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BUTYLMALONATE COUNTERACTS THE INHIBITORY EFFECT OF PROTAMINE ON SUCCINATE OXIDATION. AN ULTRASTRUCTURAL INTERPRETATION

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SUMMARY

When rat liver mitochondria were exposed to protamine or butylmalonate, succinate oxidation was inhibited. However, butylmalonate was found to release the inhibitory effect of protamine on succinate oxidation in mitochondria. Electron microscopic study carried out in the present study showed that protamine induced "orthodox" configuration in which the matrix space was maximally expanded eliminating the intracristal space, whereas butylmalonate highly contracted the matrix space thus expanding the intracristal space. Butylmalonate overcame the effect of protamine on mitochondrial configuration, specified above, expanding the intracristal space. The mechanism of the opposite action of butylmalonate in the presence and absence of protamine on succinate oxidation was correlated to the configurational changes of the mitochondrion.

INTRODUCTION

Protamine and butylmalonate are known to inhibit succinate oxidation in isolated mitochondria. This paper shows that the inhibitory effect of protamine on succinate oxidation in rat liver mitochondria, however, is released with the introduction of butylmalonate. Possible mechanism for the phenomenon is discussed in the light of the configurational changes of mitochondria.

MATERIALS AND METHODS

Mitochondria from rat liver were isolated in 0.33 M sucrose (+ 10 mM Tris chloride, pH 7.3) by the method of Loewenstein *et al.* [1], except that the last digitonin step was omitted. Oxygen uptake was measured with a Clark-type oxygen electrode at 25° as described previously [2]. For

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electron microscopy mitochondria were fixed at different metabolic states by the addition of 2% glutaraldehyde (final concentration) directly to incubation medium in the oxygen electrode chamber. The glutaraldehyde solution contained 0.25 M sucrose and 50 mM cacodylate, pH 7.3. After fixation with glutaraldehyde, the samples were centrifuged, post-fixed in osmium tetroxide and processed for electron microscopy as described by Wakabayashi *et al.* [3]. Specimens were stained with lead citrate and examined in a Tesla BS 613 electron microscope operated at 80 KV.

RESULTS

Oxidation of succinate by rat liver mitochondria was inhibited by the addition of protamine or butylmalonate (Fig. 1). After the addition of protamine or butylmalonate, mitochondria were fixed for electron microscopy at points indicated by dashed arrows on oxygen electrode traces. Ultrastructure of representative mitochondria under these experimental conditions is shown in electron micrographs A, B and C respectively. As shown in electron micrograph A, protamine-treated mitochondria had an extremely expanded matrix space and showed characteristic close appositions of adjacent cristal membranes (the "orthodox" configuration). Moreover, the inner membrane and the outer membrane were stuck together. While entire population of protamine-treated mitochondria showed above described configurational transition, mitochondria stayed in the "condensed" ("aggregated") configuration after butylmalonate treatment. However, two distinct populations were observed: 50% of mitochondria had more condensed matrix compared with the control (electron micrograph B); another 50% of mitochondria also had more condensed matrix compared to the control and the inner-membrane-matrix component was ristricted to the periphery (electron micrograph C).

Figure 2 shows another experiment in which succinate oxidation was first inhibited by protamine and then was restored by the addition of butylmalonate. At different stages of the experiment, mitochondria were fixed for electron microscopy (electron micrographs D-G). Mitochondria under state 3 condition showed the "orthodox" configuration (electron micrograph D, this is quite different from that observed in a sucrose medium in which mitochondria under state 3 condition show the "condensed" configuration). Upon addition of protamine mitochondria stayed in the "orthodox" configuration and yet both intracristal space and the space between the outer and the inner membranes were now eliminated as described earlier in

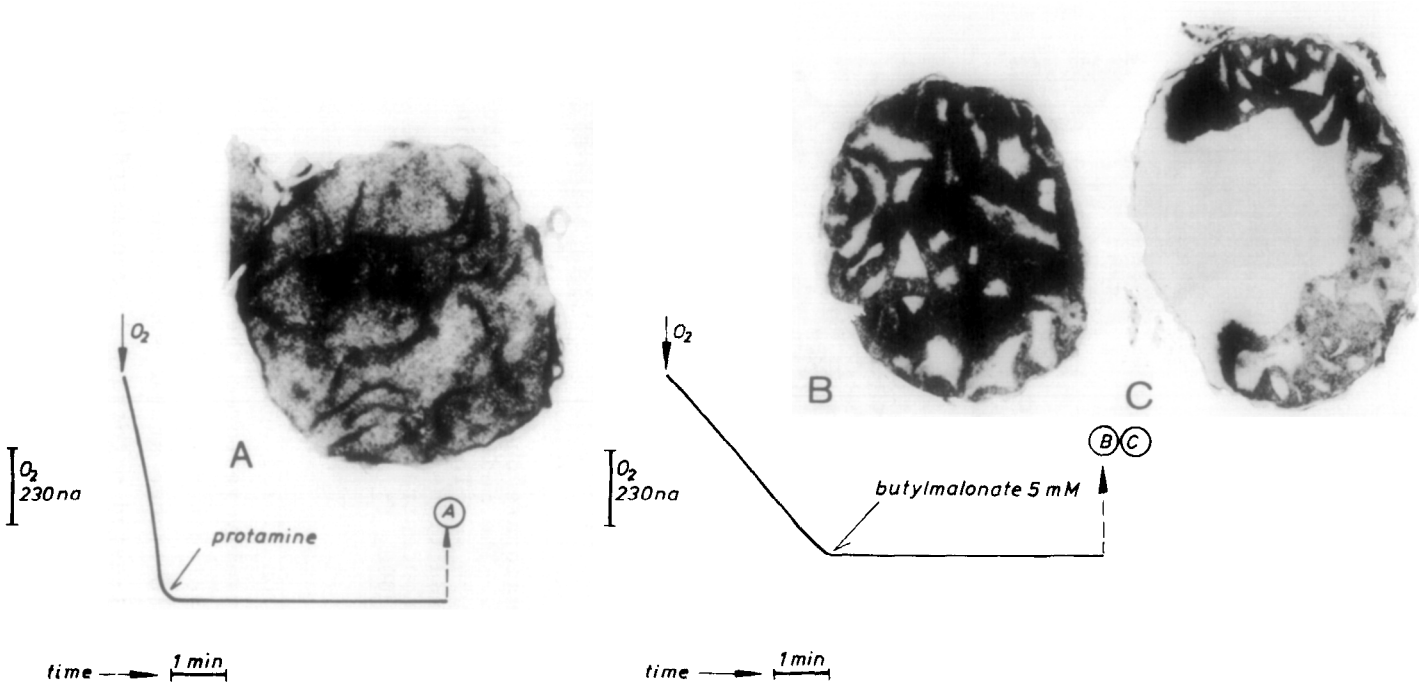

Figure 1. Differences in ultrastructural appearance of protamine (A) and butylmalonate (B and C) treated rat liver mitochondria. Respiration was measured in a medium (3.5 ml, pH 7.3) containing: 15 mM KCL, 50 mM Tris-chloride, 5 mM potassium phosphate, 2 mM $MgCl_2$, 0.2 mM EGTA, 14 µg of rotenone and 4.6 mg of biuret [4] protein. In addition, the medium was supplemented with 0.1 mM dinitrophenol. Tris-succinate was used as substrate and the final concentration was 30 mM for the experiment with protamine (170 µg/ml) or 1 mM for the experiment with butylmalonate (5 mM).
Incubation temperature was 25°C. A: x60,000, B,C: x60,000.

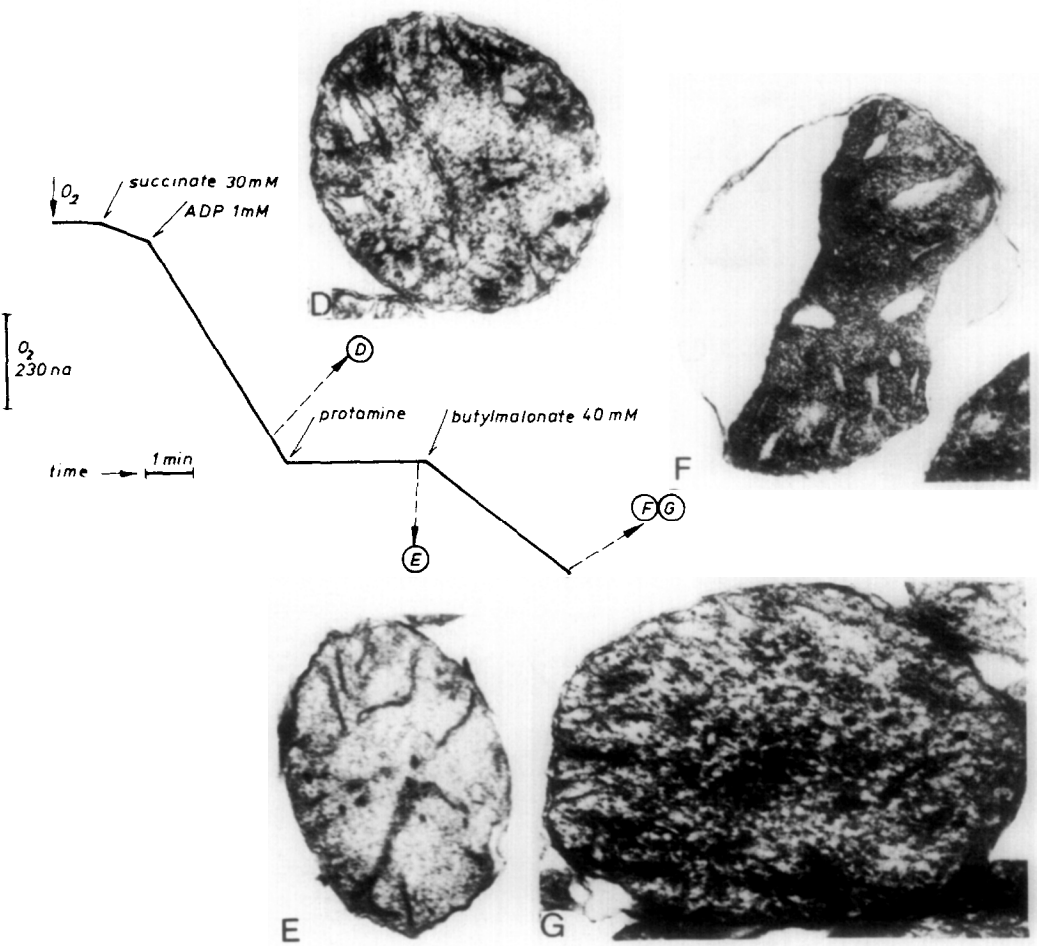

Figure 2. The effect of butylmalonate on protamine-inhibited succinate oxidation. The medium composition was the same as that in Figure 1. The mitochondrial protein was 2.6 mg. Other additions are indicated in the Figure. The final concentration of protamine was 50 μg/ml. D,E: x60,000, F: x80,000, G: x100,000.

electron micrograph A (electron micrograph E). The subsequent addition of butylmalonate partially restored the respiratory ability of mitochondria and protamined-induced alterations of mitochondrial morphology were overcome at the same time. Protamine plus butylmalonate-treated mitochondria consisted of two ultrastructurally distinct populations: 50% of mitochondria still stayed in the "orthodox" configuration, and yet both the intracristal space and the space between the inner and the outer membranes became clearly visible (electron micrograph G);

another 50% of mitochondria was transformed into the "condensed" configuration in which the matrix space was highly condensed (electron micrograph F).

DISCUSSION

Our interest in protamine action on mitochondrial respiration depends partly on the fact that this basic protein is known to induce selective permeability of artificial membrane to anions [5, 6]. Therefore, despite the fact that protamine is well established as being " a specific inhibitor of respiratory chain " acting between cytochrome c and a at the outer surface of the inner membrane [7] (see also [8]), we are still attempting to clarify the problem of whether there is a correlation between the ability of protamine to induce anion permeability and its inhibitory effect on respiration of mitochondria.

We have already shown that inhibitory effect of protamine on succinate oxidation by mitochondria is released either by gramicidin in the presence of ammonia ion, or by dinitrophenol [9, 10]. Looking for the underlying mechanism for the above described reversible effect of gramicidin and dinitrophenol, we have found in the literature that both reagents exert similar effects on mitochondrial structure and function: gramicidin in the presence of ammonia ion contracts the mitochondrial matrix space increasing the K_m for succinate oxidation [11]; dinitrophenol also reduces the matrix space [12] suppressing both succinate entry and accumulation in mitochondria (for review see [13]). Butylmalonate is also a well known classical inhibitor of succinate entry and accumulation in mitochondria (for details see ref. [14]). All these data tempted us to examine whether butylmalonate would also overcome the inhibitory effect of protamine on succinate oxidation.

It turned out that butylmalonate exerted inhibitory effect on succinate oxidation in the absence of protamine (Fig. 1), whereas caused a stimulatory effect on respiration when added after protamine (Fig. 2). Since experiments carried out in the present study are seemingly confusing we should like to refer to data from electron microscopy. At present it is generally accepted that ultrastructural transitions occur in mitochondria as secondary to changes in their metabolic

states. But at the same time, the prediction of Green and Ji [15] that via ultrastructural transformations mitochondria rearrange their metabolic states, still remains controversial. We interprete confusing effect of butylmalonate shown in the present study as follows assuming that ultrastructural transformations might precede metabolic transition of mitochondria

1. Protamine acts primarily at the level of mitochondrial membrane inducing the maximum expansion of the matrix space (see electron micrographs A and D as well as refs [10, 16 and 17]). The mechanism by which it does so remains unknown (electro-osmosis?), but as a consequence of this ultrastructural alteration anion-conductivity of the inner mitochondrial membrane increases [18] and respiration stops.
2. Butylmalonate exerts an opposite ultrastructural effect to that of protamine, condensing the matrix space (see electron micrographs B and C). In this case changes in membrane geometry reduce anion-conductivity and respiration stops due to the lack of substrate.
3. Protamine plus butylmalonate.

The ultrastructural effect of protamine is counteracted by butylmalonate (electron micrographs F and G), and therefore effect of protamine on metabolic state of mitochondria, i.e. inhibition of respiration, is partially overcome.

It is worth mentioning (data are not shown here) that butylmalonate could release protamine inhibition on succinate oxidation in different metabolic states (State 4, State 3U with dinitrophenol) but was unable to reverse protamine effect when permeability of the inner mitochondrial membrane to potassium was increased by the addition of gramicidin or valinomycin. This suggests that the ability of butylmalonate to increase the rate of succinate oxidation is limited only to the condition of anion semipermeable membrane. We have also found (not shown here) that another succinate transport inhibitor, phenylsuccinate, releases protamine inhibition on succinate oxidation. Sluse *et al.* [19] are the first to report that this anion-transport inhibitor reduces the mitochondrial matrix space. This suggests that the effect of butylmalonate on mitochondrial ultrastructure may be rather a common phenomenon to anion-transport inhibitors.

Preliminary report of this work has already been presented [20, 21].

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