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Effect of sodium oleate on the metabolism and size of rat liver mitochondria I. Uncoupling, shrinkage and their re-versal action by bovine serum albumin

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Effect of sodium oleate on the metabolism and size of rat liver mitochondria I. Uncoupling, shrinkage and their re-versal action by bovine serum albumin*

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Abstract

Effects of sodium oleate and bovine serum albumin (BSA) on rat liver mitochondrial function and structure were studied by measuring oxygen uptake, 90° light-scattering, adenosine triphosphatase activity and pyridine nucleotides fluorescence. 1. The low concentration of oleate induced the uncoupling of oxidative phosphorylation and the scattering change of mitochondria. This action of oleate differed from that of oleate at a high concentration which induces the high amplitude swelling with respect to its physiological and biochemical properties. The degrees of reversal swelling (shrinkage) and of oxygen uptake induced by oleate in the presence of Pi and succinate were altered proportionately to the concentration of oleate, and the concentration of oleate to the shrinkage coincided with that of the maximal respiratory release. 2. Antimycin A or 2, 4-dinitrophenol prevented the oleate-induced mitochondrial shrinkage, but the treatment of these agents after prior incubation with Pi and succinate allowed the shrinkage, though the degree was small in its extent compared with that in the absence of inhibitors. On the other hand, oligomycin did not affect the shrinkage with oleate. 3. BSA protected the mitochondrial phosphorylation from the uncoupling action of oleate without showing any effect of its own. A complete reversal could readily be demonstrated by a sufficient amount of BSA from the uncoupling, structural changes, and oxidation of intramitochondrial pyridine nucleotides induced by oleate in a low concentration. 4. The oleate-stimulated latent ATPase activity was proportional to the oleate-induced shrinkage of mitochondria with respect to the concentration of oleate. The latent ATPase was abolished also by the addition of a sufficient amount of BSA. 5. The action of oleate on the phosphorylation sequence of mitochondria was discussed on the basis of the present findings.

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EFFECT OF SODIUM OLEATE ON THE METABOLISM AND SIZE OF RAT LIVER MITOCHONDRIA

I. UNCOUPLING, SHRINKAGE AND THEIR REVERSAL ACTION BY BOVINE SERUM ALBUMIN

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Since the presence of uncoupler for oxidative phosphorylation as the control mechanism of cell metabolism was suggested by LARDY¹, several uncouplers have been isolated from various materials. These uncouplers have been detected including long-chain fatty acids, and the possible role of the long-chain fatty acids as the regulator of oxidation metabolism has been reported by several authors, e. g. in microsome by PRESSMAN and LARDY², in aged liver mitochondria by POLIS and SHMUKLER³, by PULLMAN and RACKER⁴, in mitochrome by HÜLSMANN and co-workers^{5,6}, in insect mitochondria by WOJTCZAK and WOJTCZAK⁷, in ascites tumor cells by SCHOLEFIELD and co-workers^{8,9} and in hepatoma mitochondria by EMMELOT and BOS^{10,11}.

LEHNINGER and REMMERT¹² also extracted uncoupling factor (so-called U-factor) for oxidative phosphorylation from aged mitochondrial fragments, which causes the swelling of mitochondria. The activity of U-factor could be simulated by sodium oleate¹², and later WOJTCZAK and LEHNINGER¹³ identified U-factor as long-chain fatty acids. The effect of fatty acid on rat liver mitochondria has been reported by many investigators^{7,8,14-18}. Concerning the swelling and uncoupling of mitochondria the author¹⁴ has demonstrated that the swelling action of mitochondria is stronger with unsaturated fatty acids than that with saturated ones, and that the extent of uncoupling activity of the fatty acids is parallel to that of the swelling action for mitochondria as a function of carbon chain length. There exists an interesting relationship between the uncoupling oxidative phosphorylation and the swelling. Besides this, the latent ATPase activity is also correlated to the uncoupling and swelling of mitochondria^{2,14}, and the uncoupling action of fatty acid for phosphorylation is protected^{12,13,19-21} or reversed^{8,4,15} by serum albumin.

For the purpose to reveal the action of sodium oleate on the mutual relationship between the function and structure of mitochondria, the changes of the respiration, oxidative phosphorylation, ATPase activity affected with oleate and

the reversal action of bovine serum albumin for uncoupling have been observed in connection with the swelling-shrinkage of rat liver mitochondria induced by oleate.

MATERIALS AND METHODS

The mitochondria were isolated from rat liver by a modified method of HOGEBOOM²² as described by UTSUMI²³, a single exception being with and without ethylenediamine tetraacetate (EDTA), and suspended finally as stock mitochondria in a volume of 0.25M sucrose so that each milliliter represented the mitochondria obtained from 2g wet weight of the liver. Mitochondrial protein content was determined by the Kjeldahl technique²⁴.

The oxygen uptake of mitochondria was measured by the rotating platinum electrode, and the swelling-shrinkage was by the 90° light-scattering at 520 m μ . Both measurements were carried out at 25° simultaneously with the use of the apparatus which was designed and constructed by UTSUMI²³. Standard incubation mixture was consisted of 0.05M sucrose, 20mM KCl, 5mM K-phosphate, 10mM Tris-HCl buffer (pH 7.4), 100 μ M EDTA and with or without 1mM MgCl₂. Other experimental details of individual experiment are given in the legends to each figure.

Adenosine triphosphatase (ATPase) activity was tested at 25° for 10 minutes with gentle shaking in air environment. The incubation medium was composed of 0.1M sucrose, 20mM KCl, 10mM Tris-HCl buffer (pH 7.4), 40 μ M EDTA and 3mM ATP. The test in varying concentrations of sodium oleate was carried out in the presence or absence of bovine serum albumin (BSA) or Mg⁺⁺. BSA and MgCl₂ when added were 4mg and 1mM respectively, making the final volume 2ml. Reaction was started by the addition of 0.1ml of the stock mitochondrial suspension and stopped with 1 ml of 24 per cent perchloric acid. After centrifugation inorganic phosphate (Pi) was determined by the method of TAKAHASHI²⁵.

Relative fluorescence intensity of intramitochondrial pyridine nucleotides was measured by a fluorometer containing oxygen electrode in the sample cell constructed by the author²⁶. Experimental detail is given in the legend to the figure.

ADP and ATP were obtained from Sigma Chemical Co., BSA from Armour Laboratories (fraction V), antimycin A from Kyowa Hakko Co. and 2, 4- dinitrophenol from Ishizu Pharmaceutical Co. Oligomycin was donated by Dr. UTSUMI. Sodium oleate was prepared with NaOH and oleic acid obtained from Tokyo Kasei Co. and the hydrogen ion concentration was adjusted to 7.4 with diluted HCl just before use. Other chemicals were of reagent grade and the distilled water to make up the solution was deionized.

RESULTS

ADP/O ratio and respiratory control index²⁷ of the intact mitochondria isolated from rat liver showed 1.9 and 4.0 respectively with Pi and succinate. The oxygen uptake of the mitochondria was increased slightly by the addition of sodium oleate ($8\mu\text{M}$), and ADP/O ratio and respiratory control index lowered to 1.25 and 2.3 respectively. With the increased oxygen uptake by oleate, the mitochondria showed an increase of light-scattering (shrinkage) with a short lag. This shrinkage was enhanced by the addition of ADP, and after phosphorylating the added ADP to ATP, the light-scattering decreased (swelling) to the level before the addition of ADP. When oxygen was exhausted, the reversal of swelling (shrinkage)²⁸ of mitochondria was observed coming back to the initial level in the scattering intensity (Fig. 1).

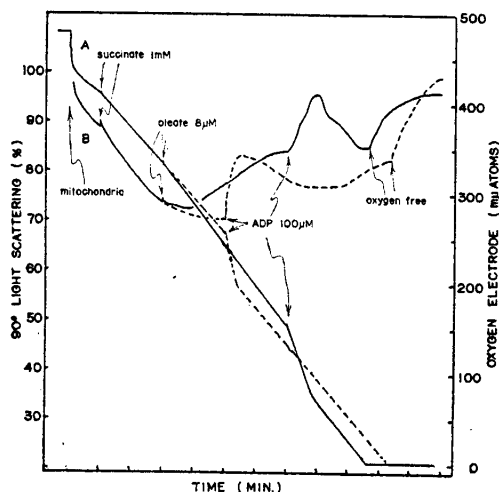


Fig. 1 Effect of sodium oleate on the respiration and swelling-shrinkage of rat liver mitochondria. The reaction mixture at 25° contained 50mM sucrose, 20mM KCl, 5mM K-phosphate, 10mM Tris buffer (pH 7.4) and 0.1mM EDTA. Reaction was started by the addition of 0.1ml of stock mitochondria (3.0mg protein) isolated in 0.25M sucrose without EDTA, the final volume, 2ml. Other additions were 1mM Na-succinate, $8\mu\text{M}$ Na-oleate and $100\mu\text{M}$ ADP. The time moves from left to right, and traces A and B indicate oxygen electrode current and 90° light-scattering changes respectively. The latter was measured at $520\text{ m}\mu$, and the initial scattering intensity of the mitochondria in the basic reaction mixture was taken as 100%. The decreases in scattering (swelling), scattering increases (shrinkage) and oxygen uptake were in the presence (—) and absence (---) of oleate.

The degree and velocity of the shrinkage induced by oleate were enhanced proportionately to the concentration of oleate up to 0.16 mM per 3mg mitochondrial protein, accompanying the proportional increment of the initial

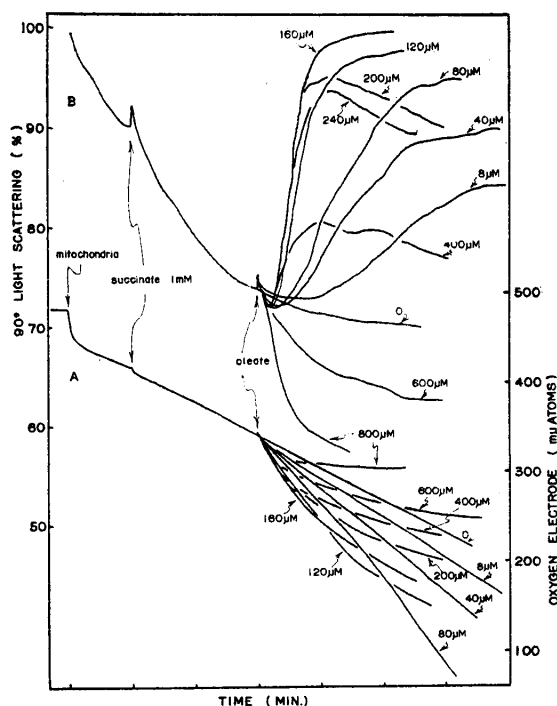


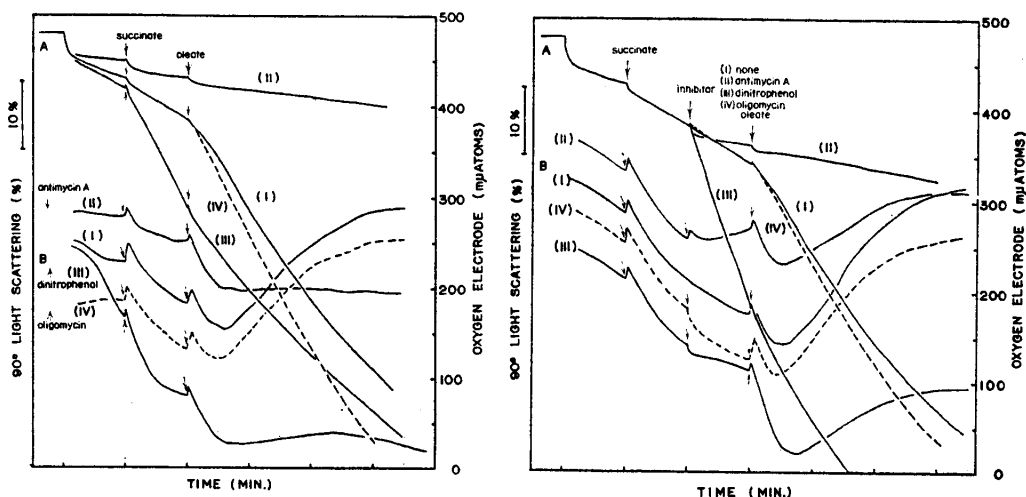
Fig. 2 Effect of various concentrations of sodium oleate on the swelling-shrinkage changes and respiration of rat liver mitochondria. Traces A and B refer to oxygram and light-scattering. The concentrations of added substances are in Fig. 1 and the reacting conditions are as in Fig. 1.

velocity of respiratory release (Fig. 2). From the data of this experiment, it was calculated that about 0.1μ mole of oleate was required to cause the greatest shrinkage of 1 mg protein mitochondria. In a higher concentration of oleate more than 0.1μ mole per mg mitochondrial protein the degree of shrinkage was lowered proportionately to the increment of oleate concentration, though the initial velocity of the shrinkage was the same as observed in the case of the maximum shrinkage. Namely, the shrinkage ceased at a certain period of incubation after the addition of oleate accompanied by the fall-off of oxygen uptake. Oleate in the concentration of more than 0.2μ moles per mg protein induced the prompt swelling of mitochondria without shrinkage, accompanied by the inhibition of the respiration.

The swelling-shrinkage change and the extent of oxygen uptake of mitochondria with oleate were affected by respiratory inhibitor, uncoupler, and inhibitor of phosphorylating respiration (Figs. 3 and 4). The period of the addition, before and after the incubation of mitochondria with Pi and succinate, affected the action of these inhibitors. On incubation of mitochondria with antimycin A,

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Figs. 3 and 4 Effect of inhibitors on the oleate-induced shrinkage and respiratory release of rat liver mitochondria. The reaction mixture at 25° contained 0.1M sucrose, 0.02M KCl, 5mM K-phosphate, 10mM Tris buffer (pH 7.4), 0.1mM EDTA and 1mM $MgCl_2$. Reaction was started by the addition of mitochondria (2.4mg protein) isolated in 0.25M sucrose with 40 μ M EDTA. Total volume was 2ml. Other additions were 3mM Na-succinate, 0.12mM Na-oleate, 4 μ g antimycin A, 0.05mM 2, 4- dinitrophenol and 4 μ g oligomycin. Traces A and B refer to oxygram and light-scattering. Fig. 3 shows the effect of inhibitors added before the incubation of mitochondria with succinate and Fig. 4 shows the one added after the incubation. Curves (I), (II), (III) and (IV) indicate those in the absence of inhibitor, in the presence of antimycin A, 2, 4- dinitrophenol and oligomycin, respectively.

2,4-dinitrophenol, or oligomycin before the addition of succinate, the mitochondrial shrinkage with oleate was inhibited by antimycin A or by dinitrophenol but not by oligomycin, and the oxygen uptake of mitochondria treated with oleate was inhibited with antimycin A or with 2, 4- dinitrophenol but increased by oligomycin (Fig. 3). These inhibitor patterns were changed by the addition of inhibitors after the pre-incubation of mitochondria with Pi and succinate (Fig. 4). The inhibitor patterns of oxygen uptake was same as in the case of preincubation with these inhibitors but the shrinkage with oleate was observed even in the presence of antimycin A or 2, 4- dinitrophenol.

By the addition of BSA, ADP/O ratio and respiratory control index of the mitochondria were not altered, and the mitochondrial structure was also not affected by BSA, as the pattern of swelling-shrinkage induced by ADP was not altered (Fig. 5) Thus, BSA has no effect on oxidative phosphorylation linked with succinate in fresh liver mitochondria under the present experimental conditions.

After addition of a small amount of oleate (0.05mM) to mitochondria which induces the increased oxygen uptake and the marked shrinkage (Fig. 6 trace I), a slight increase of respiration and shrinkage were brought about by the

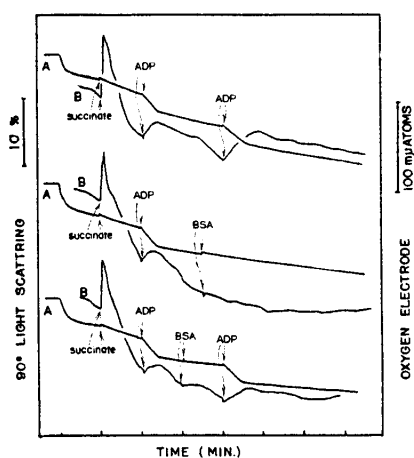


Fig. 5 Effect of BSA on the phosphorylation of rat liver mitochondria. Preparation of mitochondria and the reaction mixture are the same as in Fig. 1. Mitochondrial volume used in this experiment was 1.6mg protein and other additions were 10mM Na-succinate, 40 μ M ADP and 2mg BSA. Traces A and B refer to oxygram and light-scattering.

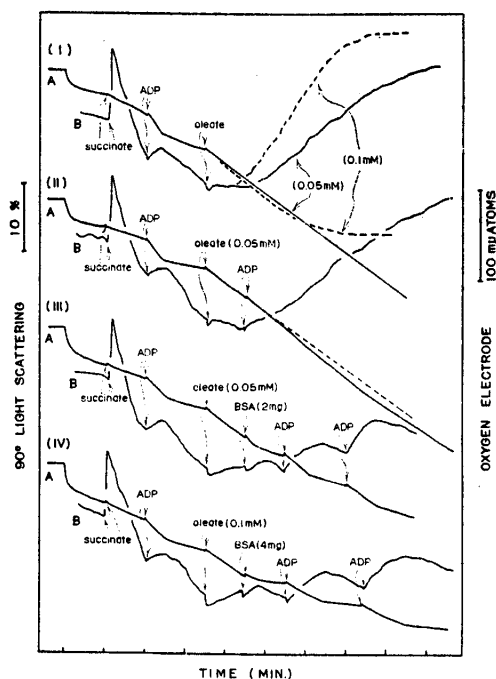


Fig. 6 Reversal by BSA from the uncoupled oxidative phosphorylation and shrinkage of rat liver mitochondria induced by sodium oleate. Preparation of mitochondria and reaction mixture are as shown in Fig. 1 except 1.6mg protein mitochondria. The concentrations of oleate and BSA are given in Fig. and of Na-Succinate and ADP are 10 mM and 40 μ M, respectively. Traces A and B refer to oxygram and light-scattering.

addition of ADP showing the decrement of the ADP/O ratio and respiratory control index (trace II). But by the addition of 2mg BSA after the treatment with oleate, the increased oxygen uptake by oleate was decreased and the swelling induced, and then the ADP/O ratio and respiratory control index were observed steadily by the addition of ADP (trace III). On the other hand, when a higher concentration of oleate (0.1mM) was added to mitochondria, the oxygen uptake was increased but was followed by fall-off, and the increased scattering was greater in extent than that of oleate at a low concentration (trace I). In this instance, it required about 4mg BSA to reverse the respiratory release and the shrinkage (trace IV). This reversal of uncoupling was not induced with 2mg BSA, however there could be observed the increased respiration without fall-

off and the decreased shrinkage. Moreover, in the presence of BSA in a sufficient amount before the treatment with oleate, the mitochondrial respiration and swelling-shrinkage were not affected by added oleate.

The ATPase activity was increased proportionately to the increasing of concentration of oleate up to about 0.1μ mole per mg protein of mitochondria and decreased gradually by that of oleate higher than 0.1μ mole (Fig. 7). The con-

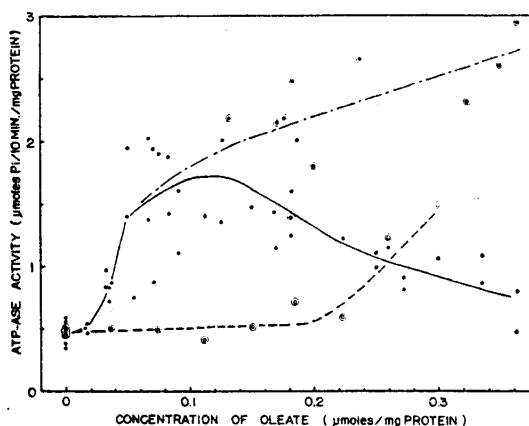


Fig. 7 The ATPase activity of rat liver mitochondria induced by sodium oleate in varying concentrations. Experimental conditions are as described in the text. ●, —, oleate; ○, ---, oleate + 4 mg BSA; ⊙, - · - · -, oleate + 1 mM $MgCl_2$

centration of oleate to induce the maximum activity of ATPase was in the same range as in the case of the maximum mitochondrial shrinkage. This oleate-induced latent ATPase activity was protected by BSA, but this protective action of BSA (4 mg) was abolished progressively with the increasing of oleate ranging 0.2 — 0.4μ moles per mg protein of mitochondria. The concentration of BSA to abolish the oleate-induced latent ATPase activity coincided with that of BSA to protect the uncoupling and shrinkage action of oleate. On the other hand, the ATPase activity induced by oleate was stimulated by the presence of Mg^{++} , and this stimulated activity was increased gradually by the increasing of oleate concentration, agreeing with the reports of other investigators^{2,15,17}.

Relative fluorescence intensity (reduced pyridine nucleotides) of rat liver mitochondria was reduced by added Pi and increased by succinate (Fig. 8). The addition of ADP induced the increase of the intensity accompanied with the increase of respiration with respect to the respiratory control. Then after phosphorylation of ADP to ATP the fluorescence intensity increased to the level of steady state, as described by CHANCE and HOLLUNGER²⁹. The decrease of fluorescence intensity and increase of respiration were induced by the addition of oleate

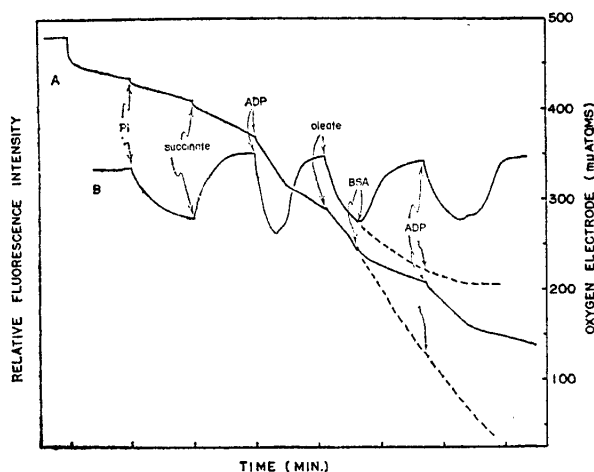


Fig. 8 Changes of relative fluorescence and respiration of rat liver mitochondria. Both measurements were carried out simultaneously at 25° in final 2 ml of a medium consisted of 0.1M sucrose, 0.02M KCl, 50 μ M EDTA, 1mM MgCl₂ and 10mM Tris buffer (pH 7.4). The reaction was started by the addition of 2.1mg protein mitochondria and other additions were 3mM K-phosphate (pH 7.4), 3mM Na-succinate, 100 μ M ADP, 0.16mM Na-oleate and 4mg BSA. Trace A indicates oxygen uptake and trace B relative fluorescence. The latter trace shows the state of oxidation-reduction of intramitochondrial pyridine nucleotides (arbitrary unit). The downward deflection refers to the oxidation and upward to the reduction. Broken line denotes the changes in the absence of BSA.

at the same time. On adding BSA, the increased respiration induced by oleate was reversed and the fluorescence intensity decreased to the level of steady state, and then the respiratory control with ADP accompanied by the oxidation-reduction of fluorescence could be seen again.

DISCUSSION

It has been clarified that the mitochondria manifest structural changes, as measured by 90° light-scattering in their membrane coupled with oxidative phosphorylation^{23,30-34}. The extent of swelling with oleate is changed not only by the concentration of oleate¹³ but also by varying incubation mixture^{18,30}. Oleate induces high amplitude swelling³⁴ in KCl-Tris buffer solution but low amplitude swelling³⁴ in sucrose-Tris buffer solution. This low amplitude swelling induced by oleate is worthy of attention with respect to the endogenous regulator of oxidation metabolism, because the activity of endogenous uncoupler can be produced by oleate¹². In fact, this uncoupling oxidative phosphorylation with oleate is closely correlated with the swelling-shrinkage of mitochondria, as can be observed from the fact that the extent of mitochondrial shrinkage induced by

oleate parallels the extent of respiratory release linked to succinate. Furthermore, there has been observed quantitative relationship between the amount of mitochondria and that of oleate manifesting the consistent activity for uncoupling and shrinkage.

The shrinkage and uncoupling of mitochondria induced by oleate are protected and readily reversed by serum albumin. The protective action of albumin has been observed in skeletal muscle mitochondria^{21,35}, in insect mitochondria⁷, in fresh isolated tumor mitochondria³⁶, and in aged mitochondria^{4,20}. As to this protective action, it may be explained that serum albumin binds to long-chain fatty acids, particularly to oleic acid, so tightly that the two could not be separated electrophoretically^{37,38}. Serum albumin, on the other hand, strongly inhibits the swelling of mitochondria caused by oleate^{12,13,18}. However, little is known^{15,20} about the reversibility of uncoupling and swelling by serum albumin after mitochondria have come in contact with oleate. The present investigation shows that a complete reversal action could be demonstrated by the addition of a sufficient amount of BSA on the uncoupling, on the structural change, and on the oxidation of pyridine nucleotides induced by oleate. Furthermore, oleate-induced latent ATPase activity was also abolished by addition of BSA. However, the mitochondrial respiration accompanied by the swelling in high amplitude by a large amount of oleate was not reversed completely by addition of a sufficient amount of BSA. Therefore, the action of oleate in a low concentration may be distinguished from the break-down of the mitochondrial structure and may have a physiological meaning such as regulation of energy metabolism. In this instance, if the control of the cell metabolism by the presence of uncoupler, fatty acids, is possible¹, the relationship between the uncoupling of such amount of oleate and the ready reversal of this uncoupling by BSA, regardless of how BSA may protect reversibility under any circumstance, may be considered as the action as regulator of cell metabolism.

Many investigators have been studying the action of oleate on mitochondria but the mechanism remains obscure. The increment of mitochondrial respiration induced by oleate may be due to the following three possibilities: First one is the oxidation of fatty acid by mitochondria. This possibility was proposed by WOJTCZAK *et al.*^{13,18} from the study of C¹⁴-oleic acid oxidation, and several investigators described that oxidation of fatty acids is increased by the formation of fatty albuminate³⁹⁻⁴⁰ without uncoupling of oxidative phosphorylation. Furthermore, as described in this paper, the reduction of pyridine nucleotides by BSA after treatment of oleate would be proposed the first possibility. The second is the oleate-induced ATPase activity. ADP increased by ATPase induces the respiratory control, increasing respiration. In fact, the ATPase activity is induced by oleate as described in the present report but the produced ADP by the oleate-

induced ATPase can not bring about the increase of respiration, because oligomycin did not inhibit this increased respiration. The third is the uncoupling oxidative phosphorylation.

Mechanism of the increased respiration by true uncouplers such as dinitrophenol and oleic acid has been accounted for by the discharge of high energy intermediate, initial step of oxidative phosphorylation^{8,41,42}. On the other hand, the mechanism of mitochondrial shrinkage by oleate is accounted for by the decrement of high energy intermediate of oxidation as observed in the shrinkage induced by ADP, dinitrophenol, antimycin A or anaerobiosis^{23,31,33}. In the present data the shrinkage with oleate is inhibited slightly by addition of antimycin A or 2, 4- dinitrophenol after prior incubation with Pi and succinate, since it would already slightly decrease the high energy intermediate by these inhibitors, but the inhibition is accelerated by preincubation with antimycin A or 2, 4- dinitrophenol before additions of Pi and succinate. In contrast to these inhibitors, oligomycin does not inhibit the oleate-induced respiration and shrinkage. Therefore, it can be considered that the energized intermediate acted on by oleate exists in an initial step of oxidative phosphorylation processes. In this case, oleate at a higher concentration would bind to the intermediate as demonstrated by HEMKER⁴² for the explanation of the uncoupling mechanism of dinitrophenol. Thus, the fall-off of increased respiration accompanied by the shrinkage ceased by oleate would be explained by the inhibition of the regeneration of intermediate in phosphorylation sequence.

With respect to the idea related to the intermediate, furthermore, it is supported by the evidence of ATPase activity: both oleate-induced ATPase and shrinkage are increased in its activity and degree proportional to the concentration of oleate, showing that the degree of shrinkage is dependent upon the quantity of intermediate. On the other hand, the decreasing ATPase activity by increasing concentration of oleate is due to disruption of the mitochondrial structure, because the Mg^{++} -stimulated ATPase activity is induced proportional to the decrement of oleate-induced ATPase activity and also to the increment of the degree of swelling (high amplitude).

The high amplitude swelling is identified from the low amplitude swelling induced by a low concentration of oleate with respect to the control mechanism of energy metabolism in the cell, and the physical and physiological actions of oleate differ by its concentration. The extent of shrinkage induced by oleate is much greater than that by other reagents. This phenomenon cannot be explained satisfactorily simply by the mechanism of uncoupling action regarding the high energized intermediate. For the action of oleate it must await further experimental examinations, but it is considered that the interaction of the synthesis of phospholipids from oleic acid is accompanied with the ATP-induced contrac-

tion of swollen mitochondria¹⁸.

SUMMARY

Effects of sodium oleate and bovine serum albumin (BSA) on rat liver mitochondrial function and structure were studied by measuring oxygen uptake, 90° light-scattering, adenosine triphosphatase activity and pyridine nucleotides fluorescence.

1. The low concentration of oleate induced the uncoupling of oxidative phosphorylation and the scattering change of mitochondria. This action of oleate differed from that of oleate at a high concentration which induces the high amplitude swelling with respect to its physiological and biochemical properties. The degrees of reversal swelling (shrinkage) and of oxygen uptake induced by oleate in the presence of Pi and succinate were altered proportionately to the concentration of oleate, and the concentration of oleate to the shrinkage coincided with that of the maximal respiratory release.

2. Antimycin A or 2, 4-dinitrophenol prevented the oleate-induced mitochondrial shrinkage, but the treatment of these agents after prior incubation with Pi and succinate allowed the shrinkage, though the degree was small in its extent compared with that in the absence of inhibitors. On the other hand, oligomycin did not affect the shrinkage with oleate.

3. BSA protected the mitochondrial phosphorylation from the uncoupling action of oleate without showing any effect of its own. A complete reversal could readily be demonstrated by a sufficient amount of BSA from the uncoupling, structural changes, and oxidation of intramitochondrial pyridine nucleotides induced by oleate in a low concentration.

4. The oleate-stimulated latent ATPase activity was proportional to the oleate-induced shrinkage of mitochondria with respect to the concentration of oleate. The latent ATPase was abolished also by the addition of a sufficient amount of BSA.

5. The action of oleate on the phosphorylation sequence of mitochondria was discussed on the basis of the present findings.

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