SWELLING STUDIES ON LIVER MITOCHONDRIA FROM ESSENTIAL FATTY ACID DEFICIENT RATS[1, 2, 3]

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EARLIER investigations concerning the biochemical changes associated with an essential unsaturated fatty acid deficiency in rats revealed that anaerobic glycolysis, employing rat liver as enzyme source, appeared normal, while aerobic glycolysis did not result in the expected uptake of inorganic phosphate from the medium. Further studies demonstrated that this was due to an uncoupled oxidative phosphorylation associated with oxidation of a number of substrates [12]. Subsequently it was reported that liver mitochondria prepared in the usual manner from fat deficient rats appeared swollen, when examined under the electron microscope. It was suggested that "essential fatty acid deficient" mitochondria might be altered easily, in size and perhaps in shape, with accompanying changes in oxidative phosphorylation capacity [16]. Work in this laboratory [10] and that of Hayashida and Portman [8] has shown that liver mitochondria from fat deficient rats do indeed exhibit a greater tendency to swell *in vitro* than those from normal rats. The studies reported here describe additional swelling characteristics of these mitochondria. These and related studies, to be reported, suggest a relationship between the swelling tendency and electron transport and/or uncoupled oxidative phosphorylation in liver mitochondria from essential unsaturated fatty acid deficient rats.

EXPERIMENTAL PROCEDURE

Male Holtzman rats were weaned at 18 days of age and placed on a fat deficient diet similar to that used in earlier experiments [11] except that the vitamin mixture employed included 0.5 g of vitamin A ester concentrate (200,000 I.U./g), 0.02 g

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[2] A preliminary account of this work was presented at the Annual Meeting of the American Society of Biological Chemists, Atlantic City, New Jersey, April 1962.

[3] Abbreviations used are: EDTA, ethylenediamenetetracetate, Tris, tris (hydroxymethyl) amino methane.

calciferol, and 10 g/kg α-tocopherol. Control animals were fed the same diet in which corn oil was substituted at a level of 5 per cent for an equal weight of sucrose. Weight gains and gross symptoms indicated that a pronounced essential unsaturated fatty acid deficiency had developed within approximately 12–14 weeks, at which time the animals were used for the experiments described.

Mitochondria were prepared in 0.25 *M* sucrose, as described earlier [17], and resuspended in fresh 0.25 *M* sucrose at 0°C, at the concentrations required to give an initial optical density of approximately 0.6 when 0.1 ml of the suspension was transferred to 2.9 ml of the medium in which swelling was to be measured. Mitochondrial swelling was determined by measuring the decrease in optical density at 520 Mμ, of a suspension of mitochondria [19–21], employing either a Bausch and Lomb Model 505 spectrophotometer or a Beckman Model DU spectrophotometer. Measurements were made at room temperature. The exact test conditions are described in the text.

RESULTS

Liver mitochondria prepared from unsaturated fatty acid deficient rats and resuspended in either 0.3 *M* sucrose or 0.154 *M* KCl usually absorbed only 70–90 per cent as much incident light at 520 Mμ, as preparations from normal animals, on a mitochondrial protein basis. This suggested either that mitochondria from fat deficient animals swell more than those from normal rats during the process of isolation, or are swollen *in situ*, or both [16]. Direct gravimetric measurements on freshly prepared mitochondria [7, 18, 21] employing dextran-^{14}C (M.W. 15,000–17,000) revealed that the preparations from unsaturated fatty acid deficient rats had an average of 30.2 mg H_2O/mg protein while those from normal control animals had 24.1 mg H_2O/mg protein.

Table I indicates that when the mitochondria are allowed to stand at 0°C in the medium used for their preparation they swell, the deficient mitochondria to a much greater degree than the normal. It seems safe to conclude from this and the direct gravimetric measurements that mitochondria, whether

TABLE I. *Swelling of liver mitochondria from essential unsaturated fatty acid deficient normal rats.*

Time lapse, hr	USFA deficient	Normal
None	0.620	0.620
2	0.508	0.579
4	0.480	0.568

Figures are optical density units, read at 520 Mμ. Swelling occurred at 0°C, mitochondria were suspended in 0.3 *M* sucrose containing 0.02 *M* Tris buffer, pH 7.4.

from normal or unsaturated fatty acid deficient animals, swell during the 1½–2 hr required to prepare them, and that deficient mitochondria swell much more than the normal. The question of whether or not mitochondria from deficient animals are swollen *in situ* remains to be answered.

It was observed early in these experiments that mitochondria from unsaturated fatty acid deficient rats which were allowed to stand for 2–3 hr at 0°C

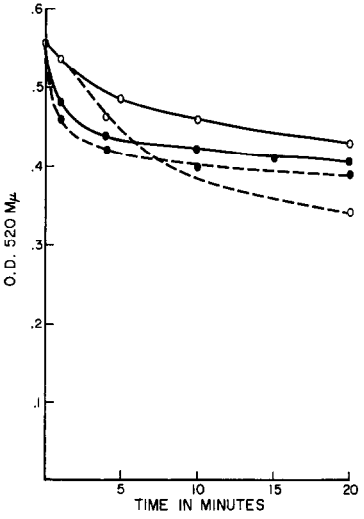

Fig. 1.

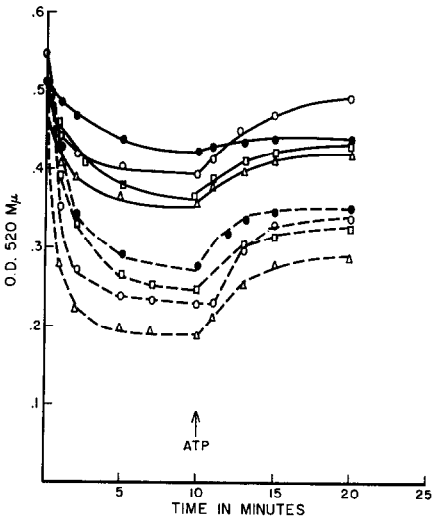

Fig. 2.

Fig. 1.—Swelling of mitochondria from normal and essential fatty acid (USFA) deficient mice, in 0.3 *M* sucrose or 0.154 *M* KCl buffered at pH 4 with 0.02 *M* Tris. Deficient mitochondria are indicated by – – – –, normal mitochondria by ——. ○, 0.3 *M* sucrose; ●, 0.154 *M* KCl.

Fig. 2.—The reversal of swelling of mitochondria by ATP. Deficient and normal mitochondria indicated as in Fig. 1. ●, 0.154 *M* KCl; ○, 0.154 *M* KCl–1×10^{-6} *M* thyroxine; △, H_2O; □, 0.154 *M* KCl–0.001 *M* Ca^{2+}. All mixtures buffered at pH 7.4 with 0.02 *M* Tris. At the point indicated by the arrow, 0.1 ml of 0.3 *M* ATP was added to cuvettes containing either 0.154 *M* KCl or 0.154 *M* KCl–1×10^{-6} *M* thyroxine, and 0.1 ml of 0.3 *M* ATP–0.09 *M* Mg^{2+}.

beyond their preparation did not respond, in the tests to be described, in a manner similar to those which were freshly prepared. This was presumed due to the fact that a considerable amount of swelling had already occurred. For this reason, the data reported concern only fresh mitochondrial preparations from both normal and essential unsaturated fatty acid deficient rats.

Deficient mitochondria, resuspended in either 0.3 *M* sucrose or 0.154 *M* KCl at room temperature, swelled much faster than normal ones (Fig. 1), in agreement with observations of Hayashida and Portman [8]. Ca^{2+}, thy-

roxine, and hypotonicity enhanced the swelling tendency of the deficient mitochondria, and ATP or ATP + Mg^{2+} produced reversals of swelling, similar to those seen in normal mitochondria. In addition, ATP alone brought about a marked reversal of the swelling occurring in 0.154 *M* KCl whereas it produced only a slight reversal—actually little more than an inhibition of further swelling—in normal mitochondria (Fig. 2).

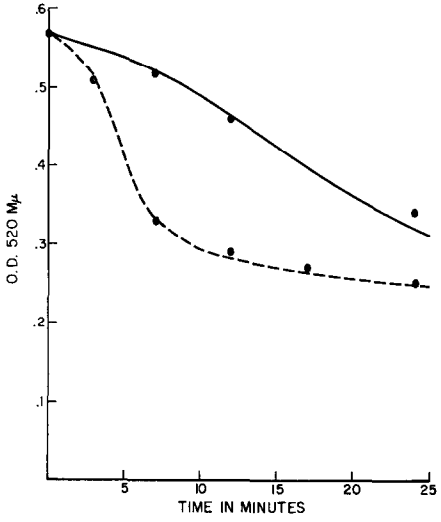

Fig. 3.

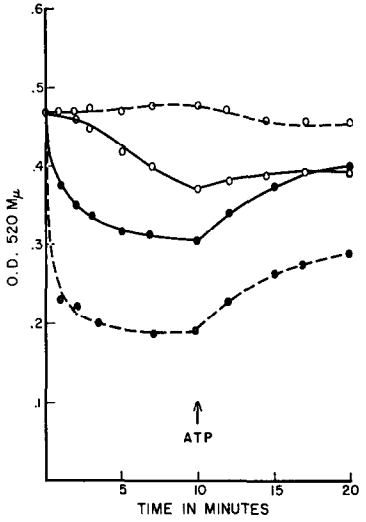

Fig. 4.

Fig. 3.—The influence of aging on mitochondrial swelling. Suspensions of mitochondria in 0.25 *M* sucrose–0.02 *M* Tris, pH 7.4 were kept at 30°C with occasional gentle stirring. At the indicated times 0.1 ml of each suspension was added to 2.9 ml 0.3 *M* sucrose at room temperature and the optical density measured. Normal and deficient mitochondria indicated as in Fig. 1.

Fig. 4.—The swelling effect of thyroxine on aged mitochondria. Normal and deficient mitochondria indicated as in Fig. 1. ○, mitochondria, suspended in 0.154 *M* KCl–0.02 *M* Tris, pH 7.4, were aged at 30°C for 30 min, after which 0.1 ml of the suspension and 0.03 ml of 0.001 *M* thyroxine was added to 2.9 ml of 0.154 *M* KCl–0.02 *M* Tris, and optical density measurements made. ●, same procedure except that the thyroxine was added to the mitochondrial suspension prior to aging. 0.1 ml of 0.3 *M* ATP was added to the cuvettes at the time indicated.

The increased swelling tendency of the essential fatty acid deficient mitochondria is further demonstrated in the following experiments. When mitochondria were aged for various periods of time at 30°C in 0.25 *M* sucrose, the deficient ones swelled much more rapidly than the normal ones (Fig. 3). When they were aged in 0.154 *M* KCl at 30°C for 30 min, normal mitochondria swelled at a slower rate, under the influence of thyroxine, than

did fresh preparations, and ATP produced a slight reversal of the swelling. Similarly aged unsaturated fatty acid deficient mitochondria, however, failed to respond either to the swelling influence of thyroxine or the contracting influence of ATP (Fig. 4).

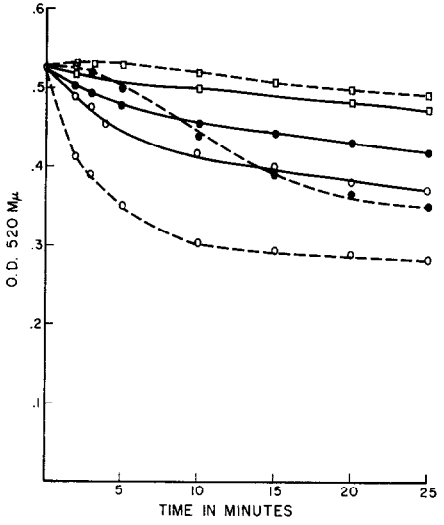

Fig. 5.

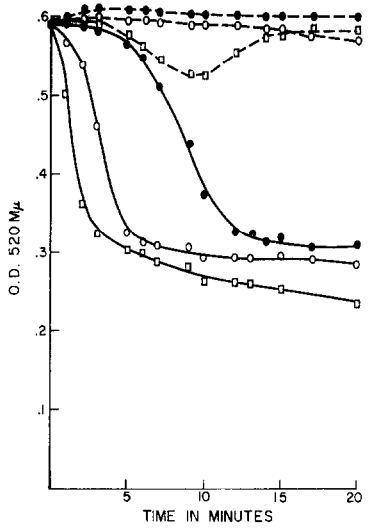

Fig. 6.

Fig. 5.—The swelling of mitochondria in different concentrations of sucrose. 0.1 ml of a suspension of mitochondria in 0.25 *M* sucrose was added to 2.9 ml of 0.3 *M* sucrose–0.02 *M* Tris, pH 7.4 as shown. USFA, essential unsaturated fatty acid. Normal and deficient mitochondria as indicated in Fig. 1. ——, normal; – – –, USFA deficient; ○, 0.25 *M* sucrose; ●, 0.50 *M* sucrose; □, 0.75 *M* sucrose.

Fig. 6.—The swelling of mitochondria in various concentrations of ATP. Swelling measurements were carried out in 0.3 *M* sucrose–0.02 *M* Tris, pH 7.4. Normal and deficient mitochondria as indicated in Fig. 1. ●, 0.01 *M* ATP; ○, 0.005 *M* ATP; □, 0.0025 *M* ATP.

Certain interesting effects of sucrose on mitochondrial swelling, presumed to be due to an effect on the coupling mechanism of oxidation to phosphorylation which controls contractibility of the mitochondrial membrane [13, 15] led us to examine the swelling of unsaturated fatty acid deficient mitochondria in it. Increasing the concentration of sucrose from 0.25 *M* to 0.75 *M* provided increasing protection against swelling in both normal and unsaturated fatty acid deficient mitochondria. Interestingly, 0.50 *M* and 0.75 *M* sucrose seemed to be slightly more protective toward the deficient than the normal mitochondria (Fig. 5).

When swelling occurred in a 0.25 *M* sucrose solution, in the presence of

0.01 *M*, 0.005 *M*, or 0.0025 *M* ATP, normal mitochondria swelled with little indication of protection. However, nearly complete protection against swelling was observed with the unsaturated fatty acid deficient mitochondria at all levels of ATP employed (Fig. 6). This was unexpected in view of the ob-

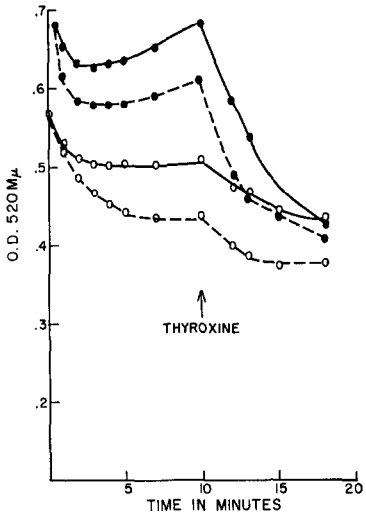

Fig. 7.

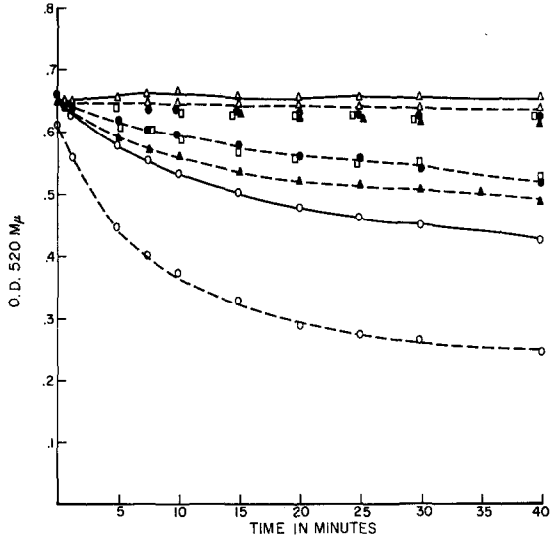

Fig. 8.

Fig. 7.—Mitochondrial swelling in a phosphorylating medium. Normal and deficient mitochondria as indicated in Fig. 1. ● 0.1 ml mitochondria suspension in 0.25 *M* sucrose added to 2.9 ml of the complete phosphorylating medium described earlier [12], employing succinate as substrate. ○, 0.154 *M* KCl–0.02 *M* Tris, pH 7.4. 0.03 ml of 0.001 *M* thyroxine added as indicated.

Fig. 8.—The swelling of mitochondria that had been prepared in the presence of various swelling inhibitors. Normal and deficient mitochondria as indicated in Fig. 1. △, 0.001 *M* EDTA; □, 0.002 *M* CN^-; ○, no addition; ▲, 0.0034 *M* amytal; ●, 0.002 *M* azide. Mitochondria were prepared and swelling measurements made in the concentrations of the agents shown, in 0.25 *M* sucrose.

served lability of the latter to swelling. Both deficient and normal mitochondria swelled, in 0.154 *M* KCl and the same concentrations of ATP, in a manner similar to that shown in Fig. 6 for the deficient ones.

When either normal or deficient mitochondria were suspended in a complete phosphorylating medium, both swelled rapidly for 2–3 min and then contracted. The swelling rate in the unsaturated fatty acid deficient mitochondria was greater, however, and the extent of contraction less, than that observed in the normal mitochondria. After being in the phosphorylating medium for 10 min, both mitochondrial preparations swelled at similar rates upon the addition of thyroxine. The swelling rates in both were

similar, and were greater than that which occurred when thyroxine was added to mitochondria that had been in 0.154 *M* KCl for the same period of time (Fig. 7).

In view of the marked spontaneous swelling tendency of mitochondria obtained from unsaturated fatty acid deficient rat livers, and the likelihood

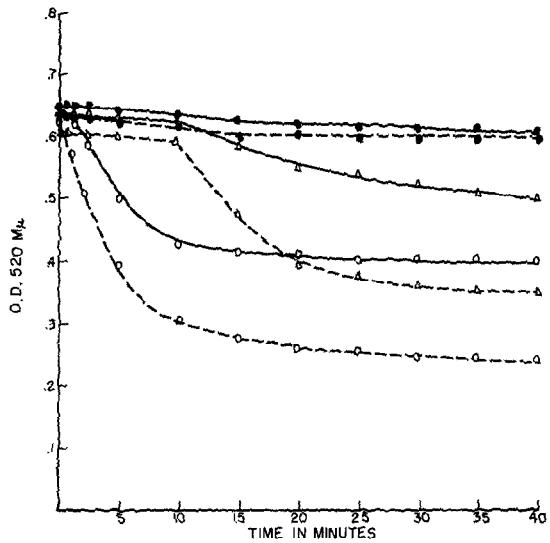

Fig. 9.—The swelling of mitochondria that had been prepared in the presence of ATP or ATP + Mg^{2+}. Normal and deficient mitochondria as indicated in Fig. 1. ○, no addition to the preparation medium; △, 0.01 *M* ATP; ●, 0.01 *M* ATP + 0.003 *M* Mg^{2+}. Mitochondria were prepared and swelling measurements made in concentrations given, in 0.25 *M* sucrose.

that swelling occurs in them during the isolation procedures, the effects of several swelling inhibitors, when they were present throughout the entire preparation of the mitochondria, were investigated. Normal and unsaturated fatty acid deficient mitochondria were prepared as usual, except that the preparation medium contained, in addition to 0.25 *M* sucrose, either 0.001 *M* EDTA, 0.002 *M* sodium cyanide, 0.0034 *M* amytal, 0.002 *M* sodium azide, 0.01 *M* ATP, or 0.01 *M* ATP + 0.003 *M* Mg^{2+}. For swelling measurements a portion of the mitochondrial suspension was then transferred either to a fresh sample of the same medium, or to 0.3 *M* sucrose, containing 0.02 *M* Tris, pH 7.4. In the case of the latter, the suspension contained the small amount of the inhibiting substance that was transferred with the original mitochondrial suspension. The final concentration of each inhibitor is indicated with the data presented.

EDTA, cyanide, azide, and amytal completely protected normal mitochondria against swelling (Fig. 8), in agreement with observations of others [8, 9]. EDTA, however, was the only one of these agents that prevented swelling of

the unsaturated fatty acid deficient mitochondria. Cyanide, azide and amytal gave only partial protection. When 0.1 ml of suspensions of mitochondria isolated in the presence of the agents shown in Fig. 8 were transferred to 2.9 ml of 0.3 *M* sucrose–0.02 *M* Tris[1] and swelling measurements made, the results were the same as those described in Fig. 8. No swelling was observed

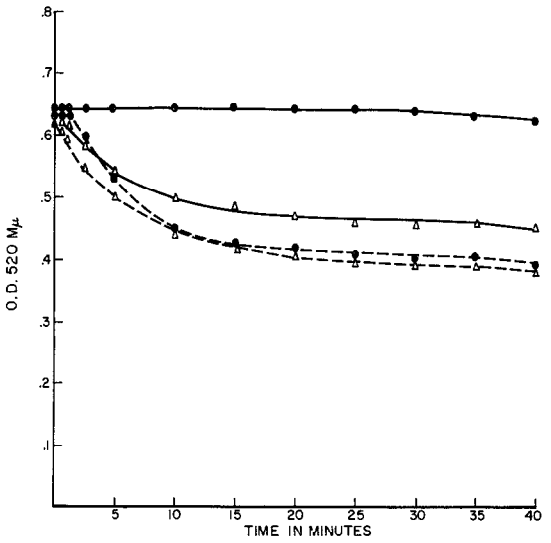

Fig. 10.—The swelling of mitochondria that had been prepared in the presence of ATP or ATP + Mg^{2+}. Normal and deficient mitochondria as indicated in Fig. 1. The preparations were made as indicated in Fig. 9. Suspensions of these preparations were transferred for swelling studies to 0.25 *M* sucrose to produce final concentrations as follows: △, 1.7×10^{-4} *M* ATP; ●, 1.7×15^{4} *M* ATP–5×10^{-5} *M* Mg^{2+}.

in any of the normal preparations over a 30 min period; the deficient mitochondria prepared in the presence of EDTA did not swell, but cyanide, azide, and amytal again gave only partial protection, approximately to the same extent as shown in Fig. 8.

Both normal and deficient mitochondria, isolated in a medium containing 0.01 *M* ATP or 0.01 *M* ATP + 0.003 *M* Mg^{2+} and examined in the same medium, were partially protected by ATP, and were completely prevented from swelling by ATP + Mg^{2+} (Fig. 9). When the mitochondria were transferred to 0.3 *M* sucrose, the normal mitochondria previously isolated in ATP + Mg^{2+} again were completely protected. The unsaturated fatty acid deficient mitochondria were not, however (Fig. 10). It appeared as though the normal mitochondria, having been isolated in the presence of either EDTA or ATP + Mg^{2+}, carried over a residual protection against swelling. Unsaturated fatty acid deficient mitochondria were stabilized similarly by

[1] The concentrations of the inhibitors were as follows: EDTA, 3.3×10^{-5} *M*; cyanide, 6.6×10^{-5} *M*; amytal, 1.1×10^{-4} *M*; 6.6×10^{-5} *M* azide.

EDTA but not by 0.01 ATP + 0.003 *M* Mg^{2+}, despite the fact that the latter when present in the preparation and swelling medium, prevented their swelling.

The possibility was considered that the two mitochondria might exhibit a different binding tendency for ATP in the presence of Mg^{2+}. To test this,

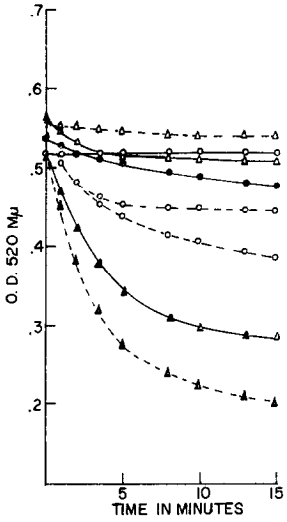

Fig. 11.—The swelling of mitochondria in dilute EDTA or ATP + Mg^{2+}. Mitochondria were prepared in 0.25 *M* sucrose as usual, and suspended in 0.3 *M* sucrose containing one of the following for swelling measurements: ○, 0.001 *M* EDTA; ●, 3×10^{-5} *M* EDTA; △, 0.01 *M* ATP + 0.003 *M* Mg^{2+}; ▲, 1.7×10^{-4} *M* ATP + 5×10^{-5} *M* Mg^{2+}. Normal and deficient mitochondria as indicated in Fig. 1.

they were allowed to incubate for 30 minutes in 0.01 *M* ATP–^{14}C + 0.003 *M* Mg^{2+}, both in phosphorylating and non-phosphorylating media. No evidence was obtained that ATP was bound to the normal to any greater extent than to the deficient mitochondria.

To ascertain whether the extremely low concentrations of EDTA or ATP + Mg^{2+} that resulted when mitochondrial suspensions were transferred to sucrose for swelling measurements might prevent swelling, the following experiment was performed. Mitochondria were prepared as usual in the absence of either EDTA or ATP + Mg^{2+}, and were resuspended in the media shown in Fig. 11. 0.01 *M* ATP + 0.003 *M* Mg^{2+} afforded protection against swelling to both normal and deficient mitochondria, but 1/60 the concentrations of these gave little or no protection for either. It will be recalled that the latter concentrations gave complete protection for the normal mitochondria that had been isolated in the presence of ATP + Mg^{2+}, but not for deficient ones (Fig. 9). Both concentrations of EDTA prevented swelling in the normal and partially prevented it in the deficient mitochondria.

DISCUSSION

The marked swelling tendency displayed by the essential unsaturated fatty acid deficient mitochondria appears, from these experiments, to be associated with electron transport and/or oxidative phosphorylation, rather than with a defect in the membranes in which swelling could be stabilized by EDTA. For example, ATP reverses the swelling of deficient mitochondria that occurs in 0.154 *M* KCl to a greater extent than it does in normal mitochondria. Also, the ability of the latter to "carry over" a protection due to ATP $+Mg^{2+}$, when they are isolated in the presence of these agents, while deficient mitochondria do not, would seem to indicate an (ATP $+Mg^{2+}$)-mitochondria relationship in the case of normal rats that does not occur in the deficient animals. This may be of importance in a consideration of the reason for the differences between the two in oxidative phosphorylating capacity, and is being investigated further.

The fact that ATP prevented the swelling of unsaturated fatty acid deficient mitochondria suspended in either 0.3 *M* sucrose or 0.154 *M* KCl, but prevented swelling of the normal ones only in the KCl medium is of interest, and may be related to the weakly coupled oxidative phosphorylation observed in deficient mitochondria. Sucrose inhibits both swelling and contraction [13, 14, 15], ATPase, DNP-activated ATPase (but not Mg^{2+} activated ATPase), and ATP–$^{32}P_i$ exchange [3, 4], and it has an uncoupling effect on oxidative phosphorylation [5]. Part of its activity, at least, has been presumed to be an inhibition of an enzymic reaction associated with oxidative phosphorylation, which in turn controls contracting and swelling.

While deficient mitochondria are protected from swelling by a complete phosphorylating medium, the protection is not as great as that afforded normal mitochondria. The significance of this observation may be obscured, however, by the fact that deficient mitochondria undoubtedly swell more throughout the process of their preparation than the normal ones, which may alter the quantitative nature of the response shown in Fig. 7.

Swelling in normal mitochondria has been assumed to be dependent in some way on the activities of the electron transport system [1, 2, 6]. In the present experiments, inhibitors of this system prevented swelling of normal mitochondria, but gave only partial protection to deficient ones, even when they were present throughout the entire isolation procedure. It has been observed in this laboratory that these agents inhibit respiration in both deficient and normal mitochondria.[1] It would seem from this, then, that defici-

[1] Unpublished experiments.

ent mitochondria can swell in the absence of electron transport. In connection with these observations it is of interest that with aging there is a loss of respiratory control in deficient mitochondria, before it occurs in the normal ones.[1]

Deficient mitochondria aged at 30°C swell rapidly, most of the swelling taking place within 10–12 min, while normal mitochondria swell relatively slowly (Fig. 3). Of possible interest in this connection is the observation that unsaturated fatty acid deficient mitochondria aged in 0.25 *M* sucrose at 30°C for as little as 7½–10 min are almost completely uncoupled, whereas a much longer aging period is required for uncoupling in normal mitochondria.[1]

SUMMARY

Liver mitochondria from essential fatty acid deficient rats swell more rapidly, both spontaneously and in the presence of a number of swelling accelerators, than do normal mitochondria. ATP reverses the swelling of deficient mitochondria in 0.154 *M* KCl to a greater extent than it does with normal ones. Reversal effects with both mitochondria in the presence of Ca^{2+} and thyroxine, are similar. Azide, amytal, and cyanide inhibit the swelling of normal mitochondria suspended in sucrose, but only partially prevent it in deficient ones. 0.01 *M* ATP prevented the swelling of deficient mitochondria in the presence of either 0.3 *M* sucrose or 0.154 *M* KCl, but prevented swelling of normal mitochondria only in the KCl. An (ATP + Mg^{2+})-mitochondrial relationship appears to exist in the case of normal mitochondria that is not found in the deficient ones. EDTA, present in the medium throughout the process of preparation of mitochondria, prevents swelling in both deficient and normal ones. It is suggested that the swelling tendency in liver mitochondria from essential fatty acid deficient rats is associated with altered respiratory and/or phosphorylating mechanisms rather than with a membrane alteration.

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