Vol. 93, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 13, 1980 Pages 141-148

NECESSITY OF IRON FOR THE ALTERNATIVE RESPIRATORY PATHWAY IN ACANTHAMOEBA CASTELLANII[1]

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Received November 16,1979

SUMMARY: Ommission of iron from the growth medium leads, within two to three generations, to a shift in respiration of trophozoites of Acanthamoeba castellanii from the cyanide-stimulated to the cyanide-inhibited pattern. Addition of iron restores the activity of the alternative respiratory pathway within one hour.

INTRODUCTION

It has long been known that some amoebae are resistant to cyanide. Cytochrome c oxidase was not detectable in total homogenate of amoebae (1) and was first reported in mitochondria isolated from Chaos chaos (2). The problem of cyanide insensitivity of amoeba respiration reappeared recently when the existance of an alternative respiratory pathway in A. castellanii was reported (3-7). On the other hand, the presence of the cytochrome c-aa_3 pathway in mitochondria of A. castellanii was shown before (8-16). This, together with the fact that the uncoupler FCCP[2] stimulates the oxygen uptake of trophozoites in a cyanide-sensitive manner (3) suggests parallel participation of a tightly coupled cytochrome pathway in amoeba respiration.

The present studies were performed to estimate the role of iron content in the culture medium in the functioning of the alternative pathway, as was suggested for yeast (15). The results strongly indicate

[1] Supported by the grant from the Polish Academy of Sciences, Committee of Cell Biology.

[2] Abbreviations used: SHAM, salicylhydroxamic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

0006-291X/80/050141-08$01.00/0
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that iron is necessary for the appearance of cyanide insensitivity of amoeba respiration. Ommission of iron from the medium leaves mainly the cytochrome c-aa_3 pathway operating.

MATERIAL AND METHODS

Agitated cultures of Acanthamoeba castellanii Neff grew at 28° in 40 ml of the medium according to Neff and Neff (18), containing about 30 μM $FeSO_4$. Besides the regular medium the following variants were used: with $FeSO_4$ replaced by $FeCl_3$ or by Na_2SO_4, with increased or decreased $FeSO_4$, and with no $FeSO_4$. Amoebae were harvested by centrifugation at 500 g x 2 min, washed twice and resuspended in the fresh medium to the density of 10^7 cells in 1 ml. 2-3 mg of amoebae protein was introduced to 1.5 ml of fresh medium used for the given type of culture and the respiration was measured with Clark-type oxygen electrode at 25°. Respiration was also measured directly in samples of the cultures diluted, if necessary, with the fresh medium to the density of 0.4 - 0.8 x 10^6 cells in 1 ml. The rate and pattern of the respiration of trophozoites was the same in both applied procedures. Protein was estimated by the biuret method (19) with bovine serum albumin, as the standard. Iron concentration in the growth medium was assayed with bathophenantroline (20).

RESULTS

Control cultures grew with generation time of 8 hours in an exponential pattern and reached the stationary phase at about 70 hours, resulting in 7 generations (Fig. 1). In the medium without $FeSO_4$ growth was slowed down after 24 hours and reached a steady level after 5 generations. Lowering

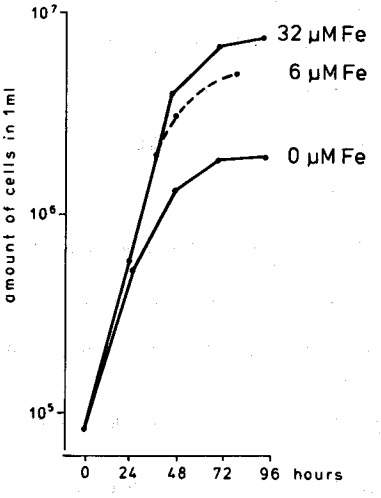

Fig. 1. Growth curves of amoebae cultures in media containing 32 μM $FeSO_4$ (control culture), 6 uM $FeSO_4$ and no $FeSO_4$.

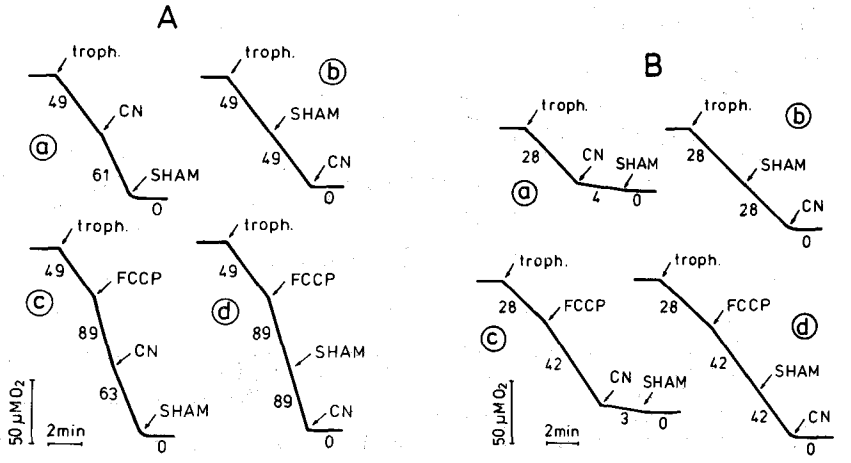

Fig. 2. Characteristics of respiration of trophozoites from stationary phase of culture
(A) Control culture, 72 hours, 6 x 10^6 cells in 1 ml,
(B) Culture without $FeSO_4$, 60 hours, 2 x 10^6 cells in 1 ml.
Oxygen uptake in $nAtO_2 \times min^{-1} \times mg^{-1}$ protein.
Final concentrations: FCCP 10 μM, SHAM 1 mM, KCN 1 mM.
Conditions of measurements as described in Methods.

$FeSO_4$ concentration to 6 μM in the culture medium resulted in only negligible reduction of growth of the culture.

The effect of cyanide, SHAM and FCCP on respiration of trophozoites from the late exponential phase (48 hours) of control culture is illustrated in Fig. 2 A. The addition of 1 mM KCN immediately stimulated the respiration. 10 μM FCCP resulted in a stimulation of amoeba respiration by 70-75 percent which was again inhibited with 1 mM KCN (Fig. 2 A c) to the level similar to that of the respiration stimulated by cyanide alone (Fig. 2 A a). Regardless of the presence or absence of FCCP, SHAM alone had no significant effect on respiration of trophozoites, but was totaly inhibitory in presence of KCN (Fig. 2 A b,d).

The effect of inhibitors and FCCP on respiration of trophozoites from various phases of growth of the control culture containing 30 μM $FeSO_4$ is illustrated in Fig. 3 A. The respiratory pattern throughout the culture growth remained the same as the one described above for the stationary phase. At the end of the culture (96 hours), just before encystation, the content of iron in the medium dropped only to 26 μM Fe.

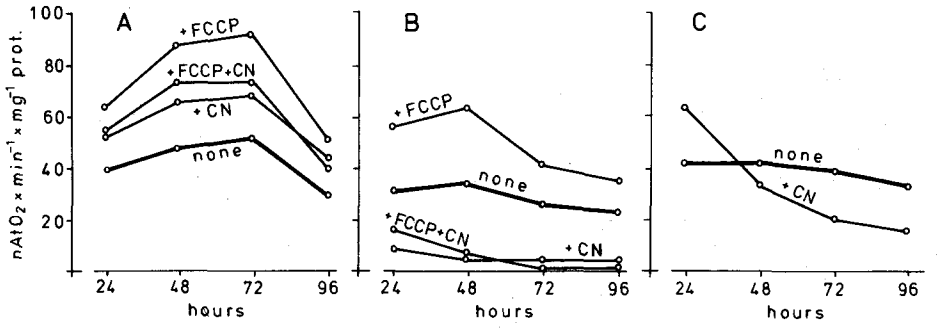

Fig. 3. Respiration rate of trophozoites from various phases of cultures and the effect of 1 mM KCN (CN) and 10 µM FCCP.
(A) Control culture (30 uM $FeSO_4$), (B) Culture without $FeSO_4$,
(C) Culture with 6 µM $FeSO_4$.
Conditions of measurements as described in Methods.

When $FeSO_4$ was omitted from the medium only traces of iron from yeast extract and iron introduced with the inoculum were available (2-3 µM Fe). Respiration of trophozoites from this type of culture was significantly lower than that from control culture and its inhibition by 1 mM cyanide increased with the age of the culture, resulting in 70 and 85 percent inhibition in trophozoites from 24 and 72 hours of culture, respectively (Fig. 3 B). While 10 µM FCCP higly stimulated the respiration, as it did in the control culture, KCN added after FCCP was strongly inhibitory (Fig. 3 B). 1 mM SHAM alone had no effect regardless of the presence or absence of FCCP, but in the presence of 1 mM KCN inhibited the respiration totally (Fig. 2 B).

With initial concentration of 6 µM $FeSO_4$ cultures grew almost normally, but the sensitivity of respiration of trophozoites to cyanide changed dramatically (Fig. 3 C) from stimulation at the early exponential phase to pronounced inhibition at the stationary phase, whilst iron in the medium only dropped to 4 µM, probably reflecting the lowest concentration of iron to be utilized by amoebae.

The trophozoites from cultures in which $FeSO_4$ was replaced by equimolar Na_2SO_4 respired and responded to inhibitors and to FCCP in the same manner as trophozoites from the culture without $FeSO_4$. Replacement of

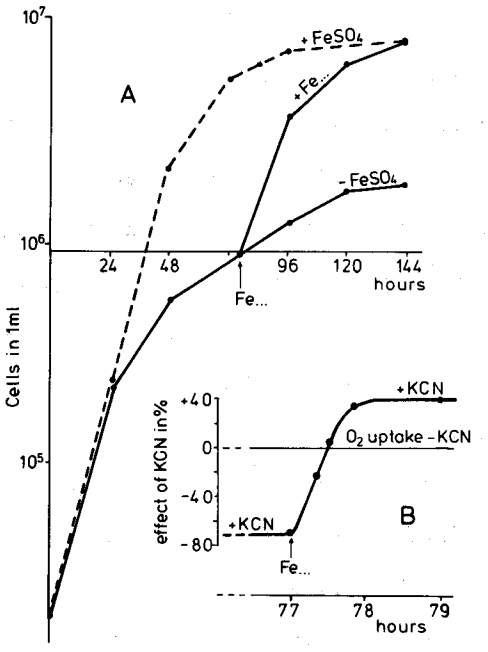

Fig. 4. Recovery of trophozoites from iron deficiency by addition of 30 μM $FeSO_4$, $FeCl_3$ or Fe-citrate.
(A) Growth curves of control culture (a) and of culture without $FeSO_4$ (b).
(B) Shift from the inhibitory (-) to the stimulatory (+) effect of 1 mM KCN on respiration of trophozoites following addition of iron. O_2 uptake without KCN - 32 nmoles x min^{-1} x mg^{-1} protein. Conditions of growth and respiration measurements as described in Methods.

$FeSO_4$ by equimolar $FeCl_3$ in the culture medium did not change either the rate of respiration of trophozoites or its response to the inhibitors and FCCP. Thus, lack of sulphate was not manifested in the respiratory pattern of the amoebae, and the valency of iron does not seem to be crucial.

In order to restore the activity of the alternative pathway 30 μM iron was added as $FeSO_4$, $FeCl_3$ or Fe-citrate to the cultures growing without iron and revealing inhibition of growth and inhibitory effect of KCN, at the end of their exponential growth. The growth was immediately reassumed (Fig. 4 A). Effect of cyanide on respiration of these trophozoites was reversed in less than 1 hour changing from previous inhibition to the stimulation of the oxygen uptake (Fig. 4 B), regardless of the kind of iron salt introduced.

DISCUSSION

The present results are in partial agreement with the data reported for cyanide sensitivity of trophozoites of A. castellanii by Edwards and Lloyd (5, 6). However, they differ significantly in the pattern of respiration during the later stages of culture growth. Change from the stimulatory effect of cyanide during early exponential phase into the inhibitory one at the later stages, reported by these authors might be explained in terms of exhaustion of traces of iron present, as no addition of iron to the culture medium was mentioned (5, 6). Moreover, the curves of cyanide sensitivity reported (6) correspond to the situation in which amoebae grew under limiting conditions of 6 μM $FeSO_4$ in the present investigations (Fig. 3 B). Therefore, the conclusions that composition of the culture medium has no influence on the activity of the alternative pathway (5) requires revision.

Henry et al. (21) reported that in yeast Saccharomycopsis lypolitica Fe^{III} was neccessary for the appearance of the alternative respiratory pathway, while Fe^{II} was not effective. In the present investigations the stimulation of the alternative pathway of A. castellanii in cultures without iron was obtained by addition of Fe^{II} or Fe^{III} salts as well.

With a limited supply, iron seems to be incorporated in this metabolic pathway which is more energetically effective. In yeasts during iron deficiency only 5-fold decrease in cytochrome titre but 20-fold decrease in iron-sulphur protein content was reported (22). Participation of the alternative pathway in the respiration of amoebae growing with high iron supply, as measured by the sensitivity to cyanide, seems to be the same in all phases of culture growth. Stimulation by FCCP of respiration of A. castellanii from cultures with iron (3) and without it, being largely inhibited by cyanide, reflects the stimulation of the cytochrome c-aa_3 pathway and might be useful for determination of the degree in which both respiratory pathways participate in the respiration of amoebae under physiological conditions.

Besides iron also oxygen supply during culture growth might have an influence on the development of the alternative respiratory pathway in amoeba because in non-agitated, less aerated and slowly growing cultures, containing 30 μM $FeSO_4$, cyanide is neither inhibitory nor stimulatory (17). Moreover, trophozoites from these cultures, left for 24 hours with no aeration, turned into a cyanide sensitive pattern of respiration (17).

Respiration of mitochondria isolated from A. castellanii trophozoites from standard cultures containing 30 μM $FeSO_4$ in the medium is always inhibited by cyanide to a degree, depending on the energy state. The presence of the alternative pathway in these mitochondria is expressed, however, in the total inhibition of the remaining oxygen uptake by 0.5 mM SHAM (3-5, 17) and in its stimulation by AMP (5, 17). Presumably, in the intact cells there is enough AMP to support the operation of the alternative pathway (6), while the pool of adenine nucleotides in amoeba mitochondria is very low (23). The possible relationship between AMP level and iron availability in A. castellanii is presently under investigation.

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