[12] M. DEMEREC, *Cold Spring Harbor Symp. Quant. Biol.*, 21 (1956) 113.
[13] G. PONTECORVO, *Trends in Genetic Analysis*, Columbia University Press, 1958.
[14] J. LEDERBERG AND E. L. TATUM, *Cold Spring Harbor Symp. Quant. Biol.*, 11 (1946) 113.
[15] E. L. WOLLMAN, F. JACOB AND W. HAYES, *Cold Spring Harbor Symp. Quant. Biol.*, 21 (1956) 141.
[16] E. S. LENNOX, *Virology*, 1 (1955) 190.
[17] A. TORRIANI, *Biochim. Biophys. Acta*, 38 (1960) 460.
[18] P. D. SKAAR AND A. GAREN, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 619.
[19] L. L. CAVALLI, *Boll. ist. sieroter, Milan*, 29 (1950) 281.
[20] R. K. MORTON, *Methodes of Enzymology*, in S. P. COLOWICK AND N. O. KAPLAN, Vol. II, Academic Press, 1955, p. 538.
[21] H. A. SOBER AND E. A. PETERSON, *J. Am. Chem. Soc.*, 78 (1956) 756.
[22] C. S. FRENCH AND H. W. MILNER, *Methods in Enzymology*, Vol. I, Academic Press, N.Y., 1956. The device is obtained from American Instrument Co., Silver Spring, Md.
[23] E. T. BOLTON, B. H. HAGAR AND D. B. BRITTON, in R. B. ROBERTS, *Microsomal Peptides and Protein Synthesis*, Pergamon Press, 1958.
[24] J. C. MATHIES, *J. Biol. Chem.*, 233 (1958) 1121.
[25] A. EHRENBERG, *Acta. Chem. Scand.*, 11 (1957) 1257.
[26] J. T. EDSALL, in NEURATH AND BARLEY, *The Proteins*, Chapter 7, Academic Press, N.Y., 1953.
[27] R. K. MORTON, *Biochem. J.*, 61 (1955) 232.
[28] M. COHN, *Bacteriol. Rev.*, 21 (1957) 140.
[29] A. B. PARDEE, F. JACOB AND J. MONOD, *J. Mol. Biol.*, in the press.
[30] C. L. MARKERT AND F. MØLLER, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 753.
[31] C. E. HALL AND H. S. SLAYTER, *J. Biophys. Biochem. Cytol.*, 5 (1959) 11.
[32] R. B. ROBERTS, P. H. ABELSON, D. B. COWIE, E. T. BOLTON AND R. J. BRITTEN, *Studies of Biosynthesis in E. coli*, Carnegie Institution of Washington, Washington, D.C., 1955.
[33] O. SMITHIES, *Biochem. J.*, 61 (1955) 629.

Biochim. Biophys. Acia, 38 (1960) 470–483

MITOCHONDRIAL SWELLING AND ELECTRON TRANSPORT

I. SWELLING SUPPORTED BY FERRICYANIDE

J. B. CHAPPELL AND G. D. GREVILLE
Department of Biochemistry, University of Cambridge (Great Britain)

(Received May 20th, 1959)

SUMMARY

The swelling of rat-liver mitochondria in presence of phosphate is inhibited by cyanide but restored by further addition of ferricyanide. The effects on swelling of inhibitors of electron transport (amytal, antimycin A) and of compounds which prevent phosphorylation (2,4-dinitrophenol, oligomycin) have been studied in presence and absence of ferricyanide and cyanide. It is concluded that, under defined conditions, swelling is supported by electron transport, even through a restricted portion of the respiratory chain, by a mechanism not directly involving the coupled phosphorylation process.

INTRODUCTION

That the swelling of isolated rat-liver mitochondria in the presence of phosphate or thyroxine is dependent on respiration is indicated by the following considerations.

With freshly-prepared mitochondria a variety of inhibitors of electron transport suppress swelling; thus, under appropriate conditions, antimycin A[1-3], amytal[1,2], oligomycin[2,4] and cyanide[1,3,5], when present in concentrations known to inhibit many mitochondrial oxidations, also prevent swelling. In the case of mitochondria which have been allowed to age by storing at 0° for one day in 0.44 *M* sucrose ("aged" mitochondria) it is necessary to add an oxidizable substrate, e.g. D-β-hydroxybutyrate, before phosphate or thyroxine can exert their effects. The inhibition by malonate of the swelling supported by succinate lends further support to the hypothesis that respiration is a necessary accompaniment of mitochondrial swelling[1].

It appears therefore that the interruption of the electron-transport chain at various points by the addition of specific inhibitors can prevent swelling. These observations do not indicate, however, whether swelling can be supported by electron transport in a restricted portion of the respiratory chain. We have therefore studied mitochondrial swelling when cyanide was present to inhibit the cytochrome oxidase and ferricyanide was added to act as terminal electron acceptor, and also when ascorbate was added to act as a substrate for the cytochrome *c*–cytochrome oxidase system.

Some parallel observations on the rate of reduction of ferricyanide by mitochondria are included in another communication[6].

MATERIALS AND METHODS

Materials

Water distilled in glass was used and, except where indicated otherwise, AnalaR reagents. Glass apparatus was cleaned with H_2SO_4. Ethanol was redistilled in glass. Tris(hydroxymethyl)aminomethane (tris) (Sigma 121) was recrystallized from ethanol. To prepare the buffer, tris was brought to pH 7.5 with HCl which had been twice redistilled in glass. 2,4-Dinitrophenol was synthesized from 1-chloro-2,4-dinitrobenzene and recrystallized 6 times; m.p. 112.5–113.5° (uncorr.). A neutral solution was prepared with addition of the requisite $KHCO_3$ and removal of the CO_2 *in vacuo*. To prepare sodium DL-β-hydroxybutyrate, the ethyl ester (Eastman Kodak) was distilled and the fraction 83 —4° (20 mm Hg) collected; this was saponified at room temperature with 0.8 *N* NaOH and the salt recrystallized 3 times from ethanol. Succinic acid was neutralized with KOH, and KCN with redistilled HCl. Potassium phosphate pH 7.5 was made from KH_2PO_4 and KOH. Antimycin A was obtained from the Wisconsin Alumni Foundation and oligomycin (a mixture of the A, B and C forms) was kindly given by Dr. B. C. PRESSMAN. L-Ascorbic acid (B.P. quality) was obtained from Roche Products Ltd.

As recommended by FONNESU AND DAVIES[7], the sucrose was deionized. 1750 ml of *M* sucrose were passed through a column, 21 mm in diameter, of Amberlite Monobed Resin MB-1 (50 ml when dry) in about 1.5 h. The acidic component (IR 120) catalyses the hydrolysis of sucrose, but when the solution was passed through at the rate stated the breakdown, as measured by the colorimetric method of SOMOGYI[8], was only about 0.2 %.

Mitochondria

Mitochondria were prepared from livers of male hooded Norwegian rats (180–250 g

body weight; fed *ad libitum*), 0.44 *M* sucrose[9] being used throughout. The preparation was carried out in a room at 4°; all containers were immersed in ice and water, and the centrifuge set at —1°. Rapidly-cooled liver was finely cut with scissors and homogenized by hand (9 strokes) in a Potter-Elvehjem homogenizer with Perspex pestle (0.4 mm clearance on the diameter). The 10% (v/v) homogenate was centrifuged for 10 min at 600 × *g*. Application of the principles of DE DUVE AND BERTHET[10,11] allowed the sedimentation of the mitochondria at the M.S.E. Major refrigerated centrifuge without recourse to the high-speed attachment. The automatic starting device routinely fitted to this machine gives reproducible acceleration and hence facilitates the application of the required total (*i.e.* integrated) *g*-min. 50-ml plastic tubes were used throughout, with not more than 25 ml of suspension in each, in swing-out metal buckets; R_{max} was 20.1 cm. The centrifuge was set to run at 3,600 rev/min. The mitochondrial fraction was sedimented by a total of 39,200 *g*-min (R_{min}, 15.7 cm; S_{min}, 19,600 *S*). The particles in each tube (2.5 gequiv.) were then washed by addition of sucrose to 8 ml (R_{min}, 18.6 cm; 34,700 *g*-min; S_{min}, 7,800 *S*), and then once more in the same volume (43,100 *g*-min; S_{min}, 6,300 *S*). After the initial sedimentation of the mitochondria the supernatant was decanted, but the washings were removed by a pipette with a bent tip. The final suspension contained 0.5 gequiv. of mitochondria/ml (approx. 2 mg N/ml). It was stored in a narrow covered cylinder which was surrounded by ice and water in a vacuum flask which was kept in a refrigerator.

Measurement of swelling

Changes in extinction at 520 mμ (1-cm light path), read in a Beckman model DU spectrophotometer, were taken as a measure of swelling. Water from a thermostat was circulated through Beckman "thermospacers" on either side of the cell compartment and kept the latter at 20°. Six experimental cells were used in each run, one set of three being put into a narrow metal box immersed in the thermostat when the other set was in the cell compartment.

Small volumes of fluid were added to the cells by means of an Agla micrometer syringe fitted with glass delivery tube. 0.05 ml of mitochondrial suspension was added to 2.95 ml of incubation medium at zero time. In every case the medium was buffered with 25 m*M* tris-chloride pH 7.5 and contained 0.3 *M* sucrose (final concentrations). DL-β-Hydroxybutyrate when added was in 2 m*M* concentration, other substrates in 1 m*M*, inorganic phosphate in 10 m*M*, and cyanide and potassium ferricyanide each in 1 m*M*.

RESULTS

Fresh mitochondria

The swelling of fresh mitochondria induced by phosphate was suppressed by the addition of cyanide and was restored by the further addition of ferricyanide (Fig. 1). The rate of swelling was then almost as great as with phosphate alone, but the extent was slightly reduced. In the absence of phosphate, ferricyanide, with or without cyanide, produced no swelling. Hence in the presence of cyanide, both ferricyanide and phosphate must be present together in order to produce an effect.

Fresh mitochondria have been shown to contain intrinsic substrates, *e.g.* citrate[12],

lactate[13] and precursors of glutamate[14], and to have a significant endogenous respiration[15]. Furthermore, endogenous respiration is still observed when ferricyanide is the terminal acceptor[6]. We suggest that the swelling of fresh mitochondria is supported by the oxidation of intrinsic substrates when either oxygen or ferricyanide is the ultimate acceptor, and that the experiment shown in Fig. 1 provides evidence that swelling may be supported by electron transport in a restricted part of the respiratory chain.

When DL-β-hydroxybutyrate was present the findings were similar to those depicted in Fig. 1, except that where swelling occurred it was somewhat more extensive. This difference has also been observed in the absence of ferricyanide[2]. The rate of reduction of ferricyanide in the presence of β-hydroxybutyrate is not affected by cyanide[6]. The failure of cyanide to decrease the rate of swelling produced by phosphate in presence of ferricyanide may be interpreted in terms of this observation.

Since mitochondria are stabilized by ethylenediamine tetraacetate[16] and other chelating agents[17] the possibility exists that cyanide may suppress swelling by combining with an essential metal. The finding that swelling, inhibited by cyanide, may be restored by ferricyanide, renders this unlikely.

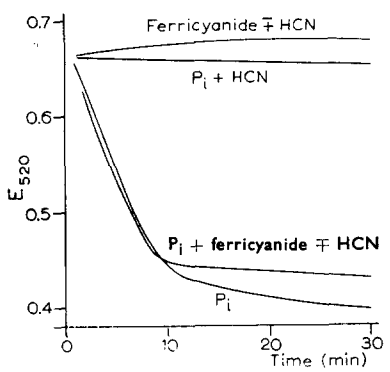

Fig. 1. The inhibition by cyanide of the swelling of freshly-prepared mitochondria in presence of phosphate (P_i), and the restoration of swelling by ferricyanide.

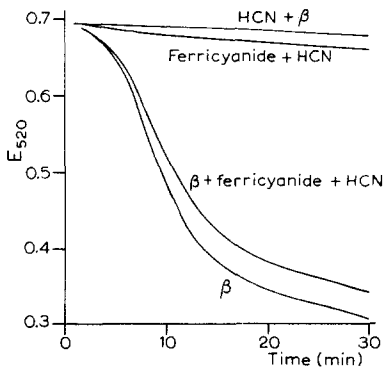

Fig. 2. The inhibitory effect of cyanide on the phosphate-induced swelling of aged mitochondria in the presence of DL-β-hydroxybutyrate (β). All cells contained phosphate. With cyanide present, swelling occurred only when phosphate, ferricyanide and substrate were all added as well.

Aged mitochondria

After the mitochondria had been stored at 0° in 0.44 *M* sucrose for 22–24 h, phosphate would not induce swelling in presence of cyanide and ferricyanide. On further addition of β-hydroxybutyrate rapid swelling occurred, almost equal in rate and extent to that obtained with phosphate and the substrate in the absence of cyanide and ferricyanide (Fig. 2). The pattern observed in the presence of substrate was therefore similar to that obtained with fresh mitochondria in absence of added substrate (Fig. 1). Succinate or L-glutamate also promoted swelling in presence of phosphate, cyanide and ferricyanide, but were much less effective than β-hydroxybutyrate (Fig. 3). In these experiments with aged mitochondria it has been shown that the process of swelling requires the simultaneous presence of a hydrogen donor

(substrate) and an electron acceptor (ferricyanide) as well as a swelling agent (phosphate). The conclusion seems inescapable that swelling was supported by electron transport, in this instance along a restricted portion of the respiratory chain.

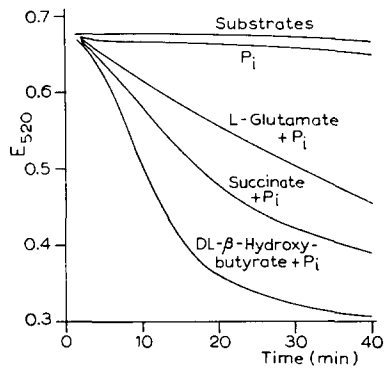

Fig. 3. The swelling of aged mitochondria in the presence of phosphate (P_i), ferricyanide, cyanide and the oxidizable substrates indicated. Swelling did not occur when phosphate or substrate was omitted.

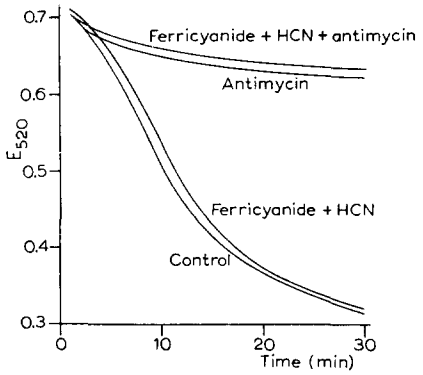

Fig. 4. The inhibition by antimycin A (20 mμg/ml) of phosphate-induced swelling of aged mitochondria in presence of β-hydroxybutyrate, with and without ferricyanide and cyanide. All cells contained phosphate.

Inhibition of swelling by antimycin A *and amytal*

With aged mitochondria the swelling induced by phosphate and supported by β-hydroxybutyrate was abolished by low concentrations of antimycin[1,2]. This is seen in Fig. 4, which shows that antimycin (20 mμg/ml) had a similar effect when ferricyanide was added as terminal electron acceptor. Swelling supported by succinate and L-glutamate was also largely suppressed by antimycin under these conditions; indeed, the inhibition was more profound than was the case when oxygen served as terminal acceptor (Fig. 5). However, with the latter acceptor and in presence of succinate, the inhibition by antimycin becomes nearly complete when amytal is added

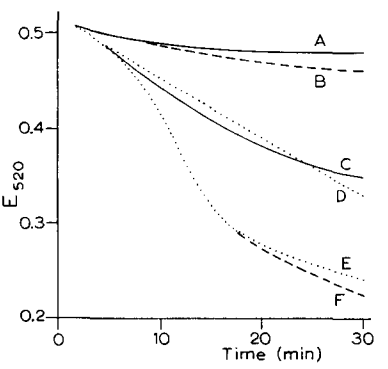

Fig. 5. The effects of amytal (2 m*M*) and antimycin A (20 mμg/ml) on the swelling of aged mitochondria in the presence of phosphate and succinate, with and without ferricyanide and cyanide. A, Antimycin, ferricyanide, cyanide; B, amytal, antimycin; C, ferricyanide, cyanide; D, antimycin; E, control; F, amytal.

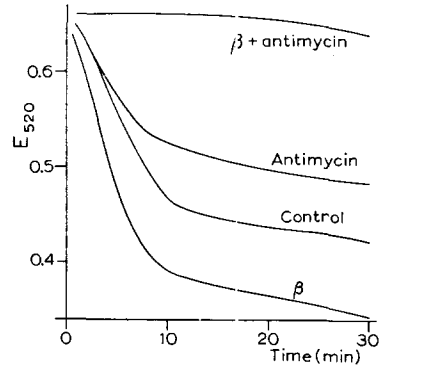

Fig. 6. The effect of antimycin A (20 mμg/ml) on phosphate-induced swelling of fresh mitochondria in presence of cyanide and ferricyanide, with and without β-hydroxybutyrate (β). Antimycin has little effect in the absence of β-hydroxybutyrate.

as well, despite the fact that amytal alone accelerates swelling (Fig. 5, 7). These findings are discussed below.

With fresh mitochondria antimycin has relatively little effect on phosphate-induced swelling in the absence of added substrate, but it becomes strongly inhibitory in the presence of β-hydroxybutyrate[2]. The effect was still obtained in the presence of added cyanide and ferricyanide (Fig. 6). This protective action of β-hydroxybutyrate together with antimycin was also obtained with aged particles with oxygen as electron acceptor; thus the phosphate-induced swelling supported by succinate or L-glutamate was inhibited by only 20 to 50% in presence of antimycin (10–50 mμg/ml)[1,2], but when DL-β-hydroxybutyrate was added as well complete inhibition resulted. These phenomena could not be investigated with ferricyanide as acceptor since, as mentioned above, the swelling supported by succinate or L-glutamate was largely suppressed by antimycin in the absence of β-hydroxybutyrate. The stabilizing action of the combination of β-hydroxybutyrate and antimycin is not easily interpreted in terms of present concepts and is being investigated further.

In contrast to antimycin, which inhibits the oxidation of succinate as well as of substrates with DPN-linked dehydrogenases by interrupting the respiratory chain in the vicinity of cytochrome *b*[18], the barbiturate amytal (5-ethyl-5-isoamylbarbiturate) suppresses the oxidation of the latter type of substrate while leaving the oxidation of succinate unaffected[19]. This compound is considered to act by inhibiting the electron-transport system between DPN and flavoprotein[18]. With ferricyanide as terminal electron acceptor the phosphate-induced swelling in the presence of β-hydroxybutyrate was completely suppressed by amytal, whereas that supported by succinate was even slightly accelerated (Fig. 7). These results parallel previous observations with oxygen as terminal acceptor[1,2].

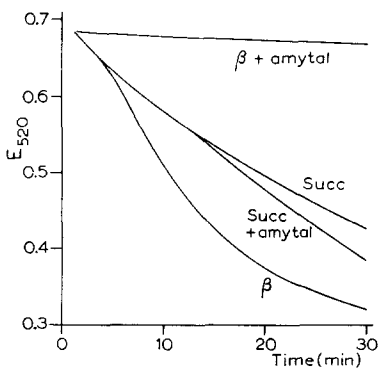

Fig. 7. The action of amytal (2 m*M*) on the swelling of aged mitochondria in presence of phosphate, cyanide and ferricyanide, and either succinate (succ) or β-hydroxybutyrate (β). Amytal fails to inhibit swelling supported by succinate.

Oligomycin and 2,4-dinitrophenol

The antibiotic oligomycin has been shown to inhibit the oxidation of β-hydroxybutyrate and glutamate in a mitochondrial system in which respiration was coupled to phosphorylation; the further addition of 2,4-dinitrophenol released the inhibition, indicating that oligomycin acts on the phosphorylation system itself[20]. Oligomycin

(0.4 μg/ml) had little or no effect on the phosphate-induced swelling of fresh mitochondria with oxygen[2,4] (Fig. 8a) or ferricyanide (Fig. 8b) as terminal electron acceptor. In the presence of β-hydroxybutyrate and still with fresh mitochondria oligomycin considerably reduced swelling, for some time, with both electron acceptor systems (Figs. 8a, b). When cyanide and ferricyanide were present the period of inhibition was shorter than in their absence, and the inhibitory effect was lost more readily with ageing of the mitochondria. 0.01 m*M* 2,4-dinitrophenol completely abolished the effect of oligomycin when added initially (Fig. 8a, b) or later[2].

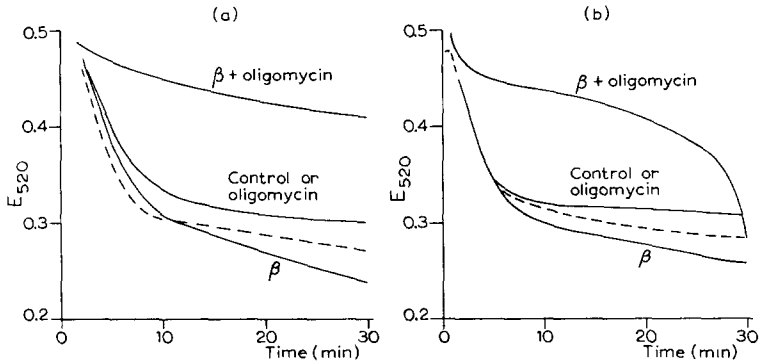

Fig. 8. The inhibition of the phosphate-induced swelling of freshly-prepared mitochondria by oligomycin (0.4 μg/ml) in the presence and absence of β-hydroxybutyrate (β), (a) without and (b) with cyanide and ferricyanide. In both (a) and (b), the broken curves show the response in presence of β-hydroxybutyrate and 0.01 m*M* 2,4-dinitrophenol, identical curves being obtained with and without oligomycin. Oligomycin inhibits only in presence of added substrate, and dinitrophenol releases this inhibition.

These results lead almost inevitably to the conclusion that it is the electron transport, and not its associated phosphorylation, which is a requirement for swelling under these various conditions. This might appear to be contradicted by the well-documented fact that dinitrophenol prevents mitochondrial swelling under some circumstances[2,3,16]. However, a closer investigation of the effects of this phenol and of dicoumarol has revealed that they only inhibit swelling when present both initially and in relatively high concentrations; in contrast, under other conditions they can increase the rate of swelling[4]. The inhibitory effect of higher concentrations of dinitrophenol, which also appears with ferricyanide as acceptor (Fig. 9), has been suggested by us to be due either to a deleterious effect on the co-factors of respiration or to an action on the mitochondrial structure itself[4].

Ascorbate

The above observations have been concerned with the support of swelling by electron transfer between substrate and a carrier above the point of action of antimycin, probably cytochrome *c*. In an attempt to demonstrate that swelling can be supported by electron transport between cytochrome *c* and oxygen, the effect of L-ascorbate on swelling of aged mitochondria in presence of phosphate was tested (Fig. 10). During 30 min either L-ascorbate alone (*cf.* ref. [21]) or phosphate alone had little effect but together they produced appreciable swelling. This was, however, much smaller than that due to phosphate together with β-hydroxybutyrate. If it can be

assumed that these mitochondria can oxidize L-ascorbate in absence of added cytochrome *c*, and there is some evidence of this from LEHNINGER, HASSAN AND SUDDUTH, Table II[22], then Fig. 10 affords some evidence that swelling may be supported by electron transport beyond cytochrome *c*.

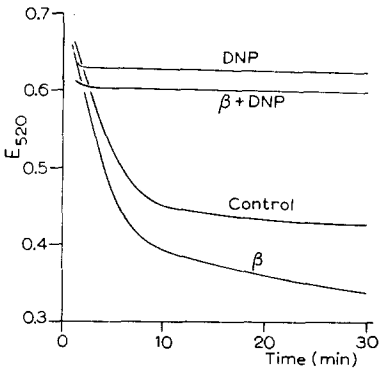

Fig. 9. Inhibition by 0.1 m*M* 2,4-dinitrophenol (DNP) of the swelling of fresh mitochondria in presence of phosphate, cyanide and ferricyanide, with and without DL-β-hydroxybutyrate (β).

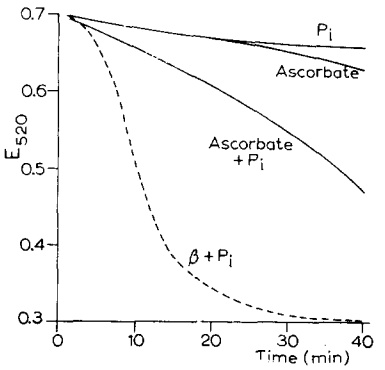

Fig. 10. Effect of 2 m*M* L-ascorbate in absence and presence of phosphate (P_i) on the swelling of aged mitochondria. The effect of DL-β-hydroxybutyrate (β) is included for comparison.

DISCUSSION

Dependence of swelling on electron transport

It seems clear that for swelling to occur in presence of the swelling agent phosphate the following conditions must be fulfilled: (a) presence of hydrogen donor, (b) presence of hydrogen acceptor and (c) electron transport through some part of the respiratory chain. The requirement for hydrogen donor is shown best by the fact that aged mitochondria will not swell unless an oxidizable substrate is added[1]. With fresh mitochondria we consider that the swelling is supported by endogenous respiration (p. 486), and strong evidence for this is provided by the inhibitory action of amytal and the restoration of swelling on further addition of succinate (Fig. 11a;

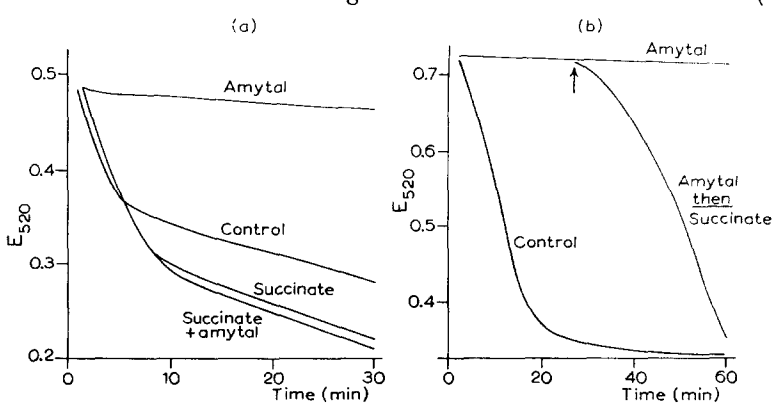

Fig. 11. (a) The phosphate-induced swelling of fresh mitochondria is inhibited by 2 m*M* amytal and restored by further addition of succinate. (b) The swelling of aged mitochondria in presence of phosphate and β-hydroxybutyrate is inhibited by 2 m*M* amytal. Addition of succinate after 25 min (arrow) initiates swelling.

see also HUNTER, FINK AND HURWITZ[23]). The requirement for hydrogen acceptor is shown by the experiments with ferricyanide in the present paper and by the abolition of swelling by the exclusion of oxygen[24]. The necessity for electron transport is established by the effects of inhibitors of the respiratory chain[1,2,5,23]. The restoration of swelling by ferricyanide after inhibition by cyanide and, possibly, the findings with ascorbic acid (p. 490) indicate that electron transport over even a restricted portion of the respiratory chain may be adequate. There is evidence, although somewhat less extensive than that for phosphate, that swelling induced by thyroxine[1,2,5,25] and phlorizin[26], and also the swelling of aged mitochondria in 0.2 *M* sucrose buffered with tris[1], are also dependent on electron transport.

LEHNINGER and his co-workers RAY AND SCHNEIDER have proposed an alternative, namely that swelling is dependent on the oxidation-reduction state of the respiratory carriers. On the basis of the suppression by cyanide, or under anaerobic conditions by β-hydroxybutyrate, LEHNINGER AND RAY[5] concluded that when the electron carriers are in the reduced state swelling cannot occur. Subsequently LEHNINGER and his colleagues[26,25], from observations with amytal and antimycin, deduced that it is the oxidation state of the mitochondrial DPN which is decisive for swelling; that the mitochondria are susceptible to thyroxine and phlorizin only when at least some of the DPN is in the oxidized state; and that the state of the other carriers appears to be irrelevant. However, all the evidence put forward by these workers is equally explicable by our thesis that the swelling is dependent on electron transport, but the suggestion that swelling is governed by the degree of oxidation of the bound DPN appears to be inconsistent with the following observations, which are explicable on the basis of the necessity for electron transport: (a) In the presence of phosphate or thyroxine, β-hydroxybutyrate or succinate enhance the swelling of fresh mitochondria and are necessary for that of aged[1].The addition of an oxidizable substrate of this kind could scarcely be expected to lead to the oxidation of bound DPNH or, for that matter, of any other component of the respiratory chain. (b) With fresh mitochondria swelling when inhibited by amytal can be restored by succinate (Fig. 11a; see also ref. [23]), and this applies also to aged particles when the swelling is induced by phosphate together with β-hydroxybutyrate (Fig. 11b). Addition of succinate in presence of amytal could hardly lead to the oxidation of bound DPNH; on the contrary, addition of succinate has been shown to cause the reduction of bound DPN[27]. (c) The irrelevance of the extent of reduction of the DPN is further illustrated by an experiment briefly reported by HUNTER, FINK AND HURWITZ[23], who found that when swelling was inhibited by amytal it was not restored on further addition of α-oxoglutarate and ammonia although this resulted in 80 % oxidation of the DPNH.

LEHNINGER, RAY AND SCHNEIDER[25] consider that information obtained from a study[21] of glutathione-induced swelling is at variance with our views. We have never maintained that swelling is dependent on respiration under all circumstances; we have, indeed, restricted our attention to swelling induced by phosphate or thyroxine and to the swelling of aged mitochondria which occurs in 0.2 *M* sucrose buffered with tris; nevertheless, the observations with glutathione are not necessarily inconsistent with our thesis. It is possible that glutathione acts as hydrogen donor as well as swelling agent. If the swelling depends on electron transport and if glutathione reacts directly with cytochrome *c*, the observed inhibition by cyanide and lack of

inhibition by amytal and antimycin would be expected. However, it is necessary to know whether glutathione was indeed being oxidized in these experiments. LEHNINGER AND SCHNEIDER[21], citing earlier work[22], state that oxidation of glutathione by mitochondria requires the addition of cytochrome *c*, but even a slow oxidation of glutathione in absence of cytochrome *c* might support the swelling. The rate of oxidation of β-hydroxybutyrate, for example, is also likely to be low with fresh mitochondria in absence of added phosphate acceptor and yet swelling is supported.

Oxidative phosphorylation and swelling

In our first communication[1], when we concluded that under various circumstances swelling is dependent on electron transport, we suggested, in view of the suppression by high concentrations of 2,4-dinitrophenol, that the effect of respiration may be mediated through the associated phosphorylation. As explained on p. 489, further experimentation has revealed that phosphorylation is unlikely to be involved in this way. Phosphorylation would seem to be involved only in so far as it controls the rate of respiration in a coupled system. This is illustrated by the inhibition of swelling by oligomycin as well as by some of the effects of dinitrophenol. It is noteworthy that after preincubation of the particles at 20° or longer storage at 0° the effect of oligomycin is no longer observed; this probably results from loss of the respiratory control. Furthermore, the inhibition by oligomycin is re-instated by the addition of magnesium ions[28] which are known to preserve the coupling of phosphorylation to respiration[29].

Swelling and hypothetical subsidiary electron-transport pathways

The overall pattern of the effects of inhibitors on swelling has been interpreted above in terms of the thesis that swelling is supported by electron transport along the accepted pathways. However, some of the effects of combinations of inhibitors, and of inhibitors in the presence of ferricyanide, cannot be fitted readily into the conventional scheme. The question therefore arises as to whether swelling may be supported by electron-transport pathways which differ from the established ones, either in the nature of the carriers themselves or in their availability to extra- or intra-mitochondrial substances. Pathways which differ from the accepted ones in the omission of one or more stages may be envisaged following COOPERSTEIN's evidence[30] that soluble DPNH-cytochrome *c* reductase can reduce cytochrome oxidase directly. The rapid reduction of mitochondrial pyridine nucleotide by succinate[27] may also be cited. That pathways for the oxidation of different substrates should be wholly or partially segregated from each other, or differ in their availability to extra-mitochondrial solutes, is consistent with the finding[20] that certain antibiotics can inhibit the oxidation of one substrate more than that of another. Furthermore, it has been deduced that there are two mitochondrial pathways for the oxidation of DPNH which differ in their sensitivity to inhibitors[11,31,32].

We set out below some relevant observations on swelling and indicate how these could be explained in terms of a hypothetical subsidiary pathway or pathways. The effects of antimycin quoted are produced by concentrations (3–50 mμg/ml) which will inhibit swelling completely in presence of β-hydroxybutyrate.

1. *Succinate-O_2*: Partial inhibition of swelling by antimycin; almost complete inhibition by antimycin + amytal. Possible interpretation: existence of subsidiary

electron-transport path which is sensitive to amytal and insensitive to antimycin.

2. *Succinate-ferricyanide*: Almost complete inhibition of swelling by antimycin alone. Possible interpretation is conjunction with 1: the subsidiary path is sensitive to cyanide and not readily available to ferricyanide.

3. *Glutamate-O_2*: Complete inhibition of swelling by amytal, partial by antimycin. Possible interpretation: as under 1.

4. *Glutamate-ferricyanide*: Complete inhibition of swelling by amytal; almost complete inhibition by antimycin. Possible interpretation: as under 2.

A hypothetical scheme based on these considerations is shown in Fig. 12. Evidence for the existence of pathways of the type envisaged is being sought by measurements of oxygen uptake and ferricyanide reduction.

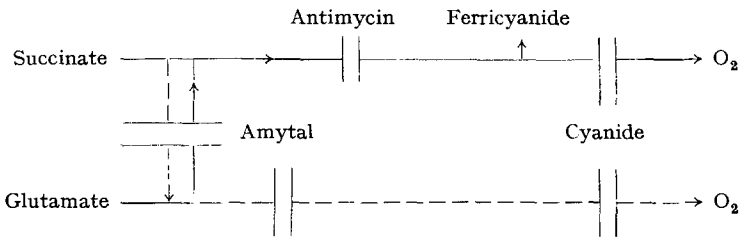

Fig. 12.

ACKNOWLEDGEMENT

We thank Mr. C. TAYLOR for skilled technical assistance.

REFERENCES

1 J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 182 (1958) 813.
2 J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 183 (1959) 1525.
3 D. F. TAPLEY, *J. Biol. Chem.*, 222 (1956) 325.
4 J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 183 (1959) 1737.
5 A. L. LEHNINGER AND B. L. RAY, *Biochim. Biophys. Acta*, 26 (1957) 643.
6 J. B. CHAPPELL AND G. D. GREVILLE, to be published.
7 A. FONNESU AND R. E. DAVIES, *Biochem. J.*, 64 (1956) 769.
8 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 61.
9 A. L. DOUNCE, R. F. WITTER, K. J. MONTY, S. PATE AND M. A. COTTONE, *J. Biophys. Biochem. Cytol.*, 1 (1955) 139.
10 C. DE DUVE AND J. BERTHET, *Nature*, 172 (1953) 1142.
11 C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
12 W. C. SCHNEIDER, M. J. STRIEBICH AND G. H. HOGEBOOM, *J. Biol. Chem.*, 222 (1956) 969.
13 G. LAUDAHN, *Z. Physiol. Chem. Hoppe-Seyler's*, 314 (1959) 95.
14 W. BARTLEY, M. SOBRINHO-SIMÕES, B. M. NOTTON AND G. MONTESI, *Biochem. J.*, 71 (1959) 26.
15 B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 383.
16 K. W. CLELAND AND E. C. SLATER, *Quart. J. Microscop. Sci.*, 94 (1953) 329.
17 J. RAAFLAUB, *Helv. Chim. Acta*, 38 (1955) 27.
18 B. CHANCE, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, Inc., New York, 1956, p. 447.
19 L. ERNSTER, O. JALLING, H. LÖW AND O. LINDBERG, *Exptl. Cell Research, Suppl.*, 3 (1955) 124.
20 H. A. LARDY, D. JOHNSON AND W. C. MCMURRAY, *Arch. Biochem. Biophys.*, 78 (1958) 587.
21 A. L. LEHNINGER AND M. SCHNEIDER, *J. Biophys. Biochem. Cytol.*, 5 (1959) 109.
22 A. L. LEHNINGER, M. UL HASSAN AND H. C. SUDDUTH, *J. Biol. Chem.*, 210 (1954) 911.
23 F. E. HUNTER, J. FINK AND A. HURWITZ, *Federation Proc.*, 18 (1959) 251.
24 F. E. HUNTER, J. DAVIS AND L. CARLAT, *Biochim. Biophys. Acta*, 20 (1956) 237.

[25] A. L. LEHNINGER, B. L. RAY AND M. SCHNEIDER, *J. Biophys. Biochem., Cytol.*, 5 (1959) 97.
[26] A. L. LEHNINGER AND M. SCHNEIDER, *Z. Physiol. Chem. Hoppe Seyler's*, 313 (1958) 138.
[27] B. CHANCE, *Proc. Internat. Symposium on Enzyme Chemistry, Tokyo and Kyoto*, Pergamon Press, London, 1958, p. 295.
[28] J. B. CHAPPELL, to be published.
[29] L. ERNSTER AND O. LINDBERG, *Ann. Rev. Physiol.*, 20 (1958) 13.
[30] S. J. COOPERSTEIN, *J. Biol. Chem.*, 234 (1959) 392.
[31] A. L. LEHNINGER, *Harvey Lectures*, 49 (1955) 176.
[32] L. ERNSTER, *Biochem. Soc. Symposia, Cambridge Engl.*, 16 (1959) 54.

Biochim. Biophys. Acta, 38 (1960) 483–494

MÉCANISME DE L'INACTIVATION DE L'OCYTOCINE PAR LE TISSU UTÉRIN*

LUCIENNE AUDRAIN ET HUBERT CLAUSER
Laboratoire de Chimie biologique, Faculté des Sciences, Paris (France)

(Reçu le 25 Juin, 1959)

SUMMARY

Mechanism of inactivation of ocytocin by uterine tissue

Ocytocin, when incubated under aerobic conditions with uterine tissue is never inactivated, whereas irreversible inactivation occurs rapidly when the incubation is performed under anaerobic conditions. The inactivation seems to proceed in two steps: the first step consists in a reduction of the disulfide bridge of ocytocin by uterine tissue; the second step is an irreversible inactivation of reduced ocytocin by an enzyme system, which is liberated into the medium during incubation. This inactivation proceeds either through proteolysis or through irreversible formation of mixed disulfides.

Similar experiments have been done with other organs, such as liver, kidney and diaphragm; it has been shown that ocytocin is inactivated by these tissues, but that inactivation in these cases proceeds equally with the reduced and the unreduced form of ocytocin. The physiological significance of these results is discussed.

INTRODUCTION

De nombreux auteurs ont étudié l'inactivation des hormones posthypophysaires par différents tissus. Les expériences ont porté soit sur l'ocytocine[1–5], soit sur la vasopressine[1,6]; une "ocytocinase" et une "vasopressinase", que les auteurs croient spécifiques** ont été décrites dans le plasma humain au cours de la gestation, par

* Un exposé préliminaire de certains résultats du présent travail a été fait au IVe Congrès International de Biochimie (Vienne, 1958).

** La spécificité de l'ocytocinase du plasma humain a été récemment établie par TUPPY *et al.*[22,23] à l'aide de substrats synthétiques.