Oxidative Phosphorylation in *in Vitro* Aged Mitochondria. II. Dinitrophenol-Stimulated Adenosine Triphosphatase Activity and Fatty Acid Content of Mouse Liver Mitochondria*

W. Chefurka and T. Dumas

With the technical assistance of S. T. Bajura

ABSTRACT: Aging mitochondria are characterized by an accumulation of free fatty acids. The major components are palmitic, stearic, oleic, linoleic, and arachidonic acids. A kinetic analysis of the release of these fatty acids shows that the greatest rate of increase occurred in the case of palmitic, stearic, oleic, and linoleic acids. Both the saturated (C_{10}–C_{18}) and unsaturated (C_{16}–C_{22}) fatty acids prevented the stimulation of the latent adenosine triphosphatase (ATPase) by dinitrophenol (DNP). No effect was obtained with the C_{22}-saturated acid. The C_{16}-unsaturated and -saturated acids were equally effective. By contrast, the C_{18}- and longer unsaturated acids were more effective than their corresponding saturated acids but as effective as the C_{16} acids. The C_{18} *cis* isomer was more effective than the *trans*. These findings may be related to the "goodness of fit" with the receptor. The data suggest that only palmitic, oleic, linoleic, and stearic acids contribute significantly to the decay of the DNP-stimulated ATPase of aging mitochondria. This may stem from an effect of the fatty acids on the reaction involving the X~P intermediate.

Previous studies from this laboratory have demonstrated that two mitochondrial reactions, *viz.*, the stimulation of the latent ATPase by dinitrophenol (DNP) and the stimulation of respiration by ADP[1] are lost during *in vitro* aging of mouse liver mitochondria (Chefurka, 1961, 1963a,b, 1966). This loss of activity was correlated primarily with one major event transpiring in aging mitochondria, namely the accumulation of free fatty acids (Chefurka, 1963a,b, 1966). The accumulation occurs at the expense of mitochondrial phospholipids and is mediated by a mitochondrial phospholipase (Chefurka, 1966).

Presumably this mechanism also serves to explain the occurrence of uncoupling material, termed U factor, (Lehninger and Remmert, 1959; Wojtczak and Lehninger, 1961) which contains free long-chain fatty acids and which decreases the P/O ratio and inhibits partial reactions of fresh mitochondria (Pullman and Racker, 1956; Polis and Shmukler, 1957; Hülsmann *et al.*, 1960; Wojtczak and Wojtczak, 1960). The present study provides additional information on the relationship between specific fatty acids and the loss of the DNP-induced ATPase during aging by considering: (1) the identity of the free fatty acids of aged mitochondria; (2) the kinetics of free fatty acid production during aging; (3) evaluation of the inhibitory capacity of various free fatty acids.

Experimental Method

Preparation of Mitochondria. The preparation of mitochondria from mouse liver has been described in the accompanying paper (Chefurka, 1966).

Inhibition of ATPase Activity by Fatty Acids. The inhibition of the DNP-stimulated ATPase activity by fatty acids was tested under experimental conditions simulating the relationship between the endogenously produced fatty acids and the ATPase in the aging experiments. Hence the fatty acids were added to the mitochondrial suspension rather than to the incubation reaction, although as will be reported later both procedures yielded comparable results. The free acids and sodium oleate were added in 50 μl of ethanol to 1 ml of 0.25 M sucrose solution. Controls contained 50 μl of ethanol. Where potassium salts were used, these were dissolved directly in the sucrose solution. These preparations were then mixed with an equal volume of freshly prepared mitochondria and after a gentle swirl, the DNP-induced ATPase was measured immediately. The ATPase assay system has already been described (Chefurka, 1963a, 1966). All acids used were rated by the manufacturer as 98+% or chromatographically pure. This was confirmed by gas chromatography of the corresponding methyl esters.

Extraction, Esterification, and Analysis of Free Fatty Acids. The free fatty acids were extracted from mitochondria aged at 37° as already described (Chefurka,

* From the Research Institute, Research Branch, Canada Department of Agriculture, University Sub Post Office, London, Ontario, Canada. Contribution No. 331. *Received July 26, 1966*

[1] Abbreviations used: ATP and ADP, adenosine tri- and diphosphates; NAD^+, oxidized nicotinamide-adenine dinucleotide.

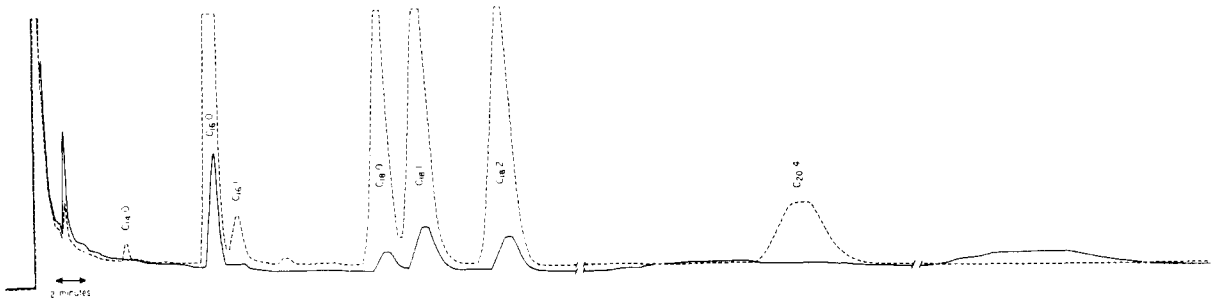

FIGURE 1: Gas–liquid partition chromatography of methylated fatty acids isolated from fresh and aged mitochondria ———, fresh mitochondria containing 154.0 mg of protein. ---------, mitochondria containing 150.0 mg of protein aged for 2 hr at 37°.

1966). The extraction method was that of Dole and Meinertz (1960). The acidified heptane–isopropyl alcohol extract was reduced to dryness *in vacuo*. The residue was dissolved in redistilled ether containing 10% methanol and methylated with diazomethane according to Schlenk and Gellerman (1960). Peroxide-free carbitol was prepared according to Eastoe (1966). The diazomethane was generated from *N,N'*-dinitroso-*N,N'*-dimethyl terepthalimide. After completion of methylation (about 12 min) the excess diazomethane was driven off by a stream of nitrogen and the esters were reduced to dryness and redissolved in the requisite amount of petroleum ether (bp 80–100°). The total fatty acids were obtained by a saponification of the mitochondria by the method of Holman and Widmer (1960).

The methyl esters were analyzed by gas–liquid partition chromatography using the Burrell Kromo-Tog Model K-7 with a flame detector. The 8-ft coiled, stainless steel column was packed with Resoflex LAC 446(P) supplied by Burrell Co. The injection block temperature was 225°, the column temperature was 190°. The carrier gas was helium at a flow rate of 45 cc/min. The sample volume was 1–2 μl of the dissolved sample. Full-scale peaks ranged from 0.7 (for myristic acid) to 7 μg (for arachidonic acid). However, the sensitivity of the instrument could be increased to give full-scale deflection for about 0.1 μg of myristic acid.

The column was calibrated with known standards of highly purified methyl esters of fatty acids. The qualitative identification of the esters in the extracts was performed by comparison of retention times relative to methyl stearate. The quantitation was performed from calibration curves relating peak height times width at one-half the peak height of the purified reference standards. All fatty acid values are expressed as millimicromoles per milligram of mitochondrial protein.

Chemicals. Capric, lauric, myristic, palmitic, stearic, linoleic, linolenic, and arachidonic acids were obtained from Nutritional Biochemicals Corp.; palmitoleic, elaidic, erucic, and arachidic acids were obtained from Mann Research Laboratories Inc.; and sodium oleate was purchased from Fisher Scientific Co. Gas–liquid partition chromatographic reference mixture of methyl esters of fatty acids were obtained from Mann Research Laboratories Inc., N. Y. All unsaturated fatty acids that were obtained in sealed vials were used up as rapidly as possible. When storage was necessary the vials were resealed and kept at 0 or −20°.

Isooctane (2,3,4-trimethylpentane) and heptane were purchased from Eastman Organic Chemicals Department. *N,N'*-dinitroso-*N,N'*-dimethyl terephthalimide was obtained from DuPont de Nemours, Wilmington, Del. All other chemicals were of reagent grade from Fisher Scientific Co., Ltd.

Chemical Determination. The analysis for orthophosphate and protein was performed according to Sumner (1944) and Lowry *et al.* (1951), respectively.

Results

Identification and Quantitation of Fatty Acids. A gas–liquid partition chromatographic analysis of the free fatty acids in the lipid extracts of fresh and aged mitochondria is presented in Figure 1. The heptane–isopropyl alcohol extracts from fresh and aged mitochondria contain saturated and unsaturated fatty acids. The major components were palmitic, stearic, oleic, linoleic, and arachidonic acids. The minor readily identified components were myristic and palmitoleic acids. The unidentified acids had retention times relative to methyl stearate as follows: 0.025, 0.080, 0.72, 1.90, and 2.8. A number of unidentified peaks were also detected in trace amounts only in very concentrated extracts.

The data in Table I and Figure 2 provide information on the changes in the fatty acid composition during aging. The average total free fatty acid level in fresh mitochondria was 9.1 mμmoles/mg of protein with a range of 4.2–10.2 mμmoles/mg of protein for three different preparations. This is considerably less than the endogenous value of 57–66 mμmoles/mg of protein obtained by titration (Chefurka, 1966). Clearly, the titration method of Dole and Meinertz (1960) was assaying acidic components other than fatty acids. A similar observation was made by Trout *et al.* (1960) who concluded that these additional com-

TABLE I: Free Fatty Acid Content of Fresh and Aged Mitochondria.

Fatty Acid	Time of Aging (minutes)								After Total Sapon	
	0		30		60		120			
Myristic	0.09[a]	1.0[b]	0.04[a]	0.16[b]	0.05[a]	0.11[b]	0.10[a]	0.17[b]	0.6[a]	0.2[b]
Palmitic	2.11	23.1	8.42	34.7	17.07	38.7	20.50	34.4	101.2	33.3
Palmitoleic	0.28	3.1	0.13	0.54	0.52	1.2	0.67	1.1	6.7	2.2
Stearic	0.94	10.3	3.60	14.8	7.42	16.8	10.58	17.7	38.3	12.6
Oleic	2.50	29.4	4.97	20.5	8.63	19.6	10.72	18.0	34.3	11.2
Linoleic	2.69	29.5	5.80	23.9	7.69	17.4	11.93	20.1	78.3	25.7
Arachidonic	0.52	5.7	1.40	5.8	2.85	6.5	4.76	8.0	45.3	14.9
Total	9.13		24.36		44.23		59.26		304.7	
Double-bond index		1.17	0.92		0.81		0.92		1.25	

[a] Millimicromoles per milligram of protein. [b] Mole per cent.

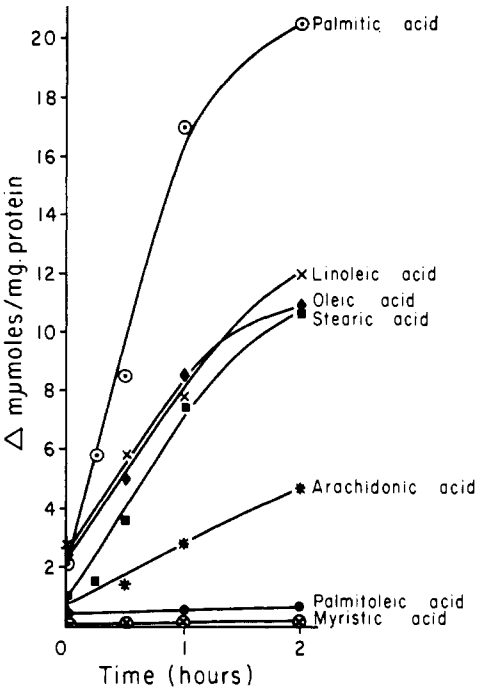

FIGURE 2: Time-course analysis of free fatty acis in aging mitochondria.

ponents were phospholipids. However, when the endogenous levels of fresh mitochondria were subtracted from those of aged miochondria, remarkably good agreement between both assay methods was obtained. This is seen by comparing the data in Table I of this study with those of Table IV in the preceding paper (Chefurka, 1966).

As seen from the data in Table I, aging results in an accumulation of free fatty acids. After 30 min at 37° the level of free fatty acids increased by 165% and after 2 hr by about 550%. This represents about 5 and 17% of the total content of mitochondrial fatty acids.

The level of each fatty acid increased linearly with time as seen in Figure 2 and Table I. The saturated palmitic and stearic acids accumulated most rapidly. They increased about fourfold in 30 min. The unsaturated oleic and linoleic acids increased about twofold while arachidonic acid increased about two- to threefold within 30 min. However the absolute amount of arachidonic acid was less than one-half that of stearic and less than a one-sixth that of palmitic acid. No increase was detected for myristic acid while palmitoleic acid increased only after a 30-min lag. The per cent of the total quantity that was released at any particular time of aging was dependent on the nature of the fatty acid. Thus only 10% of the total palmitoleic and arachidonic acids were released while about 30% of the total oleic acid was set free after 2 hr. These differential rates of release may be explained by: (1) variation in the accessability of the various phospholipids to phospholipase perhaps due to their specific localization patterns within the membrane; (2) differential suscepti-

TABLE II: Major Classes of Mitochondrial Fatty Acids.[a]

	Time of Aging (minutes)				
	0	30	60	120	After Sapon
Saturated	34.4	4.97	55.6	52.3	46.1
Monounsaturated	60.0	44.5	37.9	39.7	39.0
Polyunsaturated	5.6	5.8	6.5	8.0	14.9

[a] All values given as mole per cent and calculated from Table I.

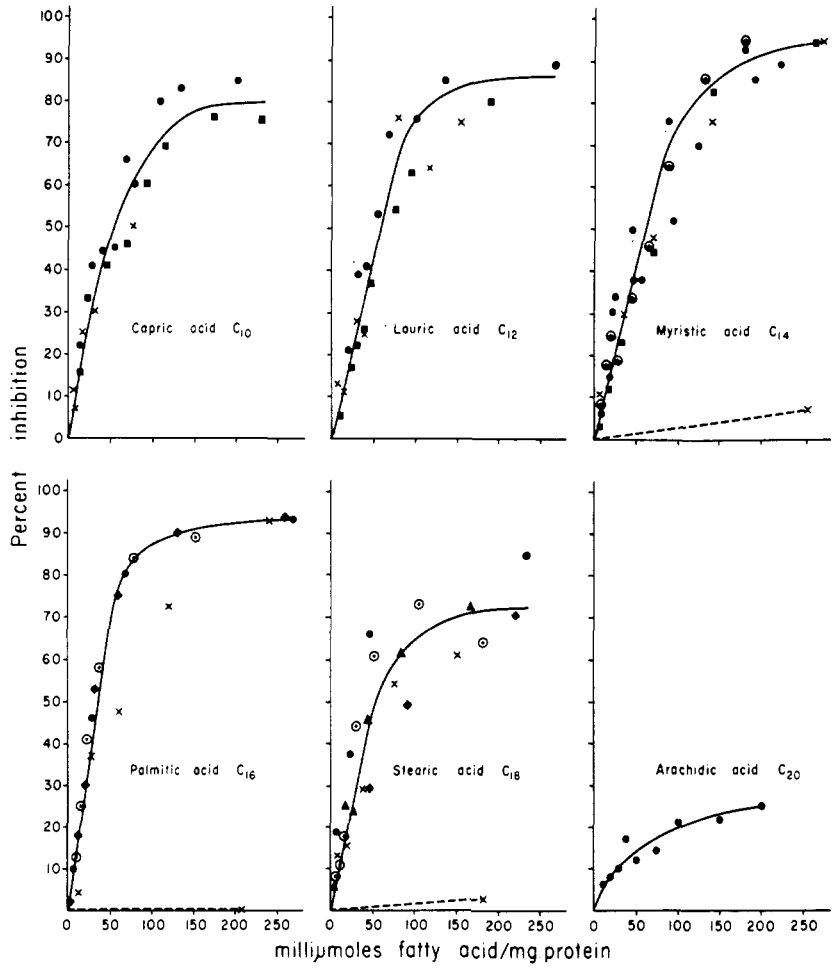

FIGURE 3: The inhibition of the DNP-stimulated ATPase activity by various concentrations of saturated fatty acids. Each set of points about the solid line represents a separate experiment with various amounts of fatty acids added to the mitochondrial suspension. The broken line represents the inhibition by fatty acids added to a mitochondrial suspension containing 2% albumin. All values were corrected for the endogenous ATPase.

bility of the ester linkages involving the fatty acyl group.

The data in Table II show that about 66% of the free fatty acids in fresh mitochondria were unsaturated. As aging progressed the degree of saturation increased due chiefly to the preferential accumulation of palmitic acid. Another way of expressing this unsaturated character of the free fatty acids is by the double-bond index defined as the sum of the product of the mole fraction of each acid and its number of double bonds. As can be seen in Table I, the double-bond index of the free fatty acids in the fresh mitochondria was 1.12. Upon aging it dropped to about 0.8–0.9 suggesting a shift to saturation. The double-bond index of the major fatty acids recovered after saponification of the mitochondria was 1.25. This is somewhat lower than 1.48 for rat liver mitochondria (Richardson *et al.*, 1961).

The data presented in the accompanying paper suggest that the failure of the ATPase to respond to DNP occurs within about 30 min of aging (Chefurka, 1966). The data in Table I show that during this time the free fatty acids increased by about 15 mμmoles/mg of protein. This represents about 5% of the total fatty acid content of the mitochondria and as already stated, palmitic, stearic, oleic, and linoleic acids comprise the bulk of this increase. The question arises whether this level of fatty acids could in fact inhibit the response of the ATPase to DNP. In order to answer this question and the one concerning the specificity of fatty acids, their inhibitory capacity was tested *in vitro*.

The Inhibition by Fatty Acids. Preliminary studies showed that the inhibition of the DNP-induced ATPase activity by palmitic acid was variable. Further investigation of this variability revealed that, as with swelling induced by oleate (Chefurka, 1966) the level of inhibition by palmitic acid was a function of the ratio of fatty acid to mitochondrial protein rather than of the concentration of fatty acid *per se*. Thus an inhibition of 80% or more of the ATPase activity by 100 μg of

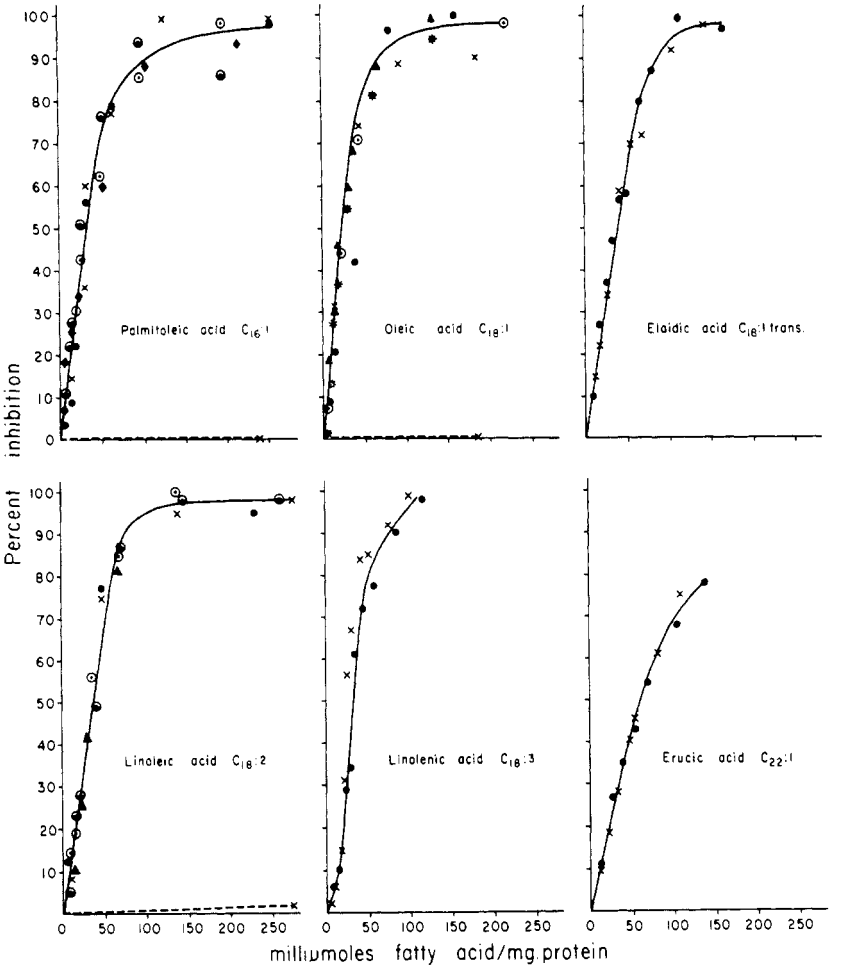

FIGURE 4: The inhibition of the DNP-stimulated ATPase activity by various concentrations of unsaturated fatty acids. As in Figure 3 each set of points about the solid line represents a separate experiment with various amounts of fatty acid added to the mitochondrial suspension except for: ◆, palmitoleic acid was added to the reaction mixture; ⊖, potassium palmitoleate was added to the mitochondrial suspension; ⊙, potassium linoleate was added to the mitochondrial suspension; *, sodium oleate was added to mitochondrial suspension containing 5 mM EDTA. The broken lines represent the inhibition by fatty acids added to a mitochondrial suspension containing 2% albumin. All values were corrected for the endogenous ATPase.

palmitic was possible with dilute mitochondrial suspension, *i.e.*, 1.5 mg of mitochondrial protein/sample or less; at 4 mg of protein/sample, inhibitions of the order of 35% were obtained. This suggests that palmitic acid combines in a stoichiometric manner with a specific receptor(s) on the mitochondria. Witter *et al.* (1957) reported a similar conclusion for the action of lysolecithin on oxidative phosphorylation and ATPase as did Edwards and Ball (1954) for the action of oleic acid on the succinoxidase system. All experiments reported in this section were performed on dilute mitochondrial suspensions.

Figure 3 illustrates the effect of members of a homologous series of straight-chain saturated fatty acids ranging from C_{10} to C_{18}. The data show that for a fatty acid of a particular chain length, similar patterns of inhibition were obtained with different mitochondrial preparations. As the chain length increased above C_{16} the maximum inhibitory levels decreased. Thus whereas myristic or palmitic acids produced maximum inhibition of about 80–90%, that by stearic acid was 70% and that by arachidic acid about 25%. Behenic acid (C_{22}) was not inhibitory at concentrations up to 200 mμmoles/mg of protein. The maximum inhibition is probably related to the solubility characteristics of the long-chain saturated fatty acids.

Figure 4 illustrates the effect of members of a homologous series of straight-chain unsaturated fatty acids ranging from C_{16} to C_{22}. By contrast with the saturated fatty acids, the C_{16} and all the members of the C_{18} group readily produced almost complete inhibition of the DNP-stimulated ATPase.

A comparison of the inhibition of the DNP-stimulated ATPase activity by saturated and unsaturated fatty acids of varying chain length is given in Figure 5 and Table III. The data in Figure 5 relate the inhibition

TABLE III: Inhibition Effect of Fatty Acids.

Fatty Acid	Short-Hand Designation[a]	Concn for 50% Inhibn (mμ-moles/mg of protein)	"Inhibn Factor" $\times 10^{-3}$ (t = 30 min)[b]
Lauric acid	C_{10}=0	58	
Capric acid	C_{12}=0	53	
Myristic acid	C_{14}=0	60	0.67
Palmitic acid	C_{16}=0	37	170
Stearic acid	C_{18}=0	53	50
Palmitoleic acid	C_{16}=1	35	7.4
Oleic acid	C_{18}=1 *cis*	25	99
Elaidic acid	C_{18}=1 *trans*	38	
Linoleic acid	C_{18}=2	37	87
Linolenic acid	C_{18}=3	33	
Erucic acid	C_{22}=1 *cis*	63	

[a] The first number indicates the chain length, the second indicates the number of double bonds. [b] Inhibition factor = K(fatty acid concentration)/(fatty acid for 50% inhibition).

produced by 50 mμmoles of fatty acid/mg of protein, a value chosen arbitrarily from the linear portion of the inhibition curves of Figures 3 and 4. Table III relates the concentration of fatty acids, obtained from Figures 3 and 4, that were necessary to inhibit the DNP-stimulated ATPase by 50%. Among the saturated fatty acids, palmitic produced the greatest inhibition at the 50-mμmole level and least of it was required for a 50% inhibition. As the chain length of the saturated fatty acids increased, the inhibition at the 50-mμmole level decreased. Conversely more was necessary for a 50% inhibition. No inhibition by behenic acid (C_{22}=O) was observed even at about 200 mμmoles/mg of protein. By contrast, maximum effects were obtained with C_{13} or C_{14} in studies of mitochondrial swelling (Avi-Dor, 1960), latent ATPase activity (Pressman and Lardy, 1956), and ATP–P_i exchange (Falcone and Mao, 1965).

A similar pattern of inhibition at the 50-mμmole level in relation to chain length was noted for the unsaturated fatty acids but with the maximum inhibition shifting to C_{18} (Figure 5). Thus oleic acid (C_{18}=1 *cis*) produced a greater inhibition than either palmitoleic acid (C_{16}=1) or erucic acid (C_{22}=1). However the inhibition by the latter was still substantial in comparison with its saturated analog. This inhibition of the ATPase activity by erucic acid is in contrast to its relative inability to induce

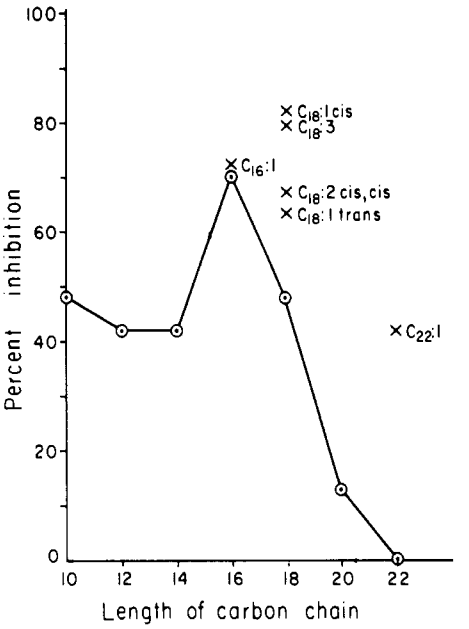

FIGURE 5: The effect of chain length on the inhibition of the DNP-stimulated ATPase by 50 mμmoles of fatty acid/mg of mitochondrial protein. ⊙, saturated fatty acids. X, unsaturated fatty acids.

mitochondrial swelling (Zborowski and Wojtczak, 1963).

Some variation in the inhibition of the DNP-stimulated ATPase activity is also evident among the various unsaturated members of the C_{18} chain length. Thus linoleic acid (C_{18}=2) seems to be somewhat less effective than either linolenic (C_{18}=3) or oleic acid (C_{18}=1 *cis*). The *trans* isomer of oleic acid, namely, elaidic acid (C_{18}=1 *trans*), was less inhibitory than oleic acid. However, this difference between the *cis* and *trans* in relation to inhibition of the DNP-induced ATPase was not as striking as it was in relation to their ability to activate the latent ATPase activity (Pressman and Lardy, 1956) or promote swelling (Zborowski and Wojtczak, 1963).

A comparison of the saturated and unsaturated fatty acids of the same chain length shows that for the long-chain fatty acids, the unsaturated were more inhibitory than the saturated. Medium chain-length acids such as palmitic and palmitoleic acids were equally effective inhibitors. Indeed, although oleic acid was somewhat of a more potent inhibitor than palmitic, the disparity between these two fatty acids under our conditions was not as pronounced as that reported by Borst *et al.* (1962).

The data in Figures 3 and 4 also clearly show that in the presence of 2% albumin the inhibition by fatty acids was either completely abolished or else reduced to a very low level. Finally the potassium salts of palmitoleic and linoleic acids (Figure 4) produced the same degree of inhibition as did the free acids and addition of palmitoleic acid (Figure 4) to the reac-

tion medium resulted in the same level of inhibition as when added directly to the mitochondria. No inhibition was observed by methyl palmitate suggesting that the carboxyl group was essential for inhibition.

Discussion

It is apparent that if a fatty acid generated *in situ* during aging is to be implicated in the loss of DNP-stimulated ATPase activity and perhaps other partial reactions during aging, it must fulfil at least two criteria. (1) It must accumulate in requisite amounts. (2) It must inhibit the enzyme system. According to these criteria, palmitic, stearic, oleic, and linoleic acids are probably the main inhibitors of the DNP-stimulated ATPase in aging mitochondria. Palmitoleic and myristic probably contribute insignificantly because insufficient amounts accumulate.

The relative contributions of those fatty acids to the inhibition of the ATPase activity could be evaluated more quantitatively by examining the "inhibition factor" for each fatty acid, which is presented in Table III. Because the rate of release of the free fatty acids is linear from $t = 0$ to $t = 60$, then the relative ratio among the inhibition factors will remain constant during this interval. These data suggest that in any mixture of fatty acids released under the conditions of these experiments, palmitic acid will be two to three times as effective as the other three major fatty acids in inhibiting the response of ATPase to DNP. However the inhibition observed *in situ* will undoubtedly result from the occurrence of the four major components. It is also recognized that these values provide only an approximation to the more complex situation that probably prevails at the receptor site. More information is necessary on the relative binding affinities of the receptor for these fatty acids and on the nature of the receptor site.

Information on the nature and localization of the receptor site would also contribute to a better understanding of the disparity between the levels of endogenous and of exogenous fatty acids which are required to produce the same degree of inhibition. Data in the accompanying paper (Chefurka, 1966) suggest that the ATPase activity almost completely fails to respond to DNP after 30-min aging, while the respiratory control is abolished within 10–20-min aging at room temperature. The assay of such mitochondria reveals an increase of, at most, 15 mμmoles of fatty acid/mg of protein. *In vitro* studies (Figures 3 and 4) suggest, however, that complete inhibition of the ATPase reaction is brought about by about 75–100 mμmoles of fatty acid/mg of protein. This discrepancy could be explained by assuming that either the free fatty acids which are generated within the mitochondria may be more readily accessible to the site of action than are exogenously added fatty acids or that the actual level of fatty acids added *in vitro* was much less since the incubation was carried out without blocking electron transport (Falcone and Mao, 1965).

The present study suggests that the main parameter in the inhibition of the DNP-stimulated ATPase activity by fatty acids is the chain length. Thus C_{16} and $C_{16}{=}1$ were equally effective. As the chain length of the saturated acids increased, the inhibition decreased. By contrast, the effectiveness of the C_{18} unsaturated fatty acids did not deviate appreciably from that of the C_{16} acids. Furthermore the *cis* isomer was more effective than the *trans*. Presumably these results are related to the "goodness of fit" with the receptor. However, that other factors may also determine the inhibition of the unsaturated fatty acids is suggested by the anomalous position occupied by $C_{18}{=}2$.

The mechanism of interaction between the fatty acids and the ATPase is not clear. It is worth recalling however that, like DNP, fatty acids are uncouplers (Borst *et al.*, 1962; Pressman and Lardy, 1955; Scholefield, 1956) and inhibit the ATP–P_i-exchange activity (Ahmed and Scholefield, 1960; Falcone and Mao, 1965). They differ from DNP in that they maximally stimulate the latent ATPase only in the presence of magnesium (Borst *et al.*, 1962; Falcone and Mao, 1965) and, as this study shows, they *block* the stimulation of the latent ATPase by DNP. Our studies extend those of Borst *et al.* (1962) and of Bos and Emmelot (1962) who showed that oleic acid inhibits the DNP-induced ATPase reaction. At present there is no definite information on the effect of fatty acids on the ATP–ADP exchange but Wadkins and Lehninger (1958) reported that this exchange reaction of digitonin subfragments prepared from rat liver mitochondria is insensitive to aging and to the U factor (Lehninger and Remmert, 1959).

In the well-known phosphorylation scheme proposed by Chance, Lehninger, and Slater

$$X + I \underset{\text{transport}}{\overset{\text{electron}}{\longleftrightarrow}} X \sim I \qquad (1)$$

$$X \sim I + P_i \longleftrightarrow X \sim P + I \qquad (2)$$

$$X \sim P + ADP \longleftrightarrow X + ATP \qquad (3)$$

$$X \sim I + H_2O \xrightarrow{DNP} X + I \qquad (4)$$

the ATP–P_i-exchange reaction is represented by (2) and (3); the ATP–ADP-exchange by (3) while the DNP-stimulated ATPase reaction by a reversal of (3) and (2) followed by (4); the latter is generally assumed to be the site of action of DNP. If we assume that the ADP–ATP exchange of intact mitochondria is insensitive to aging as is that of digitonin particles then it would seem that the inhibition by fatty acids of the DNP-stimulated ATPase must lie between reactions 1 and 3, *i.e.*, at reaction 2. The precise mechanism of inhibition in molecular terms is yet not clear. They could inhibit the enzyme-catalyzing reaction 2, or make I unavailable by combining with it. This would be compatible with the suggestion that oleic acid acts on the substrate side of the DNP site of action (Vazquez-Colon *et al.*, 1966). Furthermore by combining with I, not only would reaction 2 be inhibited but the genera-

tion of X ~ I would be impaired (reaction 1) and so explain both its inhibition of the stimulation of latent ATPase by DNP and the inhibition of respiration (Lehninger, 1945; Vazquez-Colon *et al.*, 1966) which is not reversed by DNP (Vazquez-Colon *et al.*, 1966).

The work of Falcone and Mao (1965) which demonstrated potent inhibition of an exchange of oxygen atoms between the carboxyl group of long-chain fatty acids and H_2O strongly supports the contention that fatty acids act at the level of an intermediate of oxidative phosphorylation. The stimulation by albumin of the ATP–P_i exchange (Wojtczak and Wojtczak, 1960), of phosphorylation (Sacktor, 1954; Wojtczak and Wojtczak, 1960; Helinski and Cooper, 1960) and of the ATP "jump" (Vignais, 1963) also suggests the possibility of an interaction between endogenous fatty acids and high-energy intermediates. Furthermore, because bovine serum albumin replaced ATP in the reduction of NAD^+ by succinate, it is possible that the endogenous fatty acids may determine the redox state of intramitochondrial components such as NAD^+ and flavoproteins presumably by controlling the formation of the high-energy intermediates which facilitate the reduction of NAD^+ by succinate (Klingenberg, 1961). Finally, the requirement for high-energy intermediates by mitochondria for the accumulation of Ca^{2+} and the loss of this ability in aged mitochondria (Brierley *et al.*, 1964) suggests a more direct control by fatty acids of transport phenomenon than heretofore suspected.

Acknowledgment

We wish to acknowledge our gratitute to our colleagues Drs. E. Y. Spencer and B. E. Brown for assistance with the preparation of the manuscript and to Mr. N. L. Jerry for his photographic skills.

References

Ahmed, K., and Scholefield, P. G. (1960), *Nature 186*, 1046.

Avi-Dor, Y. (1960), *Biochim. Biophys. Acta 39*, 53.

Borst, P., Loos, J. A., Christ, E. J., and Slater, E. C. (1962), *Biochim. Biophys. Acta 62*, 509.

Bos, C. J., and Emmelot, P. (1962), *Biochim. Biophys. Acta 64*, 21.

Brierley, G., Murer, E., and Bachmann, E. (1964), *J. Biol. Chem. 239*, 2706.

Chefurka, W. (1961), *Can. J. Biochem. Physiol. 39*, 1941.

Chefurka, W. (1963a), *Can. J. Biochem. Physiol. 41*, 239.

Chefurka, W. (1963b), *Life Sci. 6*, 399.

Chefurka, W. (1966), *Biochemistry 5*, 3887 (this issue; preceding paper).

Dole, V. P., and Meinertz, H. (1960), *J. Biol. Chem. 235*, 2595.

Eastoe, J. E. (1966), *Chem. Ind. (London)*, 25.

Edwards, S. W., and Ball, E. G. (1954), *J. Biol. Chem. 209*, 619.

Falcone, A. B., and Mao, R. L. (1965), *Biochim. Biophys. Acta 105*, 233.

Helinski, D. R., and Cooper, C. (1960), *J. Biol. Chem. 235*, 3573.

Holman, R. T., and Widmer, C. (1960), *J. Biol. Chem. 234*, 2269.

Hülsmann, W. C., Elliott, W. B., and Slater, E. C. (1960), *Biochim. Biophys. Acta 39*, 267.

Klingenberg, M. (1961), *Proc. Ist Intern. Symp. Biol. Struct. Function, 2, Stockholm*, Sept 1960, p 227.

Lehninger, A. L. (1945), *J. Biol. Chem. 157*, 363.

Lehninger, A. L., and Remmert, L. F. (1959), *J. Biol. Chem. 234*, 2459.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Polis, B. D., and Shmukler, H. W. (1957), *J. Biol. Chem. 227*, 419.

Pressman, B. C., and Lardy, H. A. (1955), *Biochim. Biophys. Acta 18*, 482.

Pressman, B. C., and Lardy, H. A. (1956), *Biochim. Biophys. Acta 21*, 458.

Pullman, M. E., and Racker, E. (1956), *Science 123*, 1105.

Richardson, T., Tappel, A. L., and Gruger, E. H., Jr. (1961), *Arch. Biochim. Biophys. 94*, 1.

Sacktor, B. (1954), *J. Gen. Physiol. 37*, 343.

Schlenk, H., and Gellerman, J. L. (1960), *Anal. Chem. 32*, 1412.

Scholefield, P. G. (1956), *Can. J. Biochem. Physiol. 34*, 1227.

Sumner, J. B. (1944), *Science 100*, 413.

Trout, D. L., Estes, E. H., and Friedberg, S. J. (1960), *J. Lipid Res. 1*, 199.

Vazquez-Colon, L., Ziegler, F. D., and Elliott, W. B. (1966), *Biochemistry 5*, 1334.

Vignais, P. V. (1963), *Biochim. Biophys. Acta 78*, 404.

Wadkins, C. L., Lehninger, A. L. (1958), *J. Biol. Chem. 233*, 1589.

Witter, R. F., Morrison, A., and Shepardson, G. R. (1957), *Biochim. Biophys. Acta 26*, 120.

Wojtczak, L., and Lehninger, L. (1961), *Biochim. Biophys. Acta 51*, 442.

Wojtczak, L., and Wojtczak, A. B. (1960), *Biochim. Biophys. Acta 39*, 277.

Zborowski, J., and Wojtczak, L. (1963), *Biochim. Biophys. Acta 70*, 596.