On the Mechanism of Fatty Acid Inhibition of Mitochondrial Metabolism*

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ABSTRACT: Fatty acids, at low concentrations, uncouple oxidative phosphorylation but at higher concentrations inhibit the oxidation of β-hydroxybutyrate and succinate. 2,4-Dinitrophenol (DNP), which exhibits the same action pattern, is unable to release the inhibition caused by fatty acids. These inhibitions require the intact relationship between the energy producing and energy conserving units that makes coupling of oxidative phosphorylation possible. The inhibition is markedly enhanced by the presence of phosphate acceptor. The effect is not caused by accumulation of acetate or by changes in pH; neither are the dehydrogenases of the β-hydroxybutyrate and the succinate oxidizing systems affected. The β-hydroxybutyrate oxidizing system is more sensitive to complete inhibition by low levels of the fatty acids and DNP than the succinate oxidizing system. Both nitrophenols and fatty acids appear to act at the level of the intermediate ~X step.

During the course of the investigation of the effects of snake venom (*Bungarus fasciatus*) on mitochondrial function it was noticed that fresh venom, when incubated with rat liver mitochondria, caused the development of adenosine diphosphate (ADP) inhibition of substrate oxidation (Ziegler *et al.*, 1965). This activity was different from that of phospholipase A prepared from the same venom, which only provoked uncoupling of oxidative phosphorylation. Triglycerides did not protect the mitochondria from this activity; in fact, their presence tended to enhance the effect. Albumin effectively counteracted the influence of the venom treatment and eliminated ADP inhibition of respiration. This, along with the fact that the lipids from mitochrome also acted as uncouplers of oxidative phosphorylation, which could be reversed with albumin (Elliott *et al.*, 1959; Hülsmann *et al.*, 1960), prompted the investigation of the effect of different levels of fatty acids on mitochondria.

Materials and Methods

All reagents were obtained commercially and were of the highest purity available. Oleic acid samples were obtained as chromatographic standards from Sigma Chemical Co., Mann Research Laboratories, Inc., Nutritional Biochemicals Corp., and California Biochemical Corp. Methyl oleate, linoleic, and linolenic acids (98+% pure) were obtained from Sigma Chemical Co. and Mann Research Laboratories, Inc. Phenazine methosulfate (PMS) was obtained from California Biochemical Corp. Rotenone was a gift from Dr. R. Estabrook of the Johnson Foundation, Philadelphia. The source of all other reagents were described previously (Ziegler *et al.*, 1965). The fatty acids and their derivatives were kept under liquid nitrogen until used and were held in ice and flushed with nitrogen gas during the course of the experiments.

The rat liver mitochondria used in these experiments were prepared in a sucrose–mannitol medium as described before (Ziegler *et al.*, 1965). The beef heart muscle particles (HMP) were prepared by the method of Keilin and Hartree (1947) as modified by Chance (1952). The term aged mitochondria refers to rat liver mitochondria allowed to stand at 0–2° overnight. Preincubation of mitochondria with the different lipid reagents was accomplished in the following way: A known volume of mitochondrial suspension was added to a dry polyethylene tube kept in ice. A known amount of the reagent to be incubated with the mitochondria was delivered from the tip of a microsyringe needle to the tip of a needle on a tuberculin syringe. The reagent was pulled into the syringe followed by the sample of mitochondrial preparation. The mixture was homogenized by being repeatedly expelled and drawn into the syringe. At the end of a 3-min period of incubation with continuous homogenization, an aliquot free from air bubbles was injected into the respiration vessel. If the oleic acid froze in the needle of the syringe before mixing with the mitochondria, the needle was warmed with the hand until it melted. The temperature was kept at 0–5° at all times during incubation.

In those experiments in which 2,4-dinitrophenol (DNP) was used as inhibitor, the reagent was added directly into the reaction vessel after the mitochondria

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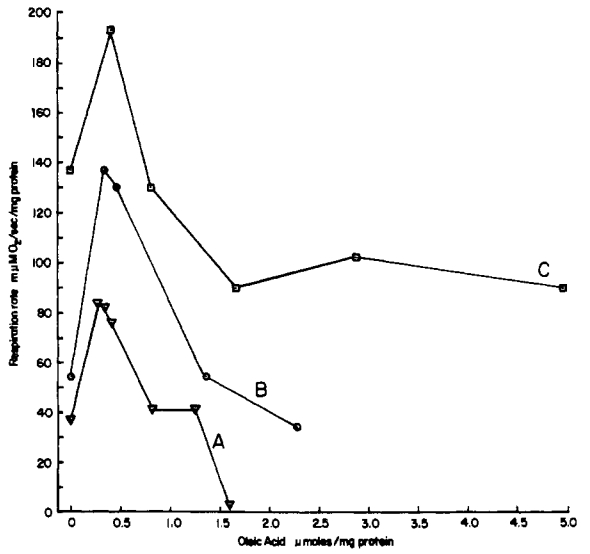

FIGURE 1: Inhibition of substrate oxidation by oleic acid in the absence of phosphate acceptor. Curve A—oxidation of β-hydroxybutyrate. Curve B—oxidation of succinate. Curve C—oxidation of succinate in mitochondria allowed to stand in ice for 4 hr.

were added. Measurement of respiration and phosphorylative activities was determined polarographically (Chance *et al.*, 1963) in a 3.2-ml sealed vessel (Strickland *et al.*, 1961) at 20°.

Results

The presence of small amounts of oleic acid accelerated the respiratory rates of mitochondria with both β-hydroxybutyrate or succinate as substrates in the absence of phosphate acceptor (Figure 1). This enhancement of respiration reached a maximum after which the fatty acid produced an inhibitory effect on the oxidation of substrate. Though the level of fatty acid necessary to reach a maximal oxidation was nearly the same for both substrates, the level necessary to inhibit substrate oxidation completely differed markedly for the substrates. While β-hydroxybutyrate oxidation was inhibited completely at oleic acid levels of about 1.7 μmoles of acid/mg of protein, succinate oxidation, in the absence of phosphate acceptor, was never inhibited completely.

Figure 1, curve C, shows the results of a representative experiment with mitochondria which had been stored for several hours at 0° that maintained good ADP–O ratios though their respiratory control decreased markedly. Though in these particles the rate of oxidation of succinate was higher than the corresponding rate in freshly prepared mitochondria, a similar effect of oleic acid was noticed.

Figure 2 shows the behavior of mitochondria upon addition of ADP at different levels of oleic acid. It should be noted that, at high concentrations of the acid, oxidation of succinate still went on at a relatively rapid rate, though upon addition of ADP respiration was completely inhibited.

That this inhibition of respiration by ADP is not an artifact of the electrode in the presence of high levels of ADP is shown in Figure 3. The response of the electrode is linear until anaerobiosis was reached.

Since the possibility existed that the effect of oleic acid was due to the presence of the carboxylic group, acetic acid was added in equimolar amounts in a con-

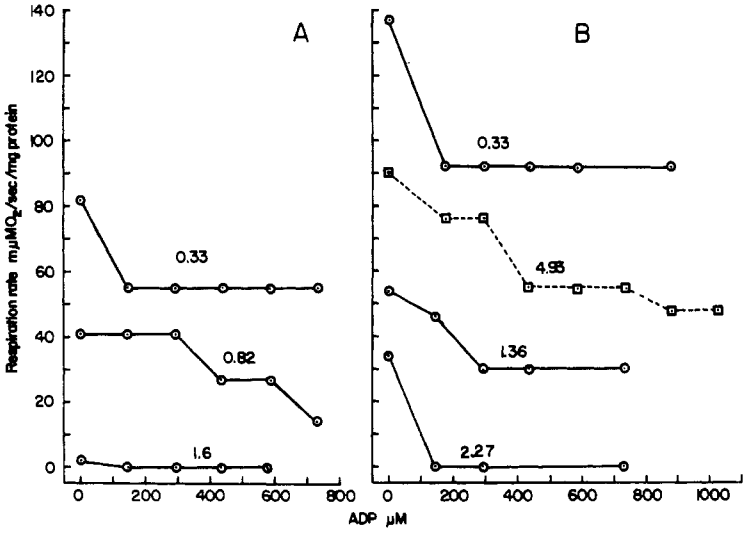

FIGURE 2: Effect of the addition of ADP to mitochondria treated with different concentrations of oleic acid, respiring on β-hydroxybutyrate (A) or succinate (B). The concentration of oleic acid, expressed as micromoles of oleic acid per milligram of protein, is shown above each curve. Curves correspond to experiments in Figure 1A and B except for the dashed curve corresponding to the experiment shown in Figure 1, curve C.

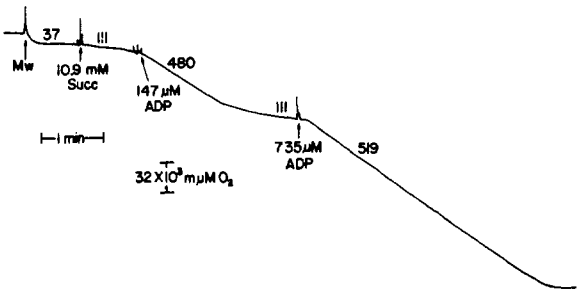

FIGURE 3: Response of the electrode to high concentrations of ADP in the absence of uncouplers or inhibitors. Numbers above the curve represent respiratory rates expressed as mμM O_2 per sec per milligram of protein. Numbers below curves represent concentrations of reagents as labeled.

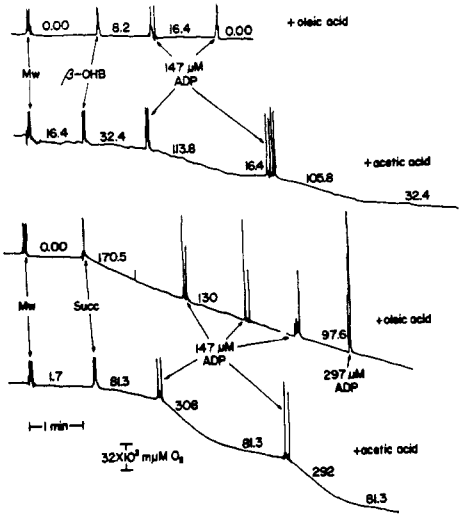

FIGURE 4: Oxidation of succinate and β-hydroxybutyrate by rat liver mitochondria in the presence of oleic acid (0.97 μmole/mg of protein) or acetic acid (0.97 μmole/mg of protein). Numbers above the curves represent respiratory rates expressed as mμM O_2 per sec per milligram of C protein.

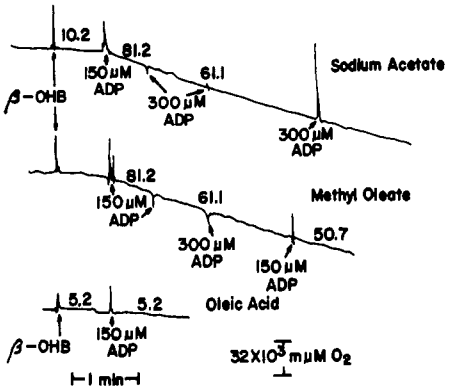

FIGURE 5: Effect of addition of oleic acid (1.2 μmoles/mg of protein), methyl oleate (3.36 μmoles/mg of protein), and sodium acetate (8.38 μmoles/mg of protein) on mitochondrial respiration. Labeling as for Figure 3.

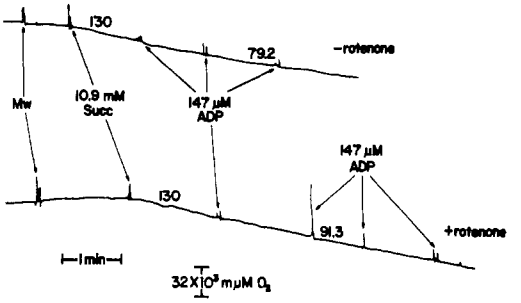

FIGURE 6: Lack of effect of addition of rotenone, at concentrations that totally inhibited β-hydroxybutyrate oxidation in control experiments (0.9 μmole/mg of protein), on the inhibition of succinate oxidation by oleic acid (1.42 μmoles/mg of protein). Labeling as for Figure 3.

trol experiment. As seen in Figure 4, while oleic acid promoted inhibition of respiration by ADP, acetic acid did not affect the ADP stimulation of respiration, nor did it affect the ADP–O ratio. The concentration of acids present in the experiments illustrated in Figure 4 is 0.97 μmole/mg of protein in all cases.

If the effect were due to an abnormal increase in the level of acetate due to oxidation of fatty acid, sodium acetate should produce similar results. As seen in Figure 5, sodium acetate, at approximately seven times the level of oleic acid used in the control, did not simulate the action of oleic acid.

Methyl oleate was used to determine whether modifying the carboxylic group, while leaving the side chain the same, would have any effect. Figure 5 shows that at three times the concentration of the free acid, the ester had little, if any, effect.

Since the inhibiting effect could be due to a fatty acid oxidation product entering the respiratory chain at the level of the pyridine nucleotides, rotenone was added to interrupt the flow of electrons from any reduced nicotinamide–adenine dinucleotide (NADH) or reduced nicotinamide–adenine dinucleotide phosphate (NADPH) producing substrate (Figure 6). Oleic acid was able to induce inhibition of succinate oxidation in the absence of reducing equivalents derived from the reduced pyridine nucleotides. Rotenone failed to interfere with normal oxidation of succinate. It did not alter the effect of the fatty acid, whether added before or after incubation with oleic acid.

However, in order to show a complete lack of interference of rotenone with the action of fatty acids on succinate respiration, the level of fatty acid present has to be high enough to completely suppress NADH-linked respiration. Otherwise the rotenone present appears to alter the effect of the fatty acids when it is

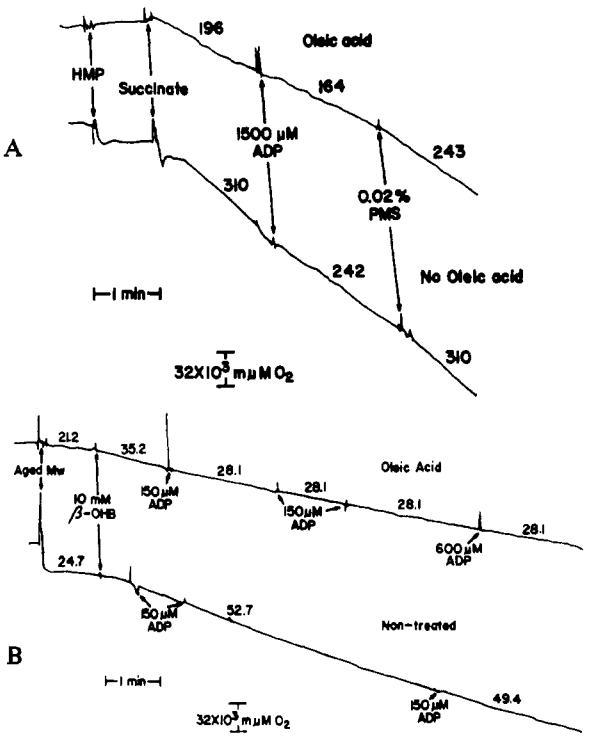

FIGURE 7: Effect of addition of oleic acid on the oxidation of succinate by Keilin and Hartree (1947) beef heart muscle particles (A) and mitochondria aged overnight at 0–2° (B). Labels as explained for Figure 3.

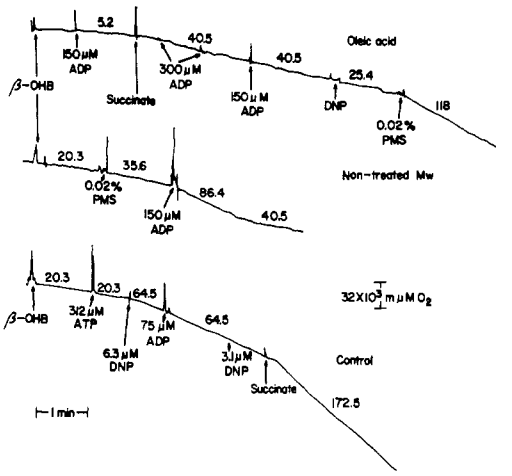

FIGURE 8: Effect of DNP (3.1 µM) and PMS on the inhibition of the oxidation of β-hydroxybutyrate and succinate by oleic acid added to rat liver mitochondria. ADP–O ratios for untreated preparations in control for these experiments is 2.93 for β-hydroxybutyrate oxidation and 2.1 for succinate oxidation. Labels as explained for Figure 3.

actually affecting the NADH-oxidizing mechanism. Linoleic and linolenic acids were also tested and were both found to be more effective inhibitors than oleic acid.

As oleic acid did not seem to affect the respiration due to the partially uncoupled oxidation, the question arose whether the intact energy transferring chain was necessary for the inhibiting action. Keilin and Hartree (1947) beef heart muscle particles (more than 1 week after preparation) and aged mitochondria were utilized to test this. As may be seen in Figure 7, A and B, even at the high levels of fatty acid and ADP used, only a very small inhibition of the HMP respiration was observed. Similar results were obtained with aged mitochondria.

In order to obtain an idea of where the energy transferring chain was affected, DNP was added to an oleic acid inhibited preparation (Figure 8). At uncoupling concentrations, DNP did not release the fatty acid inhibition. On the other hand PMS could release the inhibition effectively proving that succinate dehydrogenase remains active. It may be noticed from the lower curve in the figure that PMS did not affect the β-hydroxybutyrate dehydrogenase. DNP not only failed to release oleic acid inhibition but rather appeared to enhance it (Figure 8). Therefore, experiments were designed to test the effect of DNP in the absence of oleic acid. Its action was tested in the absence and presence of ADP. It showed a pattern of activity very similar to that of the fatty acid, as can be seen in Figure 9a and 9b.

Discussion

Several workers have reported that fatty acids cause either uncoupling of oxidative phosphorylation (Hülsmann *et al.*, 1960; Pressman and Lardy, 1956) or inhibition of respiration (Lehninger, 1945). The results presented here confirm both effects, making clear that the level at which the fatty acids are present determines whether they enhance or inhibit respiration. Myers and Slater (1957) and Hemker (1962, 1964a,b) had already observed that DNP has a biphasic action, that is, it stimulates respiration and ATPase activity at low concentrations but inhibits these functions at higher concentrations. The work reported here not only confirms the biphasic nature of DNP action, but shows the similarities between its action pattern and that of oleic acid. Moreover, DNP at uncoupling concentrations does not release the inhibition caused by oleic acid, but enhances it.

Working with mitochondrial fragments (Gregg and Lehninger, 1963; Lehninger and Gregg, 1963) described conditions under which ADP inhibited respiration. Their fragments, obtained from digitonin-treated mitochondria and already loosely coupled, required the addition of nicotinamide-adenine dinucleotide (NAD^+), besides addition of ADP, for the inhibition of β-hydroxybutyrate oxidation. In our experiments with intact mitochondria no external addition of NAD^+ is needed. Moreover, our preparation showed the same type of inhibiting effects when succinate was the substrate. In spite of these differences it is believed that the

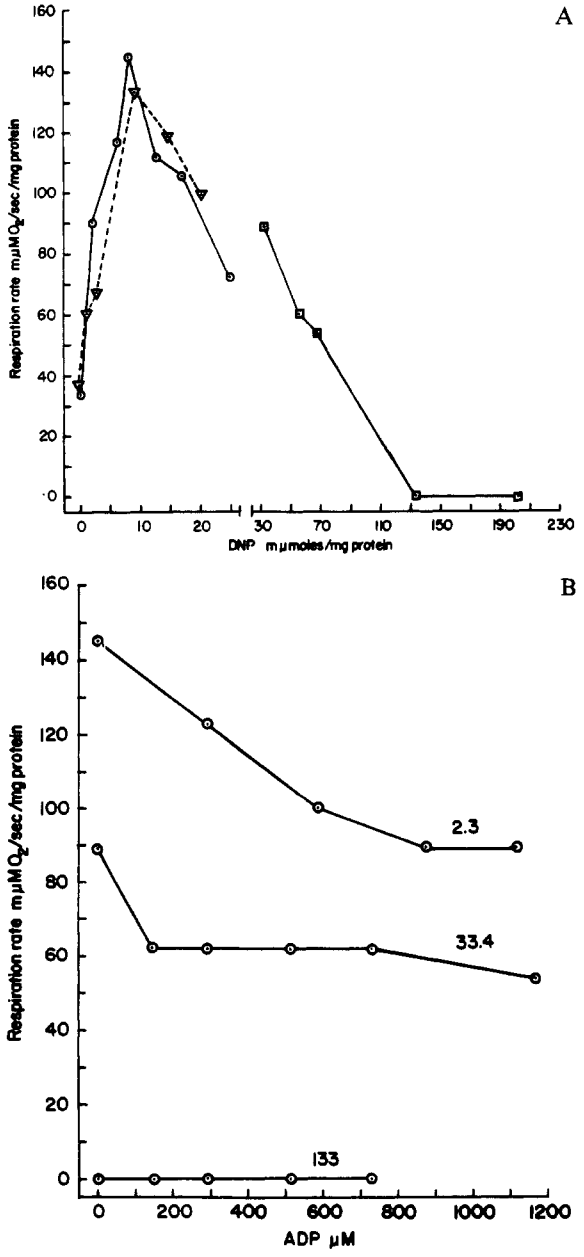

FIGURE 9: Response of mitochondrial respiration to DNP in the absence (A) and presence (B) of phosphate acceptor. The numbers above the curves in part (B) represent the concentration of DNP present expressed as millimicromoles per milligram of protein. The curves represent different experiments using β-hydroxybutyrate as substrate.

reverse acceptor effect observed by Lehninger's and our group is the same. Most probably their digitonin fragments were partially depleted of NAD^+ making its addition necessary to manifest the effect. As shown by our rotenone experiments NADH generating systems are not an absolute requirement for the inhibiting action. Rotenone did not interfere with the normal oxidation of succinate, neither did it affect the fatty acids on succinate oxidation whether it was added before or after preincubation with the lipid.

The inhibition of substrate oxidation caused by fatty acids is not due to intramitochondrial changes in pH due to the presence of the acid, as demonstrated by the fact that equimolar amounts of acetic acid are incapable of causing similar effects. Nor is their activity due to accumulation of acetate that presumably could be oxidized preferentially by the mitochondria. In the experiments with methyl oleate a slight effect is noticed that may be caused by the presence of small amounts of the free acid in the reagent used or, more likely, to a slow hydrolysis of the ester by mitochondrial lipases. A similarly slight effect was observed also in the experiments with HMP and aged mitochondria. As shown by Haas (1964), HMP contains part of the energy-conserving mechanism between the electron-transporting chain and the oligomycin-sensitive site. The mechanism for converting the energy of the high energy intermediate into ATP is not operative in these particles. However, the physiological intactness of the energy-conserving mechanism of these particles decreases with aging. Therefore, the slight effect observed with HMP and aged mitochondria could be ascribed to the presence of residual coupling of respiratory units in the preparations.

It should be emphasized that though the concentration of fatty acid necessary for maximum stimulation of respiration is approximately the same for both substrates, higher concentrations of the lipids are required for complete inhibition of respiration when succinate is used as substrate. Experimentally, one does not obtain complete inhibition of succinate oxidation unless ADP is added.

It is clear from the results that the extent of the inhibition produced by the fatty acids is dependent on the intactness of the oxidative phosphorylation chain, at least at the points where the electron-transferring units and the related part of the energy conserving units are, functionally, closely associated.

Succinate dehydrogenase is unaffected by the fatty acid or DNP as demonstrated by the experiments with PMS. Similar evidence obtained for a limited number of experiments indicates that the β-hydroxybutyrate dehydrogenase probably is not inhibited.

From the facts discussed above, and from the fact that DNP, at uncoupling concentrations, is unable to release the inhibition caused by oleic acid, it is concluded that the fatty acid acts at the DNP site of action, or at a previous step. Since the general consensus points to intermediate $\sim X(I \sim X)$ in the scheme

$$I\sim X + P_i \leftrightharpoons X\sim P + I \quad (1)$$

$$X\sim P + ADP \leftrightharpoons ATP + X \quad (2)$$

To be the site of action of DNP, and since the fatty acids do not have effect unless the energy-conserving unit is present, one is forced to believe that either both inhibitors act at the same site, or that fatty acids act at a site closer to the electron-transport chain than DNP.

In order to explain the enhancing effect of ADP on fatty acid and DNP inhibition, reactions 1 and 2 above should be considered. One can look at P_i in eq 1 as a competitor of I for a site near bound X. Similarly, in eq 2 ADP competes with X for a site near bound P_i. Therefore, essentially the function of ADP is to liberate X from I. If one postulates as a site for fatty acid interaction with X a site in which (a) I is attached to X or (b) a site where I, although not bound to X, blocks or inhibits the binding of fatty acid, then one could explain why although P_i and ADP are not necessary for the inhibitory effects studied, they enhance these effects. That is, these reagents serve to enhance the accessibility to the site near bound X at which binding of fatty acid occurs. At higher lipid concentrations, the fatty acids would interact with the I~X intermediate directly and freely. That is, they would override the competition in the binding of X.

A recent publication by van den Bergh (1965a) postulates two types of activation for fatty acids. His experiments show that P_i is required for DNP inhibition of fatty acid oxidation, which fits our postulations. However we cannot explain the oligomycin release of DNP inhibition in the presence of P_i unless it is assumed that fatty acids and DNP do not require X in a high energy state but require X to be in a conformation that allows the inhibitors to reach the X binding site. Neither are we able to explain, from his experiments, why should fatty acids inhibit the oxidation of substrates that would provide the nucleotide triphosphates required for the fatty acid activation (van den Bergh, 1965b).

Since marked inhibition of mitochondrial functions is observed only with myristic acid, or long-chain unsaturated fatty acids (Björntorp *et al.*, 1964; Avi-Dor 1960), it seems probable that the events discussed here are different from those involved in fatty acid activation as discussed by van den Bergh and are dependent on the accessibility of the affected site to these fatty acids. As mentioned above, the sensitivity to fatty acids of the two phosphorylation sites discussed is different. This fact, considered together with the fact that the effectivity of the fatty acids varies with chain length and degree of unsaturation, and the similarity with the case of the nitrophenols studied by Hemker, make us conclude that these observations reflect a difference between the two phosphorylation chains regarding (a) their nature or (b) their lipophilicity.

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References

Avi-Dor, Y. (1960), *Biochim. Biophys. Acta 39*, 53.
Björntorp, P., Ells, H. A., and Bradford, R. H. (1964), *J. Biol. Chem. 239*, 339.
Chance, B. (1952), *J. Biol. Chem. 197*, 557.
Chance, B., Williams, G. R., and Hollunger, G. (1963), *J. Biol. Chem. 238*, 439.
Elliott, W. B., Hülsmann, W. C., and Slater, E. C. (1959), *Biochim. Biophys. Acta 33*, 509.
Gregg, C. T., and Lehninger, A. L. (1963), *Biochim. Biophys. Acta 78*, 27.
Haas, D. W. (1964), *Biochim. Biophys. Acta 89*, 543.
Hemker, H. C. (1962), *Biochim. Biophys. Acta 63*, 46.
Hemker, H. C. (1964a), *Biochim. Biophys. Acta 81*, 1.
Hemker, H. C. (1964b), *Biochim. Biophys. Acta 81*, 9.
Hülsmann, W. C., Elliott, W. B., and Slater, E. C. (1960), *Biochim. Biophys. Acta 39*, 267.
Keilin, D., and Hartree, E. F. (1947), *Biochem. J. 41*, 500.
Lehninger, A. L. (1945), *J. Biol. Chem. 157*, 363.
Lehninger, A. L., and Gregg, C. T. (1963), *Biochim. Biophys. Acta 78*, 12.
Myers, D. K., and Slater, E. C. (1957), *Biochem. J. 67*, 558.
Pressman, B. C., and Lardy, H. A. (1956), *Biochim. Biophys. Acta 21*, 458.
Strickland, E. H., Ziegler, F. D., and Anthony, A. (1961), *Nature 191*, 969.
van den Bergh, S. G. (1965a), *Biochim. Biophys. Acta 98*, 442.
van den Bergh, S. G. (1965b), *Biochim. Biophys. Acta 98*, 517.
Ziegler, F. D., Vázquez-Colón, L., Elliott, W. B., Taub, A., and Gans, C. (1965), *Biochemistry 4*, 555.