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UNCOUPLING ACTIVITY OF LONG-CHAIN FATTY ACIDS

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SUMMARY

1. At all pH values between 6.0 and 9.5 unsaturated long-chain fatty acids are more effective than the saturated acids in stimulating the latent ATPase activity of fresh rat-liver mitochondria, provided Mg^{2+} is added. Oleic, linoleic, linolenic, arachidonic and elaidic acids are about equally effective.

2. Whereas the saturated fatty acids scarcely stimulate above pH 8 the unsaturated acids are maximally active at pH 9.

3. Mg^{2+} was required for maximal stimulation by oleic acid. In the absence of added Mg^{2+} oleic acid completely inhibited the ATPase stimulated by 2,4-dinitrophenol.

4. Oleic acid concentrations that gave complete loss of ADP respiratory control or phosphate respiratory control lowered the P:O ratio with glutamate as substrate below 1. With palmitic acid loss of respiratory control was only partial.

5. Uncoupling by oleic acid could be reversed by adding serum albumin.

INTRODUCTION

The interest in the uncoupling activity of long-chain fatty acids stems from investigations of naturally occurring uncoupling agents. In 1952, PRESSMAN AND LARDY[1] isolated from microsomes a heat-stable, acetone-soluble fraction which stimulated the respiration of mitochondria in a medium deficient in phosphate acceptor, and stimulated the ATPase. In 1955, the active substance was identified as a mixture of long-chain fatty acids[2,3].

In 1956, POLIS AND SHMUKLER[4] and PULLMAN AND RACKER[5] described the formation in disintegrated, aged liver mitochondria of an uncoupler, whose action on fresh mitochondria could be counteracted by serum albumin. POLIS AND SHMUKLER[4] isolated the uncoupling agent in the form of a haemoprotein to which they gave the name mitochrome. Investigations[6–12] in this laboratory in 1958 led to the separation of mitochrome into two fractions: a haemoprotein almost free from uncoupling activity, and a lipid fraction containing a mixture of long-chain fatty acids which were found to be responsible for the uncoupling activity of mitochrome. It could be shown that the protein fraction, for which the name mitochrome has been retained, is derived from cytochrome.

Following his experiments on mitochrome HÜLSMANN[9] demonstrated that

uncoupling activity could be directly extracted with isooctane from aged rat-liver mitochondria. The formation of the uncoupler was shown to be an enzymic process stimulated by Ca^{2+} and inhibited by EDTA, ATP or diisopropyl phosphofluoridate. In similar experiments LEHNINGER AND REMMERT[13] found that an enzymically formed uncoupling factor (called U factor) could also be extracted with isooctane from aged mitochondrial fragments and they suggested that this factor might be a fatty acid. Further work on U factor, which in a preliminary analysis has been identified as a mixture of long-chain fatty acids, has recently been reported by WOJTCZAK AND LEHNINGER[14].

It was shown by HÜLSMANN[9] in 1958 (see also refs. 11 and 12) that the mixture of fatty acids extracted from a mitochrome preparation by isooctane consisted of 38.5% saturated (C_{16}, 24%; C_{18}, 10%) and 61.5% unsaturated (C_{18}, one double bond, 23%; C_{18}, two double bonds, 10.5%; C_{20} and C_{22}, highly unsaturated, 21%). To determine which of these acids were responsible for the uncoupling action a series of pure long-chain fatty acids was tested for ability to stimulate the latent ATPase and uncouple oxidative phosphorylation of fresh rat-liver mitochondria. It was found that the unsaturated fatty acids as a group are far more potent as uncouplers than the saturated acids and the conclusion was drawn that the uncoupling action of the mitochrome preparation could be completely explained by the, predominantly, unsaturated long-chain fatty acids which it contained[11,12].

In this paper the experiments with pure fatty acids are reported in detail.

METHODS

Rat-liver mitochondria were prepared according to HOGEBOOM[15] as described by MYERS AND SLATER[16].

ATPase activities were measured as described by MYERS AND SLATER[16] at room temperature (22° ± 2°), in a reaction mixture containing 75 m*M* KCl, 50 m*M* Tris-acetate buffer, 0.5 m*M* EDTA, 2 m*M* ATP, 50 m*M* sucrose, 1.5 m*M* $MgCl_2$ and, when added, 0.1 m*M* 2,4-dinitrophenol. Fatty acids were added in 0.03 ml 96% ethanol (final ethanol concentration, 2%). Unless indicated otherwise all values were corrected for ATP breakdown in the presence of ethanol alone. Similarly the ATPase activity in the presence of dinitrophenol has been corrected for ATPase activity in its absence (no ethanol was present in most experiments with dinitrophenol).

P:O ratios were determined by the procedure of SLATER AND HOLTON[17]. The reaction mixture contained 14 m*M* KCl, 6 m*M* $MgCl_2$, 2 m*M* EDTA, 22 m*M* Tris-acetate buffer[16] (pH 7.5), 30 m*M* potassium phosphate buffer (pH 7.5), 0.3 m*M* ATP, 20 m*M* glucose, 100–200 Cori units of yeast hexokinase (prepared according to DARROW AND COLOWICK[18], omitting the final crystallization step*), 20 or 40 m*M* substrate, 50 m*M* sucrose and 0.03 ml 96% ethanol containing varying amounts of fatty acids. The reaction was started by the addition of 0.1 ml mitochondrial suspension (2–4 mg protein). The final volume was 1 ml, the reaction temperature 25° and the reaction time varied between 14 and 24 min. Q_{O_2} values are expressed as μl/mg protein/h.

ADP respiratory control was determined by comparing the respiratory rate in this medium with that obtained in a medium from which the glucose and hexokinase

* We are grateful to Dr. R. A. DARROW and Dr. S. P. COLOWICK for giving us the details of their procedure prior to publication.

were omitted. The respiratory-control index is defined as the ratio of the respiratory rates in the presence and in the absence of a phosphate-acceptor system or an uncoupler. Phosphate respiratory control was determined similarly, as previously described[19].

The chemicals used were obtained from the same sources as described in previous publications of this series[11]. Oleic acid was a product of the British Drug Houses, Ltd. The other fatty acids were obtained from Fluka A.G. Except arachidonic acid they were all "puriss". According to the manufacturer the linoleic acid was 98–99 % pure and the linolenic acid 97–99 % pure; both acids were practically free of peroxides and they contained only traces of trans isomers. The arachidonic acid was only 65 % pure, the main contaminants being C_{20} and C_{22} unsaturated fatty acids. With oleic, linoleic, palmitic and myristic acid the concentrations of stock solutions were checked titrimetrically[20] and found to be 100 % pure within the limits of error of the procedure.

RESULTS

Adenosinetriphosphatase

Comparison of ATPase-stimulating capacity of different fatty acids at pH 6.5

In preliminary experiments with oleic acid widely different results were obtained with different enzyme preparations. Two reasons for this were found: (a) The stimulation of the ATPase by fatty acids is strongly dependent on the amount of enzyme protein used (*cf.* ref. 3). Better reproducibility was obtained when the concentration of oleic acid was expressed as μmoles/g enzyme protein, at least in the range of protein concentrations (0.2–0.4 mg protein/tube) used in the ATPase experiments. (b) The maximal ATPase activity per milligram protein that can be obtained with oleic acid varies with different mitochondrial preparations. Since stimulation by dinitrophenol varied correspondingly, we have expressed the ATPase activity obtained with fatty acids as percentage of that obtained with 0.1 m*M* dinitrophenol.

Fig. 1 shows the concentration curves obtained with oleic and stearic acid. Curves similar to that of oleic acid were obtained for elaidic (contrast ref. 3), linoleic and linolenic acids*; a curve, qualitatively similar to that of stearic acid, was obtained for myristic, palmitic and octanoic acids and for Tween 21. There were, however, quantitative differences: at low fatty acid concentrations myristic acid stimulated the ATPase more than palmitic or stearic acid (*cf.* ref. 3). From these curves two sets of values have been calculated: the amount of fatty acid necessary to stimulate the ATPase to 50 % of that obtained with 0.1 m*M* dinitrophenol, and the stimulation given by 500 μmoles fatty acid/g protein, also expressed as a percentage of the stimulation by dinitrophenol. It is clear from the results presented in Table I that the unsaturated long-chain fatty acids stimulate the ATPase far more than the saturated acids. The degree of unsaturation and the position of the double bond appears to have little effect. Deoxycholic acid is a little more active than the saturated fatty acids; the activity shows a maximum (equal to 60–90 % of that obtained with 0.1 m*M* dinitrophenol) at 1000–1500 μmoles deoxycholate/g protein whereas higher concentrations inhibit. With fatty acids inhibition is never observed at pH 6.5 even when concentrations up to 5000 μmoles/g protein are added.

* In most experiments with the unsaturated fatty acids a more pronounced S-shaped concentration curve was obtained.

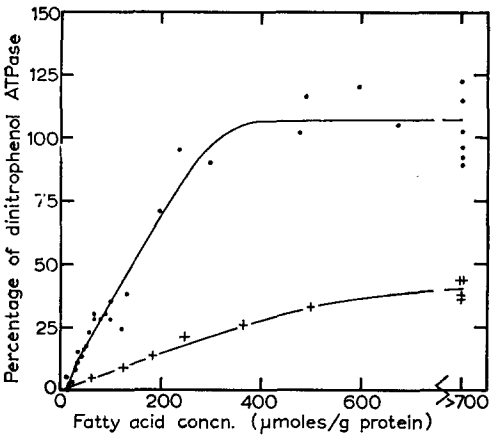

Fig. 1. Stimulation of ATPase by oleic and stearic acid at pH 6.5. 1.5 mM $MgCl_2$ present. ●—●, oleic acid (points obtained in experiments with 5 mitochondrial preparations); +—+, stearic acid (1 experiment). The ATPase activity is expressed as a percentage of the value obtained in the presence of 0.1 mM 2,4-dinitrophenol.

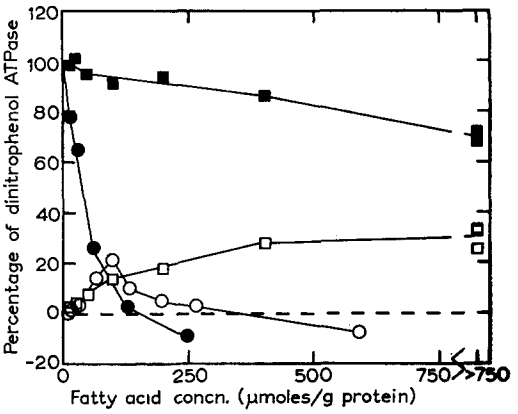

Fig. 2. Stimulation of ATPase and inhibition of dinitrophenol-stimulated ATPase in the absence of Mg^{2+} by oleic and palmitic acid. No $MgCl_2$ present; pH 6.5. ○—○, oleic acid; □—□, palmitic acid; ●—●, oleic acid and 0.1 mM dinitrophenol; ■—■, palmitic acid and 0.1 mM dinitrophenol. The values were obtained in 4 different experiments. The two negative values for high oleic acid concentrations are due to inhibition of ATPase in the presence of ethanol by oleic acid. If the values are only corrected for P_i present in the zero-time controls these two values are zero. The ATPase activity is expressed as in Fig. 1.

TABLE I

STIMULATION OF ATPase BY DIFFERENT FATTY ACIDS AND DEOXYCHOLIC ACID

ATPase was measured at pH 6.5 in the presence of 1.5 mM $MgCl_2$. The values given are calculated from concentration curves similar to those of Fig. 1.

Compound	*μmoles/g protein giving 50 % stimulation**	*Percent stimulation* obtained with 500 μmoles/g protein*
Unsaturated		
Oleic acid	140	106
Linoleic acid	120	103
Linolenic acid	160	108
Arachidonic acid		109
Saturated		
Octanoic acid	—	1
Palmitic acid	950	25
Stearic acid	—	33
Deoxycholic acid	590	38

* 100 % = ATPase with 0.1 mM dinitrophenol.

Effect of Mg^{2+}

In the experiments summarized in Fig. 2, $MgCl_2$ was omitted from the reaction medium. With palmitic acid the maximal stimulation of the ATPase was the same in the presence or absence of $MgCl_2$ (33 %). With oleic acid, however, complete inhibition occurred at higher concentrations of the acid in the absence of Mg^{2+} and the maximal ATPase activity was only 20 % of that obtained with dinitrophenol. A corresponding difference between oleic and palmitic acids was found when increasing amounts of fatty acid were added in the presence of dinitrophenol and absence of Mg^{2+}. Low concentrations of oleic acid inhibited the dinitrophenol-induced ATPase more than 100 % while palmitic acid was less effective as inhibitor. In the presence of $MgCl_2$ neither fatty acid had any effect on the dinitrophenol-induced ATPase. This suggests that the same enzyme system is responsible for ATPase activity induced by fatty acids and by dinitrophenol.

The dependence of the ATPase activity induced by oleic acid on added $MgCl_2$ for maximal activity was found at all pH values between 6 and 9 and similar results have been obtained with linoleic and linolenic acid. The ATPase activity induced by palmitic acid is independent of $MgCl_2$ only in the acid range. At pH values above 7, $MgCl_2$ is required for maximal activity. Similar results were obtained for myristic and stearic acid.

It should be noted that in these experiments only one $MgCl_2$ concentration (1.5 m*M*) was used. We have found that higher $MgCl_2$ concentrations have little effect on the ATPase activity induced by oleic acid at pH 6.5 but strongly inhibit at pH 9.0. Further work is required to establish the optimal Mg^{2+} concentration for different concentrations of fatty acids at all pH values.

Influence of pH

When a concentration of oleic acid sufficient for maximal stimulation of the ATPase activity at pH 6.5 was tested at other pH values, a typical and reproducible curve was obtained with maxima at 6.5 and 9* and a minimum at pH 7.5–8 (Fig. 3). Similar curves were obtained for linoleic, linolenic and arachidonic acids, and the fatty acids from mitochrome (*cf.* Fig. 2 of ref. 11). The saturated fatty acids, myristic, palmitic and stearic acid, however, induced very little activity in the alkaline region.

The form of the pH-activity curve obtained with oleic acid markedly depends upon the concentration of the oleic acid. Fig. 4 shows that at higher oleic acid concentrations the trough at pH 8.0 shown in Fig. 3 is filled up, and that at concentrations over 2000 μmoles/g protein inhibition sets in above pH 7.5, becoming more marked with increasing pH.

In Fig. 5 the maximal ATPase activity induced by oleic acid is plotted as a function of pH and the curve is compared with the pH-activity curve of the ATPase activity of aged mitochondria. The resemblance between the two curves is striking.

Oxidative phosphorylation

P:O ratio and respiratory control

In 1959 REMMERT AND LEHNINGER[21,22] described a factor, isolated from sonicated rat-liver mitochondria, which promoted loss of respiratory control in digitonin par-

* The maximum in the alkaline region may be somewhat underestimated since in most experiments more than 25 % of the ATP was used up during measurements of the ATPase activity.

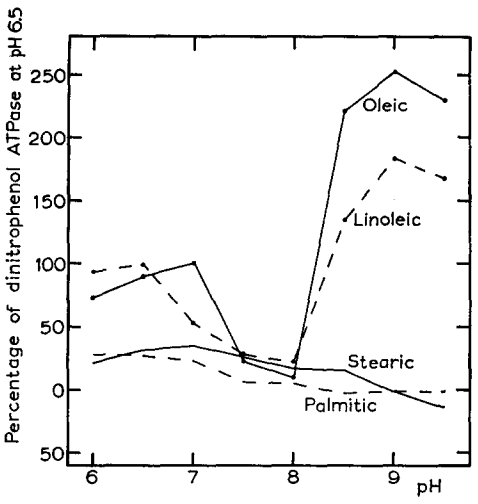

Fig. 3. pH-activity curves of ATPase stimulated by intermediate concentrations of fatty acids. 1.5 mM $MgCl_2$ present. The ATPase in the presence of 0.1 mM dinitrophenol at pH 6.5 has been set at 100. The values were obtained in 4 different experiments. ●—●, oleic acid, 430 μmoles/g protein; ●---●, linoleic acid, 340 μmoles/g protein; ——, palmitic acid, 890 μmoles/g protein; —stearic acid, 2040 μmoles/g protein.

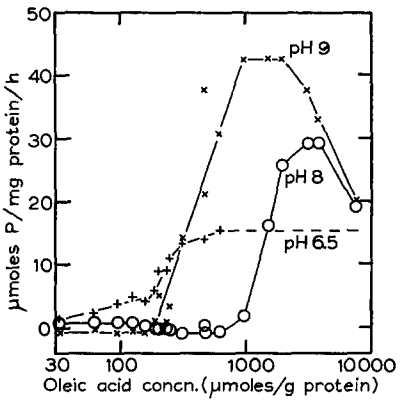

Fig. 4. Effect of oleic acid concentration on ATPase at pH's 6.5, 8 and 9. 1.5 mM $MgCl_2$ present. The values were obtained in two different experiments, the figures of which overlap between 100 and 1000 μmoles oleic acid/g protein. Note logarithmic plot of oleic acid concentration.

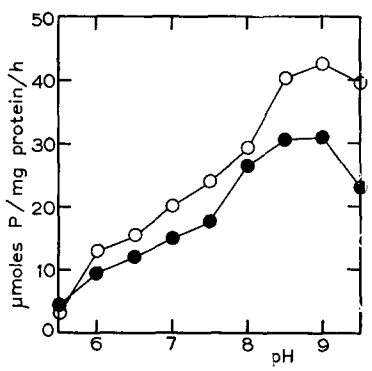

Fig. 5. Comparison of the pH-activity curves of the ATPase of aged mitochondria and the oleic acid-induced ATPase of fresh mitochondria. ●—●, liver mitochondria that had been aged and then subjected 5 times to freezing and thawing (taken from Fig. 5 of ref. 16); ○—○, maximal ATPase of fresh rat-liver mitochondria that can be obtained in the presence of oleic acid (Mg^{2+} present). The values are taken from the curves of Fig. 4 and similar curves.

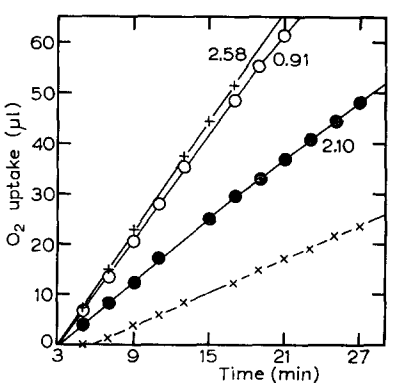

Fig. 6. Effect of oleic acid on ADP respiratory control with glutamate as substrate. Respiration measured in ADP-deficient reaction mixture with 20 mM L-glutamate as substrate, and the following special additions: ×—×, 3% ethanol; ●—●, 32 μg oleic acid + 3% ethanol; ○—○, 64 μg oleic acid + 3% ethanol; +—+, 20 mM glucose + 150 Cori units yeast hexokinase + 3% ethanol. In parallel flasks P:O ratio's were determined in the presence of glucose and hexokinase, and corresponding amounts of oleic acid; the results are indicated in the figure.

ticles without lowering the P:O ratio. As the procedure used for preparing this R factor was rather similar to procedures used in the isolation of mitochrome, we have investigated whether the properties of the R factor could be duplicated by fatty acids. Since β-hydroxybutyrate, the substrate used by REMMERT AND LEHNINGER, gave low Q_{O_2} values and poor respiratory control in our hands, glutamate was used as the substrate (*cf.* refs. 19 and 23). With this substrate ADP-respiratory-control ratio's varying between 3 and 6 were obtained. These values are a little below normal owing to the 3 % ethanol and the relatively high concentration of adenine nucleotide present, which tend to increase the rate of O_2 uptake measured in the absence of ADP. All experiments were performed at pH 7.5. An example of these experiments is given in Fig. 6. In this and similar experiments it was found that some stimulation of respiration* (measured in the absence of phosphate acceptor) could be obtained with oleic acid without a marked decrease in the P:O ratio (measured in the presence of phosphate acceptor) but that complete or near-complete loss of respiratory control was always associated with a decrease of the P:O ratio to below 1.0, unlike the results reported for the R factor[22]. Oleic acid added in the presence of 1 % serum albumin gave results similar to those obtained with oleic acid alone, the only difference being that 3–6 times more oleic acid had to be added for complete loss of respiratory control. Even high concentrations of palmitic acid gave only partial loss of respiratory control. The results of a number of experiments, in which the P:O ratio and respiratory control were measured simultaneously, are summarized in Fig. 7.

With L-proline as substrate oleic acid in concentrations sufficient to abolish ADP respiratory control also abolished P_i respiratory control (*cf.* ref. 19). Thus, as is also the case with dinitrophenol as uncoupling agent[19], respiration uncoupled by oleic acid does not require P_i.

In an attempt to see whether the different phosphorylating steps in the respiratory chain differ in their susceptibility to uncoupling by fatty acids we have compared the effect of oleic acid on the phosphorylation associated with succinate and β-hydroxybutyrate oxidation. The differences found were small and we do not attach much significance to them.

Reversibility of uncoupling by oleic acid

Partial reversibility of uncoupling by mitochrome[4,5] and the dried acetone extract from rat-liver microsomes[3] has been demonstrated. Table II shows that this

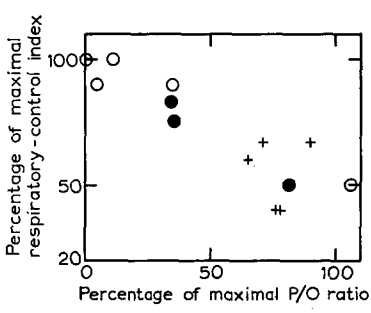

Fig. 7. Correlation between loss of respiratory control and loss of oxidative phosphorylation in the presence of oleic and palmitic acid. The effect of fatty acids on respiratory control and P:O ratio was determined in parallel flasks as shown in Fig. 6. The maximal respiratory-control index is the Q_{O_2} in the presence of glucose + hexokinase + ethanol divided by the Q_{O_2} in the presence of ethanol alone. Since this index was around 5 in most experiments 20 % is taken as the base line. The maximal P:O ratio is the P:O ratio in the presence of ethanol. ●, different oleic acid concentrations; ○, different oleic acid concentrations with 1 % serum albumin added; +, different palmitic acid concentrations. The values were obtained in experiments with 7 different mitochondrial preparations.

* For the purpose of this article it is of no importance whether the electrons were derived from the oxidation of glutamate or of the fatty acids added. In the absence of glutamate the average Q_{O_2} with oleic acid was 13, with palmitic acid 34.

is also true for uncoupling by oleic acid. Even after the mitochondria had been in contact with the oleic acid for 10 min at 25°, almost complete reversal of the uncoupling was obtained by adding serum albumin, which is known strongly to bind long-chain fatty acids[24,25]. Egg albumin was ineffective. Comparable results have recently been obtained by HELINSKI AND COOPER[26] in an extensive investigation of the effect of albumin on aged rat-liver mitochondria. The stimulatory effect of oleic acid on the ATPase was also reversed by the subsequent addition of albumin, even at pH 9.0. However, complete reversal was not obtained.

TABLE II

REVERSIBILITY OF UNCOUPLING BY OLEIC ACID

After 10 min the reaction was stopped in vessel 1, while vessels 2, 3, 4 and 7 were tipped as indicated. After 20 min vessels 2–7 were stopped. The ΔP and ΔO of vessels 2, 3, 4 and 7 have been calculated for the period after the tip by subtracting the ΔP of vessel 1 in the case of vessels 2, 3 and 4 and half of the ΔP of vessel 6 in the case of vessel 7. The latter correction was negligible.

Vessel	*Substrate*	*Oleic acid (μg)*	*Tip*	*ΔO (μatoms)*	*ΔP (μmoles)*	*P:O*
1	L-Glutamate (20 m*M*)	64	—	2.94	2.31	0.79
2	L-Glutamate (20 m*M*)	64	Water	2.78	2.11	0.76
3	L-Glutamate (20 m*M*)	64	1% Serum albumin	2.87	5.99	2.09
4	L-Glutamate (20 m*M*)	64	1% Egg albumin	2.53	2.06	0.81
5	Succinate (40 m*M*)	0	—	2.40	4.15	1.73
6	Succinate (40 m*M*)	32	—	0.93	0.01	0.01
7	Succinate (40 m*M*)	32	1% Serum albumin	1.68	2.40	1.43

DISCUSSION

The results reported in this paper have revealed four differences between oleic and palmitic acid with respect to their uncoupling activity: (a) In the presence of Mg^{2+} oleic acid induces a much greater ATPase activity at all pH values between 6 and 9.5. (b) In the same pH range maximal ATPase stimulation by oleic acid is completely dependent on the addition of Mg^{2+} to the medium. For palmitic acid this is only necessary at pH values above pH 7. (c) The ATPase induced by dinitrophenol in the absence of Mg^{2+} is completely inhibited by low concentrations of oleic acid; palmitic acid inhibits only partially even at high concentrations. (d) While oleic acid can bring about complete loss of ADP respiratory control and phosphorylation, palmitic acid can not.

These conclusions differ in two respects from those of PRESSMAN AND LARDY[3], who studied activation of ATPase by fatty acids at pH 7.5. In the first place, these authors did not find any marked differences between the effects of saturated and unsaturated fatty acids. Secondly, they did not find any effect of Mg^{2+} on the ATPase activity induced by oleic acid. Inspection of their Table II suggests, however, that this was only tested at low oleic acid concentrations. As Fig. 2 of the present paper shows, at low oleic acid concentrations no Mg^{2+} requirement can be detected. Apart from these differences our results and those of PRESSMAN AND LARDY[3] supplement one other quite satisfactorily. Table I and Fig. 2 of their paper give the relative activities of the long-chain fatty acids at low concentrations, whereas our Table I shows the relative activities at high concentrations in the presence of Mg^{2+}.

In view of the reversibility of the uncoupling by oleic acid, the inhibition of the dinitrophenol-induced ATPase by oleic acid in the absence of added Mg^{2+} can be most simply explained by assuming that oleic acid binds the intramitochondrial Mg^{2+} necessary for the dinitrophenol-induced ATPase (*cf.* ref. 27). The concentration of oleic acid required for complete inhibition is of the same order of magnitude as the Mg^{2+} concentration in fresh rat-liver mitochondria[28] (70 μmoles/g protein).

In recent years several uncouplers (or respiratory inhibitors) isolated from natural materials have been shown to contain long-chain fatty acids as the active material, *e.g.* the extract from rat-liver microsomes[3], mitochrome[11], inhibitors of electron transport isolated from rat-intestinal mucosa[29] and *Tetrahymena pyriformis*[30], and uncoupling agents present in insect sarcosome preparations[31,32]. Furthermore, the beneficial effect of serum albumin on the oxidative phosphorylation of freshly isolated tumour mitochondria[33–35] and skeletal-muscle sarcosomes[36,37] indicates that free fatty acids are possibly present in these preparations. The lipid uncoupling material present in hepatomas is under investigation by EMMELOT and coworkers[38§].

Serious consideration should be given to the possibility that other naturally occurring uncoupling agents (or respiratory inhibitors) which have been described in the literature contain fatty acids, perhaps bound to protein as in the mitochrome preparation of POLIS AND SHMUKLER[4]. The following characteristic properties of the long-chain fatty acids should make it easy to test this possibility: (a) the uncoupling activity is extracted into isooctane from acid or neutral aqueous solutions and is reextracted into the aqueous phase by alkali; (b) the activity is counteracted by serum albumin; (c) unsaturated fatty acids inhibit the dinitrophenol-induced ATPase activity of rat-liver mitochondria in the absence of Mg^{2+}; (d) they stimulate the ATPase in the presence of Mg^{2+}, giving the characteristic pH-activity relationship shown in Figs. 3 and 4. In addition long-chain fatty acids bring about loss of respiratory control, lower the P:O ratio, inhibit the ATP–$^{32}P_i$ exchange reaction[6,13,39], induce swelling of fresh rat-liver mitochondria[13,40] and inhibit the respiratory chain when added in higher concentrations[41*,43].

Uncoupling by fatty acids may be distinguished from that brought about by agents breaking up mitochondrial structure in that it is readily reversible by the subsequent addition of serum albumin. The ready reversibility of this uncoupling draws attention to the possible role of unsaturated fatty acids as regulators of oxidative metabolism. This possibility has been discussed by PRESSMAN AND LARDY[3], HÜLSMANN and co-workers[7,9,11] and LEHNINGER and co-workers[13,14]. It is further supported by the evidence of SCHOLEFIELD and co-workers[44–46] that fatty acids (only saturated acids were studied) may exert their uncoupling effects in intact ascites tumour cells.

Whether or not the uncoupling activity of unsaturated fatty acids has any physiological role, it is clear that it can be of importance in biochemical experiments *in vitro*.

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§ *Note added in proof*: In a paper[47] which appeared in January, 1962, and which reached the authors since submitting their paper, EMMELOT confirms that oleic acid inhibits the dinitrophenol-induced ATPase in the absence of Mg^{2+} and demonstrates that the low phosphorylative activity of mitochondria isolated from certain hepatomas is due to the presence in the mitochondrial preparation of unsaturated fatty acids. (Received May 5th, 1962)

* LASER's haemolytic substance studied in this paper was later found to be *cis*-vaccenic acid[42].

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