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DISAPPEARANCE OF THE CYANIDE-INSENSITIVE PATHWAY OF OXIDATION IN MITOCHONDRIA OF Mi-l MUTANT OF NEUROSPORA CRASSA IN VITRO

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Oxidation of exogenous NADH in mitochondria isolated from wild type and mi-l mutant of Neurospora crassa decreases rapidly in vitro. In mi-l mutant mitochondria the inactivation concerns the alternate pathway of oxidation whereas in the wild type it involves an unknown component of the respiratory chain. The activity of the primary NADH dehydrogenase is constant within the time of the experiments (2 - 4 h).

NADH oxidase is not inactivated if oxygen is removed from the incubation medium by nitrogen bubbling.

Succinate oxidase does not show any remarkable changes in activity within 2 - 3 h.

In fresh mitochondria of the mi-l mutant reduced ubiquinone is completely reoxidized by cytochrome oxidase but only 80% reoxidized by the alternate oxidase.

In aged mitochondria of the mi-l mutant in the presence of cyanide, ubiquinone is reduced to the level characteristic for fresh mitochondria in which respiration is completely inhibited by cyanide plus salicyldihydroxamic acid. In these mitochondria the reoxidation of the reduced ubiquinone proceeds only via the cytochrome pathway.

It is supposed that a labile component(s) of the respiratory chain present in the mi-l mutant and the wild type mitochondria may, in mi-l mutant, act as an alternate oxidase.

The cytoplasmic respiratory mutant mi-l (called poky) of Neurospora crassa has recently aroused great interest. It respires via two alternate pathways: the cyanide-sensitive cytochrome pathway, which is greatly altered by this mutation, and the cyanide-insensitive pathway which is not unique for the mi-l mutant but is also characteristic of certain plant mitochondria (Tissieres et al., 1953; Storey, 1970; Lambowitz & Slayman, 1971; von Jagow et al., 1973).

Of the few hypotheses so far presented regarding the branch point, none seems to be satisfactorily proved. It has been postulated that in the mi-l mutant of Neurospora crassa ubiquinone donates reducing equivalents equally to the cyanide-sensitive and cyanide-insensitive pathways (von Jagow et al., 1973).
Detailed studies on the redox pattern of ubiquinone seem to indicate, however, that ubiquinone is the main respiratory chain component which may be in redox equilibrium with an unknown component of the alternate pathway (Drabikowska, 1975). It is assumed that this compound may be located in the flavin region of the respiratory chain. Moreover, it is very probable that the UQ/UQH₂ ratio plays an important role in the regulation of the activity of the alternate oxidase in mi-1 mutant mitochondria (Drabikowska, 1975). In mitochondria of higher plants (Symphoricarpos foetidus) the branch point of the respiratory chain would appear to be similar to that in mi-1 mutant mitochondria. A high potential, fluorescent flavoprotein has been suggested to be the mediator of electron transport along the alternate pathway (Erecińska & Storey, 1970). Recent studies of Storey (1976) have shown that the flavoprotein with standard redox potential \( E_0 = +20 \) mV links ubiquinone to the alternate oxidase pathway.

In the present paper it is shown that NADH oxidation decreases rapidly in vitro in both mi-1 and wild type mitochondria. In mi-1 mutant mitochondria the decrease in NADH oxidation is shown to be a consequence of the disappearance of the alternate oxidase pathway. The redox changes of ubiquinone in fresh and aged mitochondria of mi-1 mutant upon inhibition of cytochrome oxidase or of the alternate oxidase are also reported.

MATERIALS AND METHODS

*Cultivation of hyphae and preparation of mitochondria.* Hyphae of the wild type and mi-1 mutant were cultivated as described by Drabikowska (1975). Mitochondria were isolated in the medium without albumin according to Weiss et al. (1970) with a grind mill used for cell disruption.

*Oxygen consumption.* Oxygen consumption was measured with a Clark oxygen electrode in the isolation medium containing 0.44 m-sucrose, 10 mM-Tris-acetate (pH 7.3) and 2 mM-EDTA. The reaction was carried out at 22°C.

*Ubiquinone determination.* Ubiquinone content was estimated by the extraction procedure described previously (Drabikowska & Kruszewska, 1972).

* Determination of intramitochondrial magnesium.* The content of magnesium in mitochondria and supernatant was measured by the use of an Evans Electroselenium Atomic Absorption Spectrophotometer. At appropriate time intervals 2-mI samples of the mitochondrial suspension were taken and centrifuged at 10 000 g for 10 min to separate the mitochondria from the medium. The pellet was resuspended in an equal volume of the same medium. Mitochondrial protein and soluble protein in the supernatant were precipitated with trichloroacetic acid. The precipitate was removed by centrifugation and the clear supernatant was used for assays.

* Determination of the release from mitochondria of the material absorbing at 260 nm.* At appropriate time intervals 2-mI aliquots of the mitochondrial suspension were taken and centrifuged at 10 000 g for 10 min in a refrigerated centrifuge. The supernatant was decanted and soluble protein was precipitated with trichloroacetic acid
and separated by centrifugation. The absorbance of the supernatant at 260 nm was estimated using a Unicam Sp500 spectrophotometer.

**Determination of enzymic activities.** NADH-ferricyanide reductase activity was determined spectrophotometrically at 420 nm. Determinations were performed at constant acceptor concentration. The composition of the assay medium is given in legends to the Figures.

The activity of membrane-bound succinate dehydrogenase in mitochondria was measured by phenazine-mediated-DCPIP\(^1\) reduction recorded at 578 nm.

**Determination of protein.** Protein was estimated by the biuret method as described by Szarkowska & Klingenberg (1963). Protein content was calculated from the decrease in absorbance at 546 nm after addition of cyanide, according to the formula: protein content (mg) = \(AE \times 17.5\) (for 5 ml volume and 1 cm light path).

**RESULTS AND DISCUSSION**

**Oxidation of NADH**

Mitochondria of *Neurospora crassa* rapidly oxidize exogenous NADH by a pathway different from that responsible for the oxidation of endogenous NADH (Weiss et al., 1970). The ease of oxidation of exogenous NADH results from the location of NADH dehydrogenase on the outer face of the inner mitochondrial membrane (von Jagow & Klingenberg, 1970).

Young cultures of the *Neurospora mi-I* mutant respire mainly via the cyanide-insensitive pathway (von Jagow et al., 1973; Drabikowska, 1975). Thus with NADH, which is very actively oxidized by these mitochondria, the contribution of the cyanide-sensitive oxidation to the overall oxidation rate is very small.

It was observed that in the *mi-I* mutant, the high activity of NADH oxidation in freshly isolated mitochondria decreases with time. At the same time, the respiration exhibits an increasing sensitivity towards cyanide. This observation led us to undertake close studies of the alterations which occur in isolated mitochondria.

Figure 1 presents the time-dependent decrease in NADH oxidation activity and indicates the extent of inactivation of the KCN-insensitive pathway. The initial NADH oxidation activity in mitochondrial preparations used in these studies is comparatively low (180 - 240 nmoles \(O_2/mg\) protein/min). This probably results from the inactivation processes which occur during the isolation procedure. Albumin, which is omitted from the isolation medium in these studies, stabilizes to some extent mitochondria and protects them against inactivation. Mitochondria isolated in the medium without albumin offer, however, the opportunity of studying the time-dependent alteration in oxidation activity *in vitro*. As shown by the use of specific inhibitors of cytochrome oxidase and the alternate oxidase, the decline in the NADH oxidation activity results from the inactivation of the alternate oxidase pathway but not of the cyanide-sensitive pathway. It was observed that the inactivation did not occur when mitochondria were kept under nitrogen. It can be, therefore,

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\(^1\) Abbreviations: FCCP, carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone; DCPIP, dichlorophenolindophenol; SHAM, salicylhydroxamic acid; PMS, phenazine methosulfate.
supposed that oxygen is responsible for the inactivation of the alternate oxidase, most likely due to the oxidation of a labile iron-sulphur center of some protein molecule. The phenomena observed were expected to occur only in \textit{mi-l} mutant mitochondria. However, the results presented in Fig. 2 indicate that, as in \textit{mi-l} mitochondria, the NADH oxidation in the isolated wild type mitochondria drops linearly with time. SHAM, which inhibits the oxidation \textit{via} the alternate pathway in the \textit{mi-l} mutant, had no effect on NADH oxidation in the wild type mitochondria.

On the basis of the considerable reduction of the NADH oxidation in \textit{mi-l} mutant and wild type mitochondria it may be assumed that in both cases the inactivation involves the same component of the external NADH oxidase system. The simultaneous disappearance of the cyanide-insensitive pathway of oxidation in \textit{mi-l} mitochondria indicates that this component may be directly involved in the oxidation \textit{via} the cyanide-insensitive pathway, or functions as an alternate oxidase. There are indications that a single component is responsible for cyanide-insensitive respiration and that it is specifically inhibited by SHAM (Edwards \textit{et al.}, 1974). A similar idea has been presented by Dizengremel \textit{et al.} (1973) who interpreted the lack of any quantitative differences in the content of iron-sulphur protein in cyanide-resistant and cyanide-sensitive mitochondria to mean that the alternate oxidase is a component common to both kinds of mitochondria. They postulated that the alternate pathway is under a control mechanism which makes enzymic proteins present in mitochondria accessible to oxygen. At present it is difficult, however, to reconcile this suggestion with the concept of the \textit{de novo} biosynthesis of the alternate oxidase protein on cytoplasmic ribosomes (Edwards \textit{et al.}, 1974).
The externally located NADH dehydrogenase, which is linked with both the cytochrome respiratory chain and the alternate oxidase pathway, does not undergo any alterations during aging of wild type and mi-1 mutant mitochondria as measured with ferricyanide as an electron acceptor (Fig. 1). Ferricyanide is a non-penetrating electron acceptor which can interact only with the component located on the outer face of the inner mitochondrial membrane, e.g. cytochrome c (Klingenberg, 1970). Since the reduction of ferricyanide is antimycin-insensitive, it is concluded that ferricyanide accepts electrons directly from the externally located NADH dehydrogenase (von Jagow & Klingenberg, 1970).

Very little is known about the components involved in the exogenous NADH oxidation (Palmer & Coleman, 1974). The lack of any inhibitory effect of rotenone and piericidin A in Neurospora mitochondria was observed by Lambowitz et al. (1972) and Drabikowska et al. (1974). This may indicate that the externally located NADH dehydrogenase, unlike the NADH dehydrogenase of animal mitochondria (Gutman et al., 1970; Singer & Gutman, 1974) lacks the binding site for these in-

![Graph](image1.png)

**Fig. 2.** Time-dependent alterations in NADH oxidation in the N. crassa wild type mitochondria. Mitochondria (4 mg of protein/ml) were incubated in the isolation medium at room temperature; oxygen consumption was measured in samples diluted to a protein concentration of 0.8 mg/ml; 1 mm-NADH and 2 mm-SHAM were used as inhibitors. Each point on the curve represents the result of a separate oxygen uptake determination. Total oxidation (●); in the presence of SHAM (△). The NADH-ferricyanide reductase activity (□) was estimated as described in Materials and Methods. The respiratory activity is expressed as a fraction of the initial rate.

![Graph](image2.png)

**Fig. 3.** Effect of aging on succinate oxidation in mi-1 mutant mitochondria. Mitochondria (3 - 4 mg of protein/ml) were incubated in the isolation medium at room temperature. Oxygen consumption was measured in samples diluted to a protein concentration of 0.8 - 1.5 mg/ml; 10 mm-succinate, 2 mm-SHAM and 1 mm-KCN were used. Each point in the graph represents the result of a separate experiment. Total oxidation (●); in the presence of SHAM (△); in the presence of KCN (▲). Succinate dehydrogenase activity (□) was measured with phenazine (0.56 mM)-mediated DCPIP (0.05 mM) reduction recorded at 578 nm in an Eppendorf spectrophotometer. The reaction was carried out in the isolation medium in the presence of 1 mm-KCN; 10 mm-succinate was used as a substrate.
hibitors. Isolation and identification of the NADH dehydrogenase located at the outer surface of the inner mitochondrial membrane or of the alternate oxidase would be desirable.

**Oxidation of succinate**

The activity of the succinate oxidation by mitochondria isolated from wild type and mi-1 mutant of *Neurospora* is very low (60 - 70 nmoles O\textsubscript{2}/mg protein/min). In mi-1 mutant mitochondria, isolated by the procedure described in this paper, the slow oxidation seems to proceed only via the cyanide-sensitive pathway. This conclusion is drawn from the observation that the cytochrome pathway always operates at its maximal activity and the alternate oxidase pathway is only partially active or inactive (Bahr & Bonner, 1973; Drabikowska, 1975).

In the present studies it was shown that the succinate oxidase activity is stable for at least 2 h of incubation of wild type and mi-1 mutant mitochondria at room temperature (Fig. 3). At the same time the NADH oxidase activity measured in the same experiment decreases by about 80%. After this time the slight decrease in succinate oxidase activity occurs, probably as a consequence of degradation unrelated to the disappearance of the alternate pathway of oxidation. The rapid decrease in the succinate oxidase activity observed by Eakin & Mitchell (1970) and Lambowitz et al. (1972) might result from the uncoupling process which greatly inactivates the succinate dehydrogenase but not the alternate oxidase (Drabikowska, 1975). A multiple control mechanism for succinate dehydrogenase in animal mitochondria has been demonstrated by Gutman et al. (1971). It is very probable that a similar regulatory mechanism is involved in the oxidation of succinate in mitochondria of wild type and mi-1 mutant of *Neurospora crassa*. Succinate dehydrogenase activity measured with PMS-DCPIP as the electron acceptor is not affected on aging of mitochondria (Fig. 3). The results of the present studies indicate that the inactivation of the oxidation processes in mitochondria does not concern dehydrogenases but some other components of the respiratory chain.

In the presence of SHAM (Fig. 3) the succinate oxidase activity in mi-1 mutant mitochondria is lowered by about 20%. The same effect was observed with wild type mitochondria in which the respiration is completely inhibited by cyanide (not shown). This observation points to some unspecific effect of SHAM on succinate oxidase in the *Neurospora* mitochondria. A similar effect of SHAM has been observed by Schonbaum et al. (1971) in potato mitochondria.

It seems reasonable to assume that SHAM affects succinate dehydrogenase but not the other component of the respiratory chain involved in succinate and NADH oxidation since no similar effect was observed with NADH as a substrate.

In mi-1 mutant mitochondria the slow electron flux from succinate is transferred only via the cytochrome pathway (Drabikowska, 1975) provided cytochrome oxidase is not inhibited by cyanide. In the presence of cyanide the reducing equivalents are directed to the alternate pathway of oxidation. As seen from Fig. 3 this pathway is inactivated on aging of mitochondria.
Ubiquinone reduction

It is generally believed that, as in animal mitochondria (Kröger & Klingenberg, 1973a,b), ubiquinone provides the acceptor system for flavoprotein dehydrogenases both in the mi-l and in the wild type mitochondria (von Jagow et al., 1973; Drabikowska, 1975). The redox pattern of ubiquinone in the mi-l mutant mitochondria in the active and controlled state with succinate as substrate differs greatly from the pattern in the uncoupled state (Drabikowska, 1975).

For the sake of simplicity, the energy-dependent processes, which might complicate interpretation of the results, were eliminated, and all experiments were carried out with uncoupled mitochondria. Use was made of succinate, the slowly oxidizable substrate, because the redox changes of ubiquinone in the presence of this substrate are easily detected in contrast to those observed with actively oxidized NADH (Drabikowska, 1975). The following criteria were used to prove the presence of the alternate oxidase: 1) low degree of ubiquinone reduction upon inhibition of cytochrome oxidase, and 2) oxidation of reduced ubiquinone upon inhibition of cytochrome oxidase and succinate dehydrogenase by cyanide and malonate, respectively.

In fresh mitochondria with the active alternate oxidase pathway, oxidation of ubiquinone should occur via both the cyanide- and SHAM-sensitive pathways. The results shown in Table 1 (Expt. no. 1 and 2) confirm our previous observation that mitochondria isolated from the mi-l mutant in the medium without albumin are completely uncoupled. In the presence of cyanide 48% of ubiquinone is reduced. This reduction is a consequence of the equilibrium established between oxidation of reduced ubiquinone by the alternate oxidase pathway, and its reduction by succinate dehydrogenase. The finding of comparatively high reduction of ubiquinone in the presence of cyanide and slight reduction in the presence of SHAM, indicates that ubiquinone is not equally accessible to cytochrome oxidase and the alternate oxidase, though the latter is several times more active than cytochrome oxidase.

On aging of mitochondria the reduction of ubiquinone in the presence of cyanide increases. In aged mitochondria it approaches the degree of reduction observed upon complete inhibition of respiration by cyanide and SHAM in fresh mitochondria (78%).

The phenomena observed may indicate that the alternate oxidase pathway undergoes either high or total inactivation in mitochondria in vitro. If this assumption is valid, then the reduced ubiquinone should not be reoxidized by this pathway upon inhibition of cytochrome oxidase. Under the special experimental conditions created, i.e. after complete anaerobic reduction of ubiquinone, both its reoxidation via the cytochrome oxidase pathway and the supply of reducing equivalents to ubiquinone are eliminated by cyanide and malonate, respectively. It was expected that in fresh mitochondria reduced ubiquinone would similarly be reoxidized, both via the cyanide-sensitive and cyanide-insensitive pathways. Results presented in Table 1 (Expt. no. 4) indicate that reduced ubiquinone can be completely reoxidized via the cyanide-sensitive pathway and only to 80-85% via the SHAM-sensitive
Table 1

Aerobic steady-state reduction of ubiquinone by succinate in the presence of respiratory inhibitors in fresh and aged mitochondria of the Neurospora mi-1 mutant

Ubiquinone content (4.0 - 4.8 nmoles/mg protein) was estimated as described in Materials and Methods. The incubation medium contained the isolation medium and mitochondria in an amount of 2 - 3 mg of protein/2 ml. Inhibitors (2 mM-SHAM and/or 2 mM-KCN) were introduced 2 min before substrate addition. The reduction of ubiquinone was initiated by addition of 10 mM-succinate to the vigorously aerated reaction mixture. Incubation with substrate was carried out for 20 sec at room temperature. Experiments with malonate were carried out as follows. Mitochondria were incubated with cyanide or SHAM to inhibit cytochrome oxidase or the alternate oxidase, respectively. After 2 min succinate was added and then the reaction mixture was flushed with nitrogen to maximally reduce endogenous ubiquinone. Afterwards malonate (30 mM) was added and the mixture was vigorously aerated for 30 sec. Where shown, 0.1 mM-FCCP was used as an uncoupler. Succinate was oxidized at a rate of 80 nmoles O₂/mg of protein/min in fresh and aged mitochondria.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Mitochondrial preparation</th>
<th>Reduction of ubiquinone (%) in the presence of</th>
<th>SHAM</th>
<th>SHAM + KCN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>none</td>
<td>KCN</td>
<td>SHAM</td>
</tr>
<tr>
<td>1</td>
<td>Fresh (state 4)</td>
<td>30</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Fresh + FCCP</td>
<td>30</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>Aged + FCCP</td>
<td>30</td>
<td>72</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>Fresh + FCCP + malonate</td>
<td>—</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Aged + FCCP + malonate</td>
<td>—</td>
<td>73</td>
<td>0</td>
</tr>
</tbody>
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oxidase pathway (15 - 20% remains reduced). These results provide further evidence for the localization of ubiquinone in the main respiratory chain and indicate that ubiquinone is not directly accessible to the alternate oxidase pathway.

In aged mitochondria reduced ubiquinone cannot be reoxidized via the alternate oxidase pathway (Expt. no. 5). The lack of reoxidation of reduced ubiquinone, the disappearance of SHAM-sensitive oxidation of NADH (reflected by the drastic decrease in the NADH oxidase activity) and complete inhibition of respiration by cyanide with both succinate and NADH as substrates prove that the alternate oxidase is inactivated during aging of mitochondria. It can also be concluded that the alternate oxidase is responsible for the drop in the total oxidation activity and for the enhanced reduction of ubiquinone upon inhibition of cytochrome oxidase by cyanide.

The results presented in this paper explain the divergent data on the oxidation activities and the contribution of cyanide-insensitive respiration to the overall rate of electron transport from substrate to oxygen in different mitochondrial preparations.

Leakage of magnesium and components absorbing at 260 nm

Fresh mitochondria contain about 100 - 120 nmoles of magnesium per mg of protein. During the aging in vitro, leakage of mitochondrial magnesium into the surrounding medium occurs (Fig. 4). The loss of mitochondrial magnesium and
the inactivation of NADH oxidase take place at the same time. However, these processes do not seem to be related to each other, since the divalent cation ionophore A23187 used at a concentration of 5 nmoles per mg protein had no effect on the oxidation activity in fresh mitochondria. This ionophore affects the efflux of magnesium from rat liver mitochondria (Reed & Lardy, 1972) and from Neurospora mitochondria (unpublished).

![Graph 1](image1)

Fig. 4. Time-dependent release of magnesium from mitochondria. Magnesium content was determined by atomic absorption as described in Materials and Methods. Mitochondrial suspension (2 - 3 mg of protein/ml) was incubated at room temperature. At appropriate time intervals samples were withdrawn and magnesium was estimated. ○, Magnesium content in mi-l mitochondria; ●, in the supernatant.

![Graph 2](image2)

Fig. 5. Gradual diffusion of components absorbing at 260 nm from mitochondria. The experiments were carried out as described in Materials and Methods. Mitochondria of the wild type (○); mi-l (●); mi-1 mitochondria isolated in the isolation medium with 0.2% albumin (□). The results are expressed in absorbance units per 1 mg of protein per 1 ml.

The aging phenomena are also manifested by leakage of mitochondrial components absorbing at 260 nm into the surrounding sucrose medium (Fig. 5). This leakage results probably from swelling of the mitochondria (not shown) and from destructive changes in their membrane. Bovine serum albumin prevents swelling by binding fatty acids liberated from phospholipids (Wojtczak & Lehninger, 1961). As a consequence of its action the leakage of components absorbing at 260 nm is also lowered.

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REFERENCES

ZANIKANIE UTLENIANIA NIEWRAZLIWEGO NA CYJANEK
W MITCHONDRIACH MUTANTA NEUROSPORA CRASSA MI-1 IN VITRO

Streszczenie

Aktywność utleniania egzogennego NADH przez mitochondria szczepu dzikiego i mutanta mi-1 Neurospora crassa spada szybko in vitro. W mitochondriach mutanta mi-1 spadek aktywności jest wynikiem inaktywacji oksydazy niewrażliwej na cyjanek. W dzikim szczepie spadek aktywności następuje w wyniku inaktywacji nieznanej składnikla łańcucha oddechowego. Aktywność dehydrogenazy egzogennego NADH nie ulega zmianie w czasie trwania eksperymentu (2-4 godz.). W mitochondriach przechowywanych w atmosferze azotu aktywność utleniania NADH nie zmienia się.

Aktywność oksydazy bursztynianowej nie zmienia się w sposób widoczny w czasie 2-3 godz. W świece wyziolowanych mitochondriach mutanta mi-1 zredukowany ubichinon jest całkowicie utleniany przez cytochromową oksydazę. Oksydaza niewrażliwa na cyjanek utlenia go tylko w 80%.

W podstawowych mitochondriach mutanta mi-1 w obecności cyjanuku ubichinon jest redukowany w tym samym stopniu co w mitochondriach ścieżek, w których oddychanie zahamowane jest całkowicie przez cyjanek i kwas salicylo-hydroksamowy. Zredukowany ubichinon jest wówczas utleniany tylko przez oksydazę cytochromową.

Otrzymane wyniki sugestują, że labilny składnik łańcucha oddechowego szczepu dzikiego jest także składnikiem łańcucha oddechowego mutanta mi-1 i że pełni on w tym mutancie funkcję oksydazy niewrażliwej na cyjanek.

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