-stimulated ATPase activity (Figure 1). EDTA (5 mM) almost completely prevented the swelling induced by serum albumin. These experiments thus dissociate the "high-amplitude swelling" produced by albumin from the protection it affords the DNP-stimulated ATPase during aging and suggest that the failure of DNP to stimulate the latent ATPase activity during aging under our conditions is not related to spontaneous swelling or gross changes in structure.

Swelling of mitochondria may also be induced by many agents including long-chain fatty acids (Lehninger, 1962; Utsumi *et al.*, 1962). Our studies indicate that the induction of swelling by oleate depends upon the ratio of mitochondria to oleate suggesting that swelling may be the result of a combination between the fatty acid and certain specific receptor sites in the mitochondrial membrane. At mitochondrial levels normally used in the aging experiments (3–4 mg of

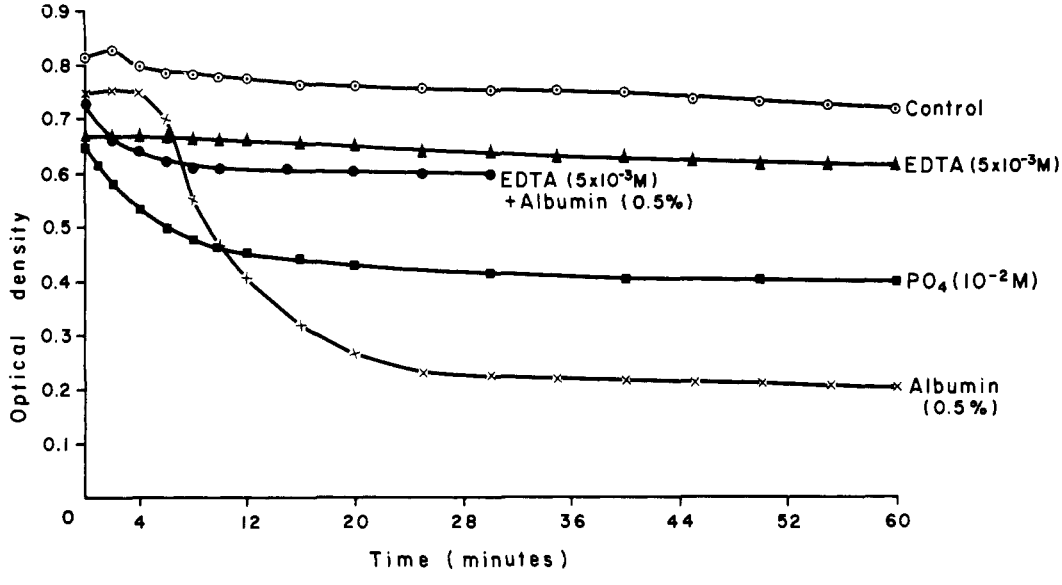

FIGURE 6: Time-course of mitochondrial swelling. The basic medium was 0.25 M sucrose. Additions were 5 mM EDTA, 10 mM sodium phosphate, 0.5% bovine serum albumin, 5 mM EDTA + 0.5% bovine serum albumin, and liver mitochondria containing about 1.2 mg of protein; final pH, 7.1. After several inversions of the cuvet for mixing, the changes in absorbancy were followed at 520 mμ. Temperature was 23.0 ± 0.5°; total volume, 3.0 ml.

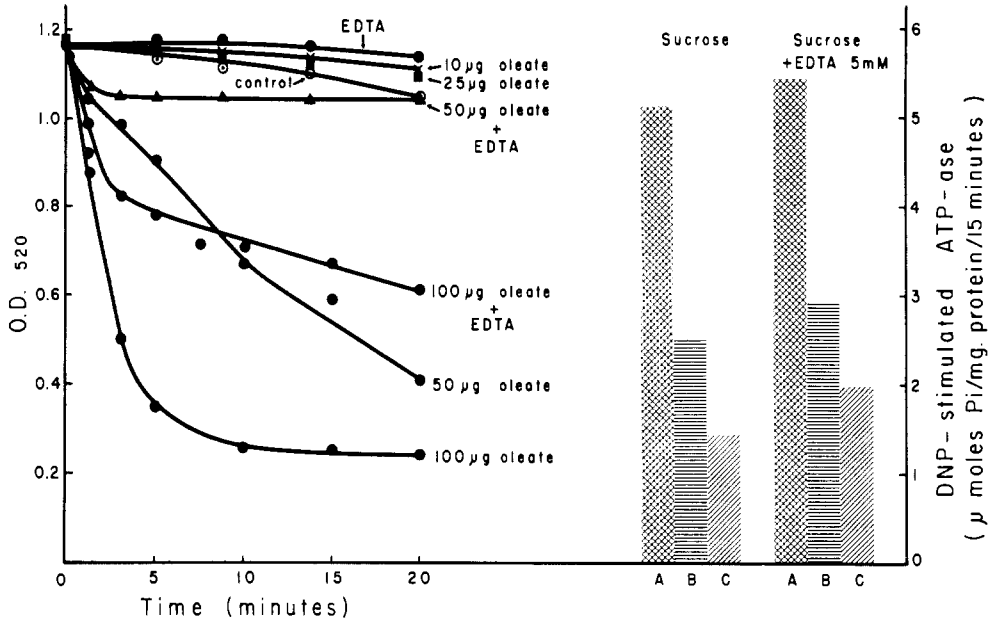

FIGURE 7: The effect of EDTA on the swelling induced by sodium oleate and on the inhibition of the DNP-stimulated ATPase activity by sodium oleate. *In the swelling experiments* the mitochondria (3.0 mg of protein/ml) were prepared in 0.25 M sucrose. To 2.0 ml of mitochondria were added the indicated amounts of sodium oleate in 0.05 ml of ethanol. Additions of EDTA (pH 7.1) were 5 mM. Swelling was inferred from changes in absorbancy which was monitored at 520 mμ in quartz cells of 5.0-mm path length and is represented by the solid lines. *In the ATPase reaction* the indicated amount of sodium oleate was prepared from a stock solution in 0.25 M sucrose pH 7.0 and added to 2.0 ml of mitochondrial suspension (2.78 mg/ml), with and without 5 mM EDTA (pH 7.1). (A) control (no oleate); (B) 25 μg of oleate; and (C) 50 μg of oleate. The DNP-stimulated ATPase activity, represented by the hatched histograms, was not corrected for the low endogenous ATPase activity which was not affected by these quantities of oleate.

protein/ml), 25 μg of oleate did not induce swelling though it readily inhibited the DNP-stimulated ATPase activity as is shown in Figure 7. On the other hand, swelling which was induced by larger amounts of oleate (50 μg) was readily prevented by 5 mM EDTA. However, this level of EDTA did not significantly relieve the inhibition of the DNP-stimulated ATPase activity caused by 50 μg of oleate. These experiments therefore suggest that a low DNP-stimulated ATPase activity is not an *a priori* indication of swollen or damaged mitochondria. Falcone and Mao (1965) were able to demonstrate that long-chain fatty acids were also capable of inhibiting the ATP–P_i exchange activity without a significant increase in ATPase activity or evidence indicative of mitochondrial swelling.

The loss of stimulation of the ATPase activity by DNP is also independent of the tonicity of the aging medium. The data in Figure 8 show that stimulation of the ATPase by DNP was lost at approximately similar rates regardless of whether the mitochondria were aged in 0.15, 0.40, or 0.88 M sucrose. Furthermore, in all instances this loss was attenuated by 2% albumin in the aging medium. The zero-time activation of the DNP-stimulated ATPase by albumin, noted in Figure 1, was also evident in hypo- and hypertonic sucrose media. It is worth recalling that Lehninger *et al.* (1959), and Neubert *et al.* (1962) observed negligible spontaneous swelling of rat liver mitochondria in 0.4 and 0.88 M sucrose. These data thus provide additional evidence against the possibility of osmotic swelling–shrinkage changes being the casual factor in the loss of the DNP-induced ATPase activity during the early stages of aging.

Loss of Intramitochondrial E_{260} Material. Siekevitz and Potter (1955) reported that acid-soluble nucleotides absorbing at 260 mμ diffuse out of rat liver mitochondria at 23 but not 0°. The stability of the DNP-stimulated ATPase at 0 but not 23° (Figure 4) suggests a possible relationship between loss of activity and loss of intramitochondrial nucleotides. Consequently the release of these nucleotides in the presence of albumin and EDTA was examined.

Figure 9 confirms the gradual diffusion of E_{260} material from aging mitochondria into the surrounding isotonic sucrose medium. However neither albumin nor EDTA added singly or together to the aging medium, had any effect on the release of acid-soluble nucleotides. Indeed albumin actually stimulated the release of the 260-mμ absorbing compounds, presumably as a direct result or the increased permeability of the mitochondrial membrane (Figure 6).

It seems clear that this release of 260-mμ material from aging mitochondria bears no direct relationship to the level of the DNP-stimulated ATPase activity.

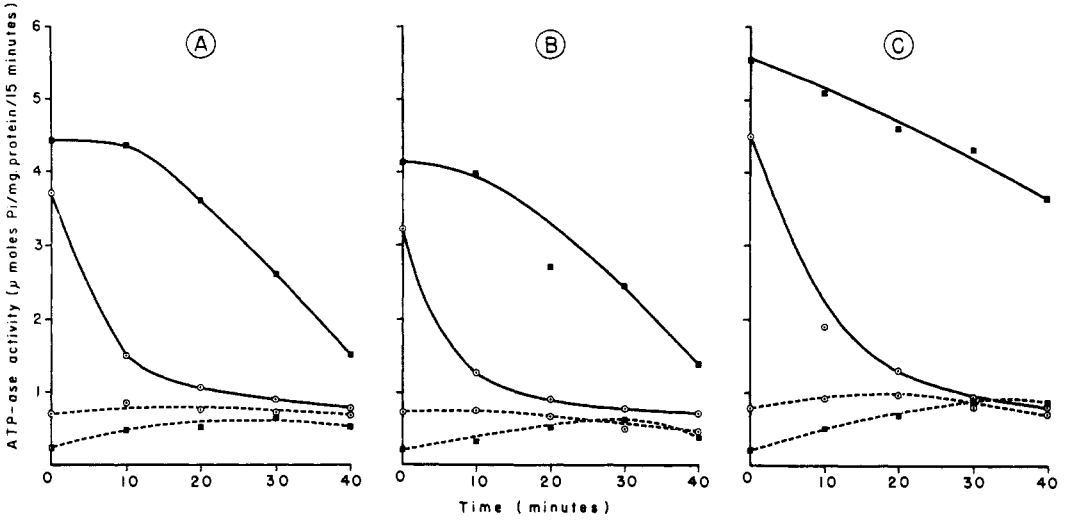

FIGURE 8: The effect of tonicity of the aging medium on the ATPase activity of mitochondria. (A) 0.15 M sucrose; (B) 0.44 M sucrose; and (C) 0.88 M sucrose. Solid lines represent ATPase activity with DNP in the reaction mixture and broken lines in absence of DNP. (○) mitochondria aged in sucrose only; (■) mitochondria aged in sucrose + 2% albumin.

It is recognized, however, that compounds present in very small amounts and which may therefore contribute negligibly to the 260-mμ absorption, may conceivably play a role in the DNP-induced ATPase.

Loss of Intramitochondrial Magnesium. The data in Table I indicate a loss of intramitochondrial magnesium to the surrounding medium. This is in contrast to the finding of Siekevitz and Potter (1955) who demonstrated no loss of magnesium from aged rat liver mito-

TABLE I: Effect of Albumin on the Magnesium Content of Aged Mitochondria.[a]

	Mg Content (mμmoles/mg of protein)			
	Mitochondria		Supernatant	
Time (min)	No Albumin	Albumin	No Albumin	Albumin
0	34.7	35.4	4.4	4.5
15	34.7	27.0	—	—
30	30.1	18.8	9.6	14.7
45	25.4	14.5	—	—
60	23.1	12.5	13.9	18.7

[a] Albumin (2%) was included in the 0.25 M sucrose aging medium, which contained 12.5–13.5 mg of mitochondrial protein/ml.

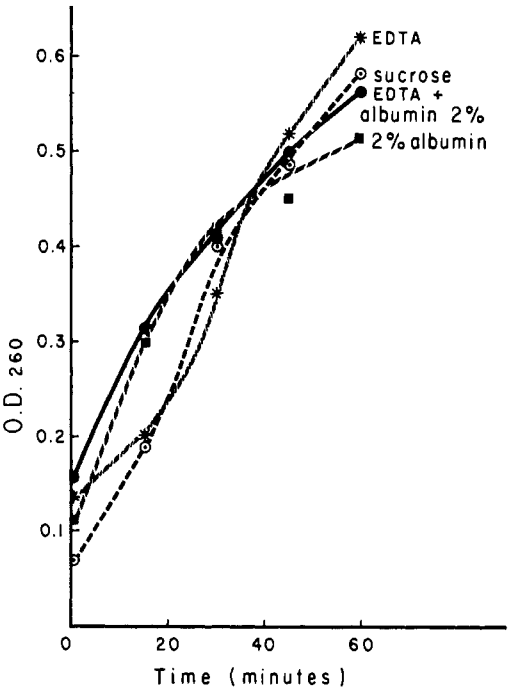

FIGURE 9: The loss of E_{260} material from mitochondria as a function of time. The mitochondria (about 300 mg of liver equivalent mitochondria) were aged in the basic sucrose medium containing 5 mM EDTA (pH 7.0) or 2% bovine serum albumin. The aged supernatants were processed as described in text and assayed at 260 mμ.

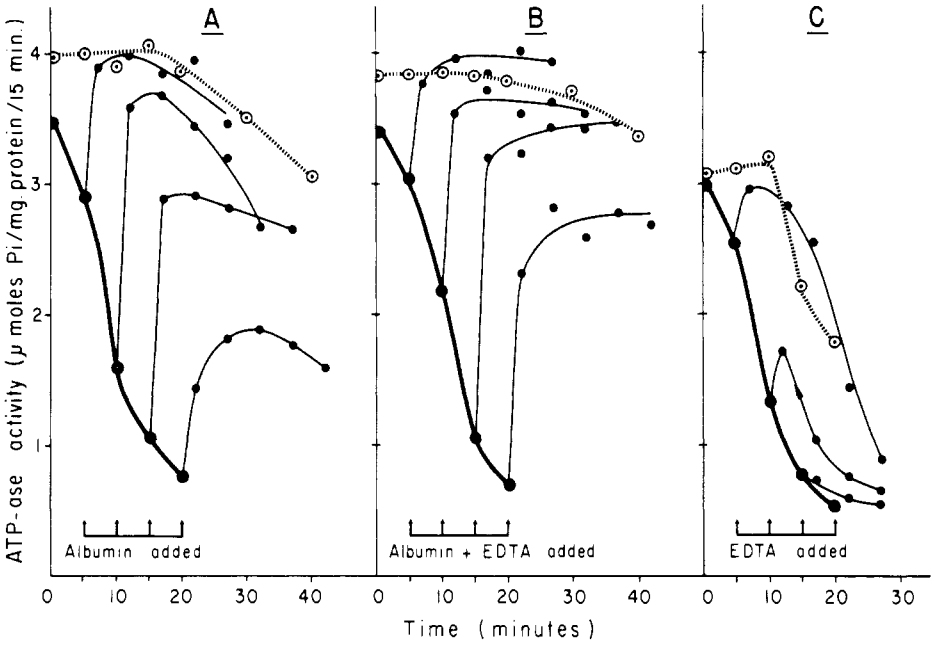

FIGURE 10: Reactivation of DNP-stimulated ATPase activity of aged mitochondria. The *continuous* curve represents the course of decay of DNP-stimulated ATPase activity of mitochondria aged only in 0.25 M sucrose; the *broken line* shows the DNP-stimulated ATPase activity of mitochondria aged in isotonic sucrose plus: (A) 2% albumin, (B) 2% albumin plus 5 mM EDTA, and (C) 5 mM EDTA (all pH 7.0) added to the medium before aging. At times indicated on the normal aging curve, an aliquot of mitochondrial suspension maintained as another control except with twice the protein content was added to albumin, albumin plus EDTA and EDTA also at twice the above concentrations. One minute later and periodically as indicated, the DNP-stimulated ATPase of this reactivated preparation was assayed.

chondria. The loss of magnesium in our preparations was enhanced by aging in the presence of albumin probably as a result of its swelling-inducing property (Figure 6). Thus after 60 min of aging, the intramitochondrial magnesium content was reduced by about 65% and yet the ATPase of such mitochondria in 2% albumin showed an unimpaired response to DNP (Figure 1). These experiments suggest that the bulk of the intramitochondrial magnesium is probably inactive with respect to the ATPase activity. Cooper (1960) similarly concluded that the magnesium content of submitochondrial particles bears no relationship to the level of the DNP-stimulated ATPase activity of the particles.

Endogenous Fatty Acids and Aging

Reactivation of the DNP-Stimulated ATPase Activity. In the experiments of Figure 10 the time of addition of 2% albumin, 2% albumin plus 5 mM EDTA, and 5 mM EDTA to aging mitochondria was varied to determine how long the mitochondria could age at 23° and the ATPase still respond to DNP. Addition of albumin or albumin plus EDTA to mitochondria, which has lost about 80–90% of their capacity to respond to DNP produced a rapid and almost complete reactivation of this response. However, it became increasingly difficult to reverse completely the activity of this inhibited enzyme system in excessively aged mitochondria. Furthermore, the *rate of reversal* by albumin was slower as aging was prolonged. By contrast to the effects of albumin or albumin plus EDTA, 5 mM EDTA alone could not significantly reactivate this inhibited enzyme system in aged mitochondria suggesting that its mode of action in stabilizing the ATPase activity is different from that of albumin.

These experiments permit a separation of the aging process into two phases: (a) early reversible phase due probably to production of an inhibitor absorbed by albumin, and (b) late irreversible phase probably due to secondary changes which are not affected by albumin.

At present the conditions that govern the onset of these irreversible changes are not completely understood, but it is reasonable to expect them to be more prominent at higher temperatures. They may, therefore, underly the failure of albumin to effectively protect mitochondrial aging at higher temperatures (Figure 4). These observations may also explain the inability of albumin to restore the DNP-stimulated ATPase activity of beef heart mitochondria which were aged at 38° (Brierley *et al.*, 1964) particularly when a partial restoration of the phosphorylative activity of mildly

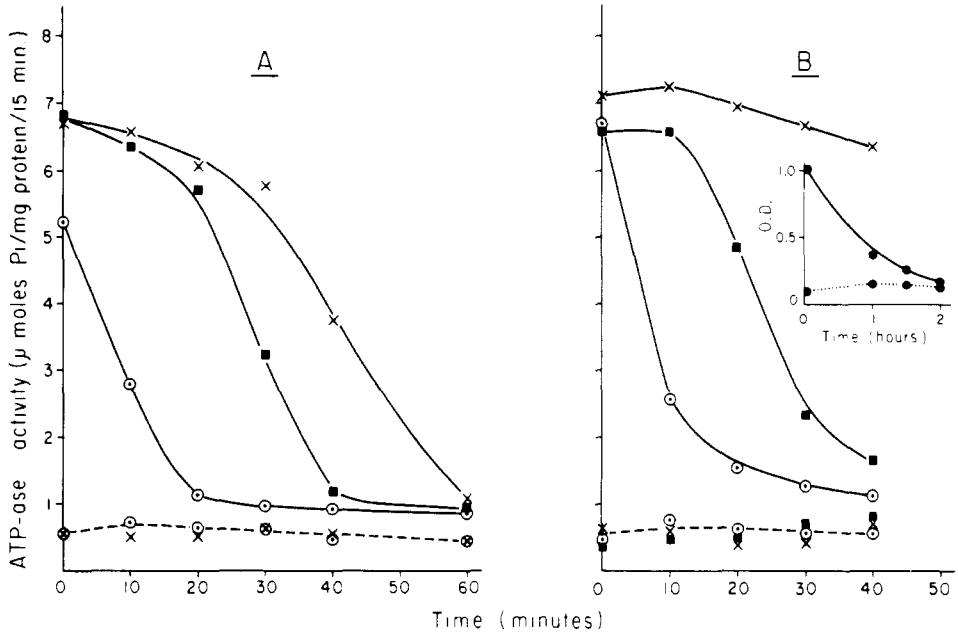

FIGURE 11: Effect of fresh and once-aged albumin on the DNP-stimulated ATPase activity of aging mitochondria. Solid lines represent ATPase in presence of DNP in the reaction mixture and broken lines in absence of DNP. (A) Temperature, 23°, (⊙) control, no additions; (×) 0.25% albumin; (■) 0.25% albumin aged once. (B) Temperature, 35°, (⊙) control, no additions; (×) 1% albumin; (■) 1% albumin aged once; (●) 2% albumin (insert).

aged beef heart and of rat liver mitochondria by albumin has been reported (Lester and Hatefi, 1958; Weinbach, 1959).

Production of Endogenous Inhibitors. If, as the data in Figure 10 suggest, albumin is serving as a "trap" for an inhibitor(s) produced during aging, then presumably after prolonged aging, albumin should adsorb sufficient inhibitor(s) to decrease its effectiveness against aging of fresh mitochondria. The data in Figure 11A show that at 23°, albumin (0.25%) failed to protect the mitochondria after 60-min aging. At this time, the mitochondria were centrifuged out of the albumin aging medium and the latter was used as a suspension medium for fresh mitochondria. The data clearly show that this once-aged albumin was less effective in attenuating the loss of the DNP-stimulated ATPase of fresh mitochondria. Presumably the inhibitor(s) adsorbed during the first round of aging decreased the availability of sites for binding of the inhibitor(s) produced during the second round of aging.

This effect was accentuated when the first round of aging was carried out at 35°. As seen in the insert of Figure 11B, the protection of 2% albumin was completely lost after aging for 2 hr. The mitochondria were then centrifuged out of the albumin aging medium which was used as the aging medium for a fresh mitochondrial preparation. Figure 11B shows the effect of this aged preparation as well as a fresh albumin solution, both now at 1%. Clearly the once-aged albumin at 35° is considerably less effective than either fresh albumin or that aged at 23°.

These data confirm that albumin serves as a trap for inhibitors produced during aging. Furthermore, the amount that was trapped was temperature dependent. However, these results also show that the failure of albumin to protect against prolonged aging was not due to complete saturation of the inhibitor binding sites. Once-aged albumin (Figure 11A) still provided freshly prepared mitochondria considerable protection. Clearly, secondary irreversible changes eventually set in which also affect the activity under study. From the point of view of the time scale of aging, however, these secondary effects set in well after the ATPase has lost its response to DNP. Consequently we conclude that the loss of the DNP-stimulated ATPase activity during aging is intimately associated with the accumulation of an endogenous inhibitor(s) and and that this enzyme system is presumably sensitive to low levels of this inhibitor(s) generated *in situ*.

The data in Table II (expt 2) indicate that mitochondria aged for 2 hr at 35° produced a substance(s) which when added back to fresh mitochondria inhibited the stimulation of the ATPase activity by DNP. This inhibition was readily counteracted by albumin. Negligible amounts of the inhibitor were released into the aging medium (Table II, expt 3). Hence the inhibitor was probably retained within or at the surface of the mitochondria. The mitochondrial membrane as the site of production of lipidlike uncouplers is strongly suggested by the findings of Lehninger and Remmert (1959) who demonstrated that the major portion of the lipid substances which inhibit the ATP–P_i exchange activity and decrease the P:O ratio was recovered from the particulate residue of sonicated

TABLE II: Effect of Lipid Extracts from Fresh and Aged Mitochondria on the ATPase Activity of Fresh Mitochondria.[a]

Expt	Addn to Suspension of Fresh Mitochondria	ATPase Act. (μmoles of P_i/mg of protein 15 min)	
		No DNP	DNP
1	None	0.51	4.73
	Extract from fresh mitochondria	0.60	3.63
2	None	0.11	3.56
	Albumin (1%)	0.03	4.08
	Extract from aged mitochondria	0.19	0.28
	Extract (dilution 1:2) from aged mitochondria	0.15	0.31
	Extract (dilution 1:5) from aged mitochondria	0.72	1.38
	Extract (dilution 1:10) from aged mitochondria	0.57	2.72
	Extract from aged mitochondria + albumin (1%)	0.20	1.99
	Extract (dilution 1:2) from aged mitochondria + albumin (1%)	0.04	3.68
	Extract (dilution 1:5) from aged mitochondria + albumin (1%)	0.16	4.31
	Extract (dilution 1:10) from aged mitochondria + albumin (1%)	0.04	4.27
3	None	0.51	4.73
	Extract from aged supernatant	0.63	3.56
4	None	0.28	5.16
	Extract of residue from acidic isooctane	—	5.16

[a] Mitochondria were prepared from five mouse livers as described in text and aged in 0.25 M sucrose (approximately 15 ml) for 2 hr at 35°. They were then centrifuged at 80,000*g* for 10 min at 0° in a Spinco Model L centrifuge, suspended in 25 ml of 5% acetic acid in isooctane and extracted overnight at 1°. The supernatant was extracted three or four times by shaking for about 2 min with an equal volume of acidic isooctane. Isooctane extracts from fresh mitochondria were prepared as described for aged mitochondria except that aging was omitted. The isooctane extracts were then processed as described in text. As a control, an equivalent volume of acidic isooctane was also processed as described. The residues were dissolved in absolute ethanol (0.30 ml) of which 0.05 ml was added to 1 ml of ice-cold 0.25 M sucrose with or without 2% bovine serum albumin. To this was added 1 ml of freshly prepared mitochondria (3–4 mg of protein/ml) of which 0.1 ml was then added to a reaction mixture for determination of the ATPase activity.

aged mitochondria. Only trace quantities of inhibitory substances were extracted from equivalent amounts of fresh mitochondria (Table II, expt 1). Appropriate solvent controls showed no inhibition (expt 4). Significantly much less inhibitory substances were extracted from mitochondria which were aged in the sucrose–albumin medium (Table III, expt 3). However, as the data in Table III (expt 2) show, extracts of this aged albumin strongly inhibited the DNP-stimulated ATPase activity of fresh mitochondria suggesting a transfer of the inhibitor(s) from the mitochondrial surface to the albumin during aging. Extracts of an equivalent quantity of albumin which was not exposed to aging mitochondria, were only slightly inhibitory (expt 1).

The active substance(s) in our extracts was readily soluble in several organic solvents but insoluble in water and this suggests that the inhibitory substance was probably a lipid. Our inability to solubilize the inhibitor in water also eliminates the possibility of interference by lysolecithin, which we have shown (W. Chefurka, 1966, unpublished data) to have a similar pattern of inhibition and reversal by albumin as these lipid substances extracted from aged mitochondria.

Effect of EDTA and Albumin on the Accumulation of Fatty Acids. The data in Table IV show that free fatty acids were readily recovered from aged mitochondria. The accumulation of free fatty acids in the aged preparations was a function of time as well as the temperature of aging. Furthermore, this increase in titratable-free fatty acids in sucrose-aged mitochondria is totally accountable by a corresponding decrease in phospholipids. The molar relationship of 1:1 between these mitochondrial components in the early phases of aging suggests that the hydrolysis of phospholipids was probably mediated by phospholipase A. In the presence of albumin, the loss of phospholipids was enhanced, suggesting that phospholipase A may be inhibited by the products of phospholipid hydrolysis. EDTA inhibited both the accumulation of free fatty acids and the loss of phospholipids. This inhibition of

TABLE III: Effect of Lipid Extracts from Fresh and Aged Albumin on the ATPase Activity of Fresh Mitochondria.[a]

Expt	Addn to Suspension of Fresh Mitochondria	ATPase Act. (μmoles of P_i/mg of protein 15 min) No DNP	DNP
1	None	0.28	5.16
	Extract from native albumin	0.32	5.12
2	None	0.44	4.15
	Extract from "aged" albumin	0.43	0.54
	Extract (dilution 1:5) from "aged" albumin	0.77	2.70
	Extract (dilution 1:10) from "aged" albumin	0.63	2.90
3	None	0.44	4.15
	Extract from mitochondria aged in albumin	0.31	3.33

[a] Mitochondria were prepared as described in text from eight livers and aged in 2% bovine serum albumin in 0.25 M sucrose for 2 hours at 35°. They were then centrifuged at 80,000 *g* for 10 min at 0° in a Spinco Model L centrifuge. The mitochondria were washed twice by resuspension in 0.25 M sucrose and centrifugation. The washings were added to the bulk albumin supernatant. The mitochondrial fatty acids were then extracted as described in Table II. The supernatant was used as the suspension and aging medium for another batch of mitochondria (from eight livers). After 2 hours of aging at 35°, the mitochondria were centrifuged and the supernatant was then extracted with acidic isooctane as already described for the supernatant in Table II. For details of processing these extracts and their additions to fresh mitochondria see Table II. An equal quantity of freshly prepared albumin was extracted with acidic isooctane as controls.

TABLE IV: The Accumulation of Fatty Acids and Loss of Phospholipids in Aged Mitochondria.

Aging Time (hr)	Temp (°C)	+Δ-Fatty Acids[a] Sucrose	Sucrose + EDTA	−Δ-Phospholipid P[b] Sucrose	Sucrose + Albumin	Sucrose + EDTA
1	0	6.0	—	—	—	—
2	0	6.0	—	—	—	—
4	0	9.4	—	—	—	—
1	23	19.3	—	—	—	—
2	23	33.5	—	—	—	—
3	23	43.5	—	—	—	—
0.5	35	19.5	6.0	17.0	19.1	4.0
1	35	31.0	11.0	32.4	40.3	5.8
1.5	35	40.7	18.0	44.2	53.6	—
2	35	54.6	26.0	43.7	67.5	13.3
3	35	—	—	51.1	—	—

[a] Milliμmoles of fatty acid per milligram of protein. The average amount of fatty acids in freshly prepared mitochondria was 61 (57–66) mμmoles/mg of protein. [b] Milliμmoles of phospholipid P per milligram of protein. The average amount of phospholipid P in freshly prepared mitochrondria was 205 (190–226) mμmoles/mg of protein. Composition of aging media 0.25 M sucrose, 0.25 M sucrose + 5 mM EDTA, pH 7.0, and 0.25 M sucrose + 2% bovine albumin as indicated above.

phospholipase by EDTA probably stems from its chelation of Ca^{2+} which is an activator of this enzyme (Kates, 1960). This possibility is strengthened by the data in Figure 12 which show that while the di-Na–EDTA and Mg–EDTA were effective in attenuating the loss of the ATPase activity, the protection by the Ca–EDTA was relatively ineffective presumably because of its inability to chelate the endogenous Ca^{2+}. On the other hand the rate of loss of the DNP-stimulated ATPase activity was increased by Ca^{2+} in the

TABLE V: Effect of Ca^{2+} on the Accumulation of Fatty Acids in Mouse Liver Mitochondria.[a]

Ca^{2+} (M × 10^{-3})	μl of NaOH Consumed
0	82.2
0.1	82.7
1.0	82.1
5.0	90.3
10	96.9

[a] $CaCl_2$ was added to a mitochondrial suspension containing 5.28 mg of protein/ml. Incubation time was 60 min at 23°. Aliquots (1 ml) were extracted and titrated for fatty acids according to Dole and Meinertz (1960) with 0.0025 N NaOH.

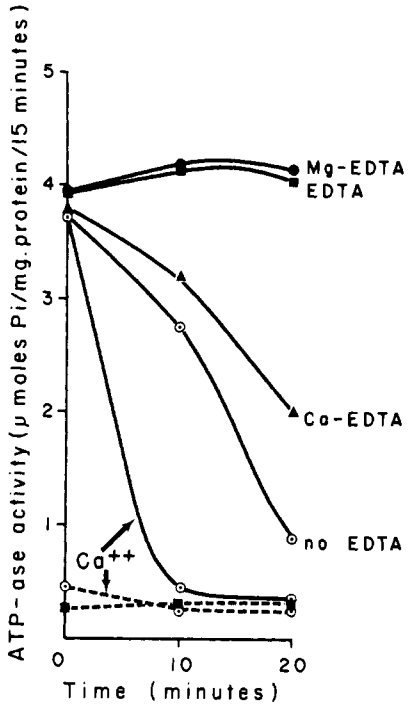

FIGURE 12: Effect of Ca^{2+} and salts of EDTA on the ATPase activity of aging mitochondria. The solid lines represent ATPase activity with DNP in the reaction mixture; broken lines in absence of DNP. Final concentration of metal chelates and Ca^{2+} added to the aging medium was 5 and 1 mM, respectively; final pH, 7.0; temperature, 23°.

TABLE VI: The Effect of Bovine Serum Albumin on the Phospholipase-Treated ATPase Activity.[a]

Aging Time (min)	BSA	DNP	Phospholipase (μg/ml) 0	12.5	25	50	100
0	—	—	0.65	0.66	1.15	1.30	0.87
	−	+	4.95	4.37	2.32	1.30	0.93
	+	−	1.11	0.80	0.80	0.76	0.80
	+	+	6.22	6.36	6.13	5.85	6.38
10	−	−	0.69	0.68	0.92	0.92	0.84
	−	+	3.89	1.03	1.10	0.97	0.84
	+	−	0.64	2.11	1.76	1.47	1.20
	+	+	6.75	8.34	8.10	6.02	6.90
20	−	−	0.67	0.67	0.90	0.70	0.63
	−	+	1.08	1.02	0.98	0.67	0.61
	+	−	0.79	1.06	2.10	1.19	1.26
	+	+	8.05	7.18	7.74	5.93	3.08

[a] The venom phospholipase was added to 0.25 M sucrose solution with and without bovine serum albumin (BSA) to which was added an equal quantity of mitochondrial suspension at 1°. The final concentration of BSA was 2% and of mitochondria 2.65 mg of protein/ml. About 1–5 min elapsed before the initial ATPase activity of the ice-cold mitochondria was assayed in the presence and absence of DNP in the reaction mixture. The mitochondrial suspensions were then aged for 10 and 20 min at 23° with the assay of the ATPase activity as given.

aging medium (Figure 12). The data in Table V show that 1 mM Ca^{2+} in the aging medium did not activate the production of fatty acids. Only when the Ca^{2+} level was increased to 10 mM did it weakly activate (18%) the mitochondrial phospholipase activity. The mitochondrial phospholipase is therefore probably nearly fully activated by the endogenous Ca^{2+}. By contrast, Ca^{2+} was a powerful activator of the U-factor formation in sonicated mitochondria (Wojiczak and Lehninger, 1961).

Effect of Phospholipase on ATPase Activity. The data in Table VI show the effect of phospholipase on the latent ATPase and on the stimulation of the latent ATPase by DNP. Pretreatment with increasing amounts of phospholipase caused (1) a progressive stimulation of the latent ATPase which never exceeded twice that of the controls; (2) a reduction of stimulation of the latent ATPase by DNP. Thus, whereas in the absence of phospholipase the latent ATPase was stimulated by DNP about eightfold, a short-term preincubation at 1° with 50 μg/ml of phospholipase essentially abolished the stimulation by DNP. As expected, a pretreatment at 23° for 10 min or longer decreased the concentration of phospholipase necessary to abolish the stimulation of the latent ATPase by DNP. Virtually no stimulation by DNP was now evident at 12.5 μg/ml.

The interference by phospholipase with the stimulation of the latent ATPase by DNP was reversed by albumin under all conditions of pretreatment except the most drastic (100 μg/ml for 20 min). This suggests that the decay of the DNP-stimulated ATPase activity does not result from the hydrolysis of phospholipids as much as from the resulting accumulation of fatty acids. It would therefore seem that hydrolysis of mitochondrial phospholipids and a high DNP-stimulated ATPase activity are not mutually exclusive provided the resulting fatty acids are "trapped."

It is also significant that albumin did not reduce the stimulation of the latent ATPase by phospholipase (Table VI). In fact, the data indicate a potentiation of this activity in the presence of albumin, suggesting that an inactivation of the DNP-stimulated ATPase is not necessarily a consequence of an active endogenous ATPase.

Discussion

The results of the experiments reported in this study strongly suggest that the loss of the DNP-stimulated ATPase activity and respiratory control in aged mitochondria is associated primarily with the

accumulation of free long-chain fatty acids. This conclusion rests upon several lines of evidence. Fatty acids were readily recovered from mitochondria aged in sucrose. However, when they were aged in a solution of sucrose–albumin, fatty acids were recovered mainly from albumin, suggesting a transfer of fatty acids from the mitochondria to albumin. Under these conditions of aging there was no impairment of respiratory control or the DNP-induced ATPase activity. The endogenously produced fatty acids inhibited the stimulation of the ATPase activity by DNP in fresh mitochondria and this inhibition was counteracted by albumin thus duplicating the events that transpire during aging. In the accompanying paper (Chefurka, 1966), it is demonstrated that this inhibition by the endogenously produced fatty acids is duplicated by a variety of exogenously added saturated and unsaturated long-chain fatty acids (Chefurka, 1966). Finally, the reactivation of the DNP-induced ATPase activity of aged (Figure 10) and phospholipase-treated mitochondria by albumin (Table VI) without any effect on the loss of phospholipids (Table IV) eliminates the possibility that loss of phospholipids might be the underlying cause of the inhibition of the DNP-stimulated ATPase in aged mitochondria. Indeed the possibility that uncoupling in phospholipase-treated mitochondria might be due mainly to fatty acids was first raised by the experiments of Ziegler *et al.* (1965) who demonstrated reversal of uncoupling by albumin.

The present study suggests a wide species and tissue variation in the stability of the DNP-induced ATPase activity. Weinbach (1959) has reported a species variation in the stability of oxidative phosphorylation and Kielley (1952) reported that oxidative phosphorylation was more sensitive to aging at 0° in mitochondria from mouse hepatomes 98/15 than from mouse liver. In at least the mouse liver and housefly thoracic muscle mitochondria this instability is positively correlated with the intensity of their fatty acid releasing capacity (Chefurka, 1963b). It is also possible that the strikingly lower rate of inactivation of the ATP–P_i exchange reaction, the DNP-stimulated ATPase and of oxidative phosphorylation in aged *submitochondrial fragments* (Cooper and Lehninger, 1957) is related to the decreased capacity of these particles to accumulate fatty acids because most of the requisite enzymic activity for phospholipid hydrolysis appears to be localized in the soluble mitochondrial matrix; the membrane providing the substrate (Lehninger and Remmert, 1959). Hence these variations in stability may reflect species differences in the activity of phospholipase, and the failure to inhibit the accumulation of fatty acids may therefore serve as one major common denominator for many reports in the literature relating decreased efficiency of oxidative phosphorylation and related reactions to mitochondrial aging.

It has been suggested that the accumulation of fatty acids in aged mitochondria arises from hydrolysis of mitochondrial phospholipids (Lehninger and Remmert, 1959). Rossi *et al.* (1964) have demonstrated a breakdown of phospholipids, particularly phosphatidylcholine in aged mitochondria, but were unable to relate this decrease to a stoichiometric increase of fatty acids. The present study shows that the increase in fatty acids in aged mitochondria totally accounts for the corresponding decrease in phospholipids. Furthermore, this 1:1 molar stoichiometry between the loss of phospholipids and yield of free fatty acids strongly suggests a phospholipase A activity, which has been recently reported in mitochondria (Scherphof and van Deenan, 1965; Rossi *et al.*, 1965). However, Scherphof and van Deenan have suggested that the breakdown of phospholipids observed by Rossi *et al.* (1964), in rat liver mitochondria which were aged for 24 hr at 22°, might be due more to a drastic lowering of the pH (7.0–4.7) than phospholipase activity. No shift in pH was ever noted under our conditions of aging. Thus in one experiment the initial pH remained at 7.15 after aging for 0.5 hr at 23° and in another, the initial pH of 6.60 increased to 6.75 after aging for 2 hr at 23°.

As mentioned earlier, it is well established that during aging, mitochondria may swell and lose many components, particularly nucleotides and magnesium, and also suffer a loss in phosphorylation efficiency. Though it has been generally assumed that phosphorylation is dependent on the absence of swelling and on a normal intramitochondrial complement of cofactors, no means have been available to date to test the extent of dependency between these phenomena. The present study suggests an approach which permits a direct evaluation of the degree of dependency between the various lesions produced during aging and the loss of function in aged mitochondria. The data indicate that under circumstances that permitted a high level of DNP-stimulated ATPase activity, *i.e.*, in the presence of albumin and EDTA, there was no corresponding attenuation of the loss of several mitochondrial components. Indeed, in the presence of albumin, the diffusion of these components into the surrounding medium was accelerated without serious impairment of the stimulation of the ATPase activity by DNP. The use of mouse liver mitochondria which, by contrast to rat liver mitochondria, are very refractive to spontaneous swelling, also permits an evaluation of the importance of swelling in the loss of this enzyme activity during aging. Although it is well known that fatty acids promote alterations in mitochondrial structure (Lehninger, 1962; Utsumi *et al.*, 1962), our data suggest that the formation of fatty acids is not necessarily an essential prerequisite to swelling. Furthermore, fatty acids may inhibit mitochondrial reactions without induction of swelling.

The use of albumin as a protective agent also permits an evaluation of the relative importance of the accumulation of fatty acids and the loss of phospholipids in the decay of the DNP-stimulated ATPase activity during aging. It has been generally maintained that phospholipids are essential components for such mitochondrial functions as active transport across membranes, electron transport, oxidative phosphorylation, and presumably also the partial reactions (Fleischer

et al., 1962; van Deenan, 1965; Green and Fleischer, 1963). The fact that albumin supports an active DNP-stimulated ATPase activity of phospholipase-treated and aged mitochondria without inhibiting the breakdown of phospholipids argues against a casual relationship between loss of phospholipids and loss of ATPase activity in aged mitochondria. It is reasonable to infer therefore that the resulting alteration in the composition of the membrane phospholipids was not critical to the activity of the DNP-stimulated ATPase. This also raises the question as to whether an inhibition by phospholipase constitutes sufficient evidence for the involvement of phospholipids. It would thus seem appropriate to reinvestigate many of the previously reported inhibitory effects by phospholipase (Braganca and Quastel, 1953; Nygaard and Sumner, 1953; Aravindakshan and Braganca, 1961a,b; Tookey and Balls, 1956) in the presence of albumin.

The present study does suggest, however, that during prolonged aging at room or higher temperatures (Figures 4 and 11), other important secondary lesions not reversed by albumin may effect the DNP-stimulated ATPase activity (Figure 10). Excessive alteration and loss of the phospholipids pattern as well as other yet unknown changes may therefore explain the inability of albumin to restore the inactivated DNP-stimulated ATPase of beef heart mitochondria (Brierley *et al.*, 1964) and the lowered P:O ratio of rat liver mitochondria (Petrushka *et al.*, 1959; Helinski and Cooper, 1960; Rossi *et al.*, 1964) under rather severe conditions of aging. On the other hand, the low P:O ratio of beef heart and rat liver mitochondria and submitochondrial particles (Lester and Hatefi, 1958; Lehninger and Remmert, 1959; Weinbach, 1959; Ziegler *et al.*, 1965), and low ATP–P_i exchange reaction of submitochondrial particles (Lehninger and Remmert, 1959) resulting from mild aging or phospholipase treatment, were substantially restored to their normal levels by albumin. Clearly, reversal of activity by albumin is a sufficient but not a necessary diagnostic test for involvement of fatty acids in impairment of mitochondrial function (Chappell and Greville, 1963; Rossi *et al.*, 1964; Brierley *et al.*, 1964).

The importance of EDTA in maintaining the integrity of the oxidative and phosphorylative capacities of mitochondria emerges from numerous previous investigations (Slater and Cleland, 1952, 1953; Chappell and Perry, 1953; Zielger *et al.*, 1956; Lester and Hatefi, 1958). Slater and Cleland (1953) ascribed the beneficial effects of EDTA to its stabilization of structure resulting from the chelation of Ca^{2+}. The results of our studies suggest that the protective effect by EDTA on the DNP-stimualted ATPase activity in aged mitochondria is unrelated to structural changes. On the other hand, the association between inhibition of the release of fatty acids and preservation of the DNP-induced ATPase by EDTA is too impressive to ignore. The occurrence of phospholipase A in mitochondria (Table IV, also Scherphof and van Deenan, 1965; Rossi *et al.*, 1965) and of Ca^{2+} (Griswold and Pace, 1956; Slater and Cleland, 1953), and the requirement of Ca^{2+} for phospholipase A activity (Kates, 1960) provide a reasonable basis for the possibility that EDTA stabilizes mitochondrial activity *in vitro* primarily by inhibition of phospholipase activity as a result of chelation of Ca^{2+}.

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