PARTICIPATION OF A NON-RESPIRATORY FERROUS COMPLEX DURING MITOSIS IN ROOTS

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ABSTRACT

A systematic survey was undertaken, of the effects of carbon monoxide and hydrogen cyanide (in the presence of 20 per cent oxygen), in darkness and light, on the relative rates of respiration, mitosis, and interphase in pea root tips. The inhibition of respiration by carbon monoxide was light-sensitive, but the inhibition by hydrogen cyanide was light-stable. The inhibitions were presumably due to combination of the inhibitor with the iron of cytochrome oxidase, in its divalent and trivalent forms respectively. In contrast, the inhibitions of mitosis by both poisons proved to be light-sensitive. The light-sensitive inhibition of mitosis by carbon monoxide shows that an iron complex is responsible for the process. That the inhibition of mitosis by hydrogen cyanide is also light-reversible shows that, in contrast with cytochrome oxidase, the mitotic iron complex remains always in the divalent state.

The relative affinities of the mitotic ferrous complex, in molar units, were 0.68 for CO/O2, and 0.37 for HCN/O2. The properties of the complex are analogous to, yet distinct from, Gastrophilus haemoglobin and reduced cytochrome oxidase. It is considered that the arrest of mitosis by oxygen lack, carbon monoxide, and hydrogen cyanide is definitely due to interference with this unidentified, non-respiratory ferrous complex.

The preceding paper (Amoore, 1962 b) has demonstrated that, in strictly anaerobic conditions, the relative rate of mitosis in pea root tips fell to about 15 per cent of the rate in air. The most likely explanation, that lack of oxygen arrested mitosis by stopping the generation of respiratory energy, has been shown to be untenable on quantitative grounds (Amoore, 1961 b).

Nevertheless, throughout this work, a striking resemblance has been observed between mitosis and respiration. For example, sodium cyanide, which is a potent inhibitor of cytochrome oxidase, is also capable of arresting mitosis by stopping the generation of respiratory energy, has been shown to be untenable on quantitative grounds (Amoore, 1961 a). When a process both requires oxygen and is poisoned by cyanide, it may be suspected that a heavy metal is involved. This was Warburg's theory when he was studying the Atmungsferment (see his monograph, 1949). However, there are other reactions by which cyanide might interfere with metabolism, so Warburg (1926) also employed the far more selective inhibitor, carbon monoxide. This gas does not normally react with any cellular constituents other than certain heavy metals. Furthermore, if iron should happen to be the metal concerned, it may be positively identified by the unique light-sensitivity of the iron carboxyls.

Warburg was taking advantage of Haldane and Smith's (1896) discovery that carboxy-haemoglobin is sensitive to light. Nowadays, additional information might perhaps be obtained by applying Kellin and Hartree's (1953) discovery that the cyanide compounds of some ferro-haemoproteins are also photo-dissociable.

A systematic survey was undertaken, of the effects of carbon monoxide and hydrogen cyanide
in the presence of 20 per cent oxygen), in darkness and light, on the relative rates of respiration, mitosis, and interphase in pea root tips. The results permitted more detailed discrimination between the three processes. Furthermore, it was definitely established that the arrest of mitosis by oxygen lack, carbon monoxide, or hydrogen cyanide, is due to interference with a heavy metal. The metal is iron; it is present in a ferrous complex, and it is non-respiratory.

A preliminary account of these results was given at the 413th meeting of the Biochemical Society (Amoore, 1962 a).

METHODS
The general experimental methods were as previously described (Amoore, 1961 a, b, 1962 b).

MANOMETRY IN DARKNESS AND LIGHT. Two identical manometer baths of the rectangular pattern were employed. They were set at the same temperature (25.0°C) and shaking rate (90 cycles/minute). A thermobarometer was placed in each bath. One bath was shrouded throughout the incubation by a black cloth. The other bath was fitted by the suppliers (A. Gallenkamp and Co., London) with a "light trough," containing four Osram 40 w, 2-feet white fluorescent lamps, mounted horizontally in a vertical row, and cooled by a blower. A light intensity of about 600 foot-candles was registered at the manometer flasks.

EXPERIMENTS WITH CARBON MONOXIDE. Nitrogen (oxygen-free) and oxygen were supplied by the British Oxygen Company Ltd., London. Carbon monoxide was obtained from the Imperial Chemical Industries Ltd., London. The apparatus and methods of preparing the gas mixtures were developed from those described previously (Amoore, 1961 b). All gas mixtures contained 20 per cent oxygen. Mixtures containing over 10 per cent carbon monoxide were prepared by first filling the Warburg flask with oxygen, then evacuating to 1/2 atmosphere, and adding the desired pressure of carbon monoxide, finally adding nitrogen to restore atmospheric pressure. Mixtures containing less than 10 per cent carbon monoxide were made by serial dilution of carbon monoxide with air in a 1 liter bottle, and then transferred to the Warburg vessel.

EXPERIMENTS WITH HYDROGEN CYANIDE. For measuring the respiration of root tips in the presence of cyanide, mixtures of calcium hydroxide and calcium cyanide were prepared and used as described by Robbie (1948). The appropriate mixture (0.4 ml) was placed in the center well, and 1.0 ml of a corresponding neutralized sodium cyanide solution was placed in the main well. For whole seedlings, an additional 0.6 ml of Ca(OH)₂/Ca(CN)₂ mixture was placed in the side-arm.

CALCULATION OF RELATIVE RATES OF MITOSIS AND INTERPHASE. The methods of calculating relative rates of mitosis and interphase were explained in the previous paper (Amoore, 1962 b).

RESULTS
The rates of respiration, mitosis, and interphase in pea root tips have already been shown to be sensitive to oxygen tension (Amoore, 1961 b, 1962 b). Accordingly, in this survey, the oxygen tension was kept constant, while the concentrations of carbon monoxide and hydrogen cyanide were varied. The oxygen tension in air (20 per cent oxygen) was selected as the standard.

Effects of Carbon Monoxide and Hydrogen Cyanide on Respiration of Root Tips, in Darkness and Light
In 80 per cent carbon monoxide, in the dark, the respiration of isolated pea root tips was inhibited by about 25 per cent (Fig. 3, in Discussion section). The inhibition was reversed by light, and actually gave way to a stimulation of respiration to 22 per cent above that in air. The inhibition of respiration by carbon monoxide, and its reversal by light, may be explained in terms of the inhibition of cytochrome oxidase, by the combination of carbon monoxide with the reduced (ferrous) form of the oxidase (Warburg, 1926). The inhibition is competitive, and it is largely light-reversible. The oxidized form of the enzyme probably does not combine with carbon monoxide at all.

The effects of hydrogen cyanide upon the respiration were the same in darkness or light (Fig. 4, in Discussion). There was about 80 per cent inhibition of respiration in 10⁻⁶ M hydrogen cyanide. The inhibition of respiration may be accounted for by combination of the oxidized form of cytochrome oxidase with undissociated hydrogen cyanide (Warburg, 1927; Stannard and Horecker, 1948). The inhibition is non-competitive. The affinity of the reduced form of the enzyme for cyanide is apparently too low to contribute appreciably to the inhibition. Only the ferro-haemoprotein cyanide complexes seem to be photo-dissociable (Keilin and Hartree, 1955). This explains why light does not influence the inhibition by cyanide of respiration through cytochrome oxidase.
Effect of Carbon Monoxide on Mitosis in Root Tips, in Darkness and Light

It has been found that carbon monoxide is able to inhibit mitoses in pea roots. The effects of 0.1 to 80 per cent carbon monoxide on the mitotic index are shown in Fig. 1. After 3½-hour exposure, in darkness, the mitotic indices were still substantially higher than that in the control tips incubated in air. The curve showed a definite peak at 10 per cent, and fell steeply to a lower value in 80 per cent carbon monoxide.

![Figure 1](image)

**Figure 1**
Effect of carbon monoxide on mitotic index. 3½-hour exposure. ●, darkness; ○, light.

This curve was compared with that showing the mitotic index after a 4-hour incubation in a wide range of oxygen tensions (Amoore, 1961 b, Fig. 2). The peak mitotic index observed at 10 per cent carbon monoxide probably corresponds with the peak observed in 0.05 per cent oxygen. The low mitotic index in 80 per cent carbon monoxide may correspond with the trough noted in 0.005 per cent oxygen. Application of the principles developed in the preceding paper (Amoore, 1962 b) showed that the relative rate of mitosis must have been appreciably slowed by 1 per cent carbon monoxide, and progressively slowed still more by increasing the carbon monoxide tension to 10 and 80 per cent.

It has been observed that high values of mitotic index, possibly representing a partial arrest of mitosis, persisted after 3½-hour exposure to 0.01 per cent or even 0.001 per cent carbon monoxide. This curious result seems to have no analogy in the oxygen cure, so no estimates of rates of mitosis could be made below 1 per cent carbon monoxide.

The light had very little effect upon the mitotic index in air or in 0.1 per cent carbon monoxide. However, above 0.1 per cent carbon monoxide, the mitotic index curve in the light followed a completely different course from that in the dark. Instead of rising to a peak in 10 per cent carbon monoxide, the mitotic index fell progressively to a low value in 30 per cent carbon monoxide. Thus, the light had completely prevented the partial arrest of mitosis observed in 1 to 30 per cent carbon monoxide in the dark.

It may be noted that the low value in 30 per cent carbon monoxide in the light was substantially below the mitotic index observed after a corresponding incubation in air in light or darkness. These low mitotic indices possibly represent a stimulation of mitosis.

In 80 per cent carbon monoxide, in the light, the mitotic index curve turned upwards again, reaching a value close to that observed after a corresponding incubation in air in the dark. This probably represents the beginnings of an inhibition of mitosis, and shows that in the light a concentration of carbon monoxide at least 80 times higher than that which is effective in the dark is necessary to inhibit mitosis.

**Calculation of the Relative Rates of Mitosis in Carbon Monoxide**

So far as possible at present, the mitotic indices recorded in Fig. 1 and in another similar experiment were converted into relative rates of mitosis, using the methods of calculation worked out for low oxygen tensions (Amoore, 1962 b). The results of the calculations are shown in Fig. 3. The shape of the mitotic index curve in the light was unlike any curve previously encountered, so no estimates of rate were generally possible. However, it was noted that in 80 per cent carbon monoxide in the light the mitotic index happened to be nearly the same as that observed in the absence of carbon monoxide. The assumption was made that this equality of mitotic index represented equal rates of mitosis.

**Effect of Hydrogen Cyanide on Mitosis in Root Tips, in Darkness and Light**

It has already been shown that sodium cyanide can inhibit mitoses (Amoore, 1961 a, b). In the following experiment the effects of hydrogen cyanide (10⁻⁴ to 10⁻⁴ M) on the mitotic index were tested in both darkness and light (Fig. 2). In darkness, the results were comparable with those previously observed (Amoore, 1961 a, Table 1; 1961 b, Table 2). That is, a high mitotic index was main-
tained for 4 hours in $10^{-3}$ or $10^{-4}$ M hydrogen cyanide. This peak probably corresponds with the peak in 0.05 per cent oxygen (Amoore, 1961 b, Fig. 2). In $10^{-2}$ M hydrogen cyanide (Fig. 2) the mitotic index fell to a value rather below the control in air. This resembles the effects of 80 per cent carbon monoxide (Fig. 1) or 0.005 per cent oxygen (Amoore, 1961 b, Fig. 2).

Light had no effect on the mitotic index in air or in $10^{-4}$ M hydrogen cyanide, but at all higher concentrations of cyanide the curve representing mitotic index in the light followed a quite different course from that in the dark. At $10^{-3}$ to $10^{-2}$ M hydrogen cyanide, the mitotic index in the light remained well below that in the dark (Fig. 2).

This shows that light prevented the arrest of mitosis by cyanide which occurred in the dark.

Between $10^{-3}$ and $10^{-2}$ M hydrogen cyanide, despite the light, the mitotic index curve turned sharply upwards to a value well above the initial control at the start of the experiment. This must represent inhibition of mitosis, and shows that in the light a concentration of hydrogen cyanide at least 100 times higher than is adequate in the dark is necessary to arrest mitosis.

**Calculation of Relative Rates of Mitosis in Hydrogen Cyanide**

Calculations of rates of mitosis were made, using not only the data in Fig. 2, but also all available information on mitotic indices in published experiments involving cyanide (Amoore, 1961 a, b). The results are shown in Fig. 4.

**Calculation of Relative Rates of Interphase in Carbon Monoxide and Hydrogen Cyanide**

From the available data, relative rates of interphase could only be calculated for 1, 10, and 80 per cent carbon monoxide, and for $10^{-5}$ M hydrogen cyanide. The results are included in Figs. 3 and 4. The accuracy of these estimates is probably low, but they do provide some indication of the susceptibility of interphase to these poisons.
DISCUSSION

Relative Rates of Respiration, Mitosis, and Interphase in Pea Root Meristem, as Functions of Carbon Monoxide Tension

The relevant data have been assembled in the form of a graph (Fig. 3). This demonstrates qualitatively that both respiration and mitosis are inhibited by high tensions of carbon monoxide, in darkness. In the light, both these inhibitions are virtually totally prevented. As regards respiration, Warburg (1926) considered that the light reversibility of the inhibition due to carbon monoxide constitutes proof that the respiration is catalysed by iron. No exception has since been found to this rule (Warburg, 1949, p. 81). Concerning mitosis, it appears justifiable to apply the same reasoning, and assume that the light reversibility of the inhibition of mitosis due to carbon monoxide constitutes proof that mitosis likewise is dependent upon iron.

Quantitatively, the graph shows that mitosis is more sensitive to carbon monoxide than is respiration. The effects of carbon monoxide on respiration and mitosis are in contrast with the effects of oxygen lack, for mitosis was found to be less sensitive to oxygen lack than was respiration (Amoore, 1961 b, 1962 b). Therefore, the iron atoms promoting mitosis are not the same as those catalysing respiration.

Fig. 3 also shows that the rate of interphase is more susceptible to carbon monoxide than is respiration. In 1 per cent carbon monoxide both mitosis and interphase appeared to be about equally inhibited, but at higher tensions of carbon monoxide interphase became more susceptible than mitosis.

Relative Rates of Respiration, Mitosis, and Interphase in Pea Root Meristem, as Functions of Hydrogen Cyanide Concentration

The information has again been collected in the form of a graph (Fig. 4). Qualitatively, it appears that both respiration and mitosis are inhibited, in darkness, by high concentrations of hydrogen cyanide. However, light relieved only the inhibition of mitosis; the inhibition of respiration by hydrogen cyanide was quite unaffected by illumination. This result is in striking contrast with the effects of light in the presence of carbon monoxide, when the inhibitions of both respiration and mitosis were relieved.

The immunity to light of the inhibition of respiration by hydrogen cyanide was considered in the first section of the Results. The explanation is that hydrogen cyanide inhibits respiration by combining preferentially with trivalent iron (of cytochrome oxidase), the resulting complex being unaffected by light. The converse of this argument may be applied to the present results to obtain an interesting conclusion. The fact that the inhibition of mitosis by hydrogen cyanide is completely prevented by light shows that the iron atoms promot-
Relative dependencies of respiration, mitosis, and interphase in pea root tips upon molar concentrations of oxygen, carbon monoxide, or hydrogen cyanide (in darkness). Continuous lines represent oxygen, broken lines carbon monoxide, and dotted lines hydrogen cyanide. ●, respiration; ■, mitosis; ▲, interphase. 20 per cent oxygen was present in all experiments with carbon monoxide or hydrogen cyanide.

**TABLE I**

Molar Concentrations ($c_{50}$) of Oxygen, Carbon Monoxide, and Hydrogen Cyanide Required to Give 50 per cent Relative Rates of Respiration, Mitosis, and Interphase

<table>
<thead>
<tr>
<th>Process</th>
<th>Oxygen (M)</th>
<th>Carbon monoxide (M)</th>
<th>Hydrogen cyanide (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td>$1.0 \times 10^{-4}$</td>
<td>$1.4 \times 10^{-2*}$</td>
<td>$3.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>Mitosis</td>
<td>$4.2 \times 10^{-6}$</td>
<td>$3.7 \times 10^{-4}$</td>
<td>$6.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Interphase</td>
<td>$1.0 \times 10^{-4}$</td>
<td>$5.7 \times 10^{-4}$</td>
<td>$5.7 \times 10^{-4*}$</td>
</tr>
</tbody>
</table>

* Extrapolated values.
presence of 20 per cent oxygen $(2.5 \times 10^{-4}$ M oxygen). The $c_{50}$ values for carbon monoxide and hydrogen cyanide may, therefore, be compared with the $c_{50}$ value represented by $2.5 \times 10^{-4}$ M oxygen. The inverse ratio of the $c_{50}$ values provides an estimate of the relative affinities; i.e. the molar concentration ratios of $O_2/CO$ or $O_2/HCN$ present when the relative rates were decreased to 50 per cent. The relative affinities of the respiratory, mitotic, and interphase systems for carbon monoxide, hydrogen cyanide, and oxygen are listed in Table II. The data were taken from the last two columns of Table I. It is apparent that the relative affinities of respiration, mitosis, and interphase are quite different from each other, which allows more exact discrimination between the three processes.

Comparison of the Properties of the Unknown Mitotic Ferrous Complex with Those of Known Ferro-Haemoproteins

Among the known, naturally occurring complexes of iron, the divalent ferro-haemoproteins

### Table II

**Relative Affinities of the Respiratory, Mitotic, and Interphase Systems for Carbon Monoxide, Hydrogen Cyanide, and Oxygen**

<table>
<thead>
<tr>
<th></th>
<th>Carbon monoxide</th>
<th>Hydrogen cyanide</th>
<th>Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respiration</strong></td>
<td>0.18</td>
<td></td>
<td>10^{-4} M oxygen</td>
</tr>
<tr>
<td><strong>Mitosis</strong></td>
<td>0.68</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td><strong>Interphase</strong></td>
<td>4.4</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

* No value, because in respiration hydrogen cyanide does not compete with oxygen.

### Table III

**Chemical Properties of the Unknown Mitotic Ferrous Complex, Compared with Those of Some Representative Ferro-Haemoproteins**

The figures in parentheses indicate the references (listed below) from which the original data were obtained. The unknown mitotic ferrous complex (data printed in heavy type) has been inserted in what appears to be its natural position in the gradations of properties running across the table.

<table>
<thead>
<tr>
<th></th>
<th>Oxygen carriage</th>
<th>Oxygen storage</th>
<th>Mitosis</th>
<th>Oxidation (respiration)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haemoglobin</td>
<td>Chlorophyll</td>
<td>Myoglobin</td>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Valency of iron</strong></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>s in Hill's equation</strong></td>
<td>2.2</td>
<td>(2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Relative affinity, CO/O_2</strong></td>
<td>300</td>
<td>760</td>
<td>49</td>
<td>26</td>
</tr>
<tr>
<td><strong>Relative affinity, HCN/O_2</strong></td>
<td>&lt;0.001</td>
<td>?</td>
<td>?</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Hill's (1910) equation not obeyed (Amoore, 1962 b).
‡ No competition.

References and notes.

alone seem to be capable of reversible combination with oxygen, carbon monoxide, and hydrogen cyanide. In Table III are assembled some representative ferro-haemoproteins, arranged in a sequence from the oxygen-carriers on the left, through the oxygen-storers in the middle, to the oxidizing enzyme on the right. This table may be regarded as an expansion of that given by Keilin and Wang (1946), who point out that the autoxidizability of the iron increases progressively towards the right. The other chemical properties also exhibit a consistent gradation from left to right across the table. Thus, the natural valency of the iron increases, the value of \( n \) in Hill's equation decreases, the relative affinities for CO/O\(_2\) decrease, and the relative affinities for HCN/O\(_2\) increase. The two relative affinities are particularly interesting, as they cover a range of over 1000-fold, and in opposite directions.

Despite this wide variety of properties, it is possible to place the data for the unknown mitotic iron complex in a single column of the table, where its chemical properties fit very satisfactorily into all the natural gradations running across the table. These properties show that the mitotic iron complex is very closely analogous to \( \textit{Gastrophilus} \) haemoglobin, and to the reduced form of cytochrome oxidase. However, the mitotic ferrous complex is not naturally autoxidizable, which distinguishes it from reduced cytochrome oxidase. Furthermore, it does not obey Hill's (1910) equation (Amoore, 1962 b), which distinguishes it from both reduced cytochrome oxidase and \( \textit{Gastrophilus} \) haemoglobin.

**Other Evidence that Iron is Involved in Cell Division**

The results of three other investigations appear to be especially relevant, because they all implicate iron in the cell division process of root meristems. Kihlman, Merz, and Swanson (1957) suggested that iron may act as a structurally linking material in chromosomes. They considered that its state of oxidation, and whether or not it is complexed with an added reagent, determine the lability of chromosomes in bean roots to ionizing radiations and other forms of interference. Brown and Possingham (1957) demonstrated, by culturing excised pea roots in iron-deficient media, that iron is required for the cell division process itself, and not just for the respiratory support of division. They further showed (Possingham and Brown, 1958) that radioactive iron is accumulated to a greater extent in the nucleus than in the cytoplasm. Burström (1961) found that cell multiplication in excised wheat roots was inhibited by ethylenediaminetetraacetic acid (EDTA) in darkness, and that the inhibition was relieved by light. He also found that in the dark, ferrous ions were the most effective for reversing the inhibition by EDTA.

Unfortunately, it does not appear possible to make direct comparisons between these studies and the present work. Many of the earlier results of Kihlman \( et \ al. \) (1957) were later satisfactorily reinterpreted according to a different hypothesis (Kihlman, 1959). Brown and Possingham (1957) and Burström (1961) measured cell multiplication over a known interval, without studying interphase and mitosis separately.

The present work shows that mitosis is arrested by oxygen lack, carbon monoxide, and hydrogen cyanide, through interference with an unidentified ferrous complex, which must be closely analogous to certain ferro-haemoproteins. However, there is still no evidence as to how this previously unsuspected ferrous complex operates in the mitotic process.

**Effects of Carbon Monoxide in the Light**

Not all the effects of carbon monoxide on respiration and on mitosis were counteracted by light. The inhibitions of cytochrome oxidase and the mitotic ferrous complex were completely removed, because the ferrous carbonyls are photodissociable. Yet, in the light, 0.1 per cent carbon monoxide apparently caused about 15 per cent inhibition of respiration, whereas 30 per cent carbon monoxide caused about 20 per cent stimulation (Fig. 3). Furthermore, also in the light, 0.1 per cent carbon monoxide caused the persistence of a high mitotic index, whereas 30 per cent resulted in an unusually low mitotic index (Fig. 1); these results have been provisionally interpreted to mean inhibition and stimulation of mitosis respectively. If Warburg's rule (1949, p. 81) is applied to these anomalous results, they must presumably also be due to interference with one or more endogenous heavy metals, probably other than iron.

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