

THE EFFECTS OF CARBON MONOXIDE INHIBITION ON ATP LEVEL AND THE RATE OF MITOSIS IN THE SEA URCHIN EGG

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ABSTRACT

The requirements for ATP synthesis during the various phases of mitosis were investigated in the oxygen-requiring eggs of the sea urchin, *Strongylocentrotus purpuratus*. CO in the dark, a specific inhibitor of respiration, was used to inhibit ATP synthesis. The kinetics of respiratory inhibition were determined by analyzing ATP levels with the luciferin-luciferase assay. The kinetics of mitotic inhibition were determined by analysis of the rate of mitosis. It was found that CO inhibition resulted in a decrease in the normal ATP level. Coincident with this decrease was a decrease in the rate of mitosis which stops completely when the ATP drops below 50 per cent of the normal level. With the use of various degrees of CO inhibition, the rate of mitosis is shown to be related to the resultant ATP level. These results contradict the basic premise of the energy reservoir hypothesis, and also disagree with other reports that cells in mitosis are insensitive to inhibitors of energy metabolism. Data are presented which demonstrate that these conflicting reports result from insufficient inhibition of ATP synthesis. The above findings all indicate that mitosis depends on the continuous synthesis and utilization of ATP.

Three contradictory hypotheses exist to explain the linkage between energy metabolism and mitosis. Each of these is based on studies of the effects of inhibitors of ATP synthesis on mitosis.

First, there are numerous experiments (reviewed by Bullough, 1952; and Swann, 1957) which indicate that mitosis is not affected by these inhibitors. These results have been interpreted as showing that the energy for mitosis is supplied by an energy reservoir, synthesized during interphase, and used specifically for mitosis (Swann, 1957).

A second group of studies indicates that all phases of mitosis except anaphase can be arrested by these inhibitors (Förster and Örstrom, 1933; Fry, 1950; Hughes, 1950). Mazia (1961), in his monograph on mitosis, suggested that this insensitivity could best be explained by assuming that the energy expended in constructing the mitotic apparatus resulted in an activated structure,

which then provided the energy for anaphase movement.

Finally, a third group of studies indicates that every phase of mitosis can be arrested by inhibitors of ATP synthesis (Blumenthal, 1930; Harvey, 1927). The simplest interpretation of this result is that the continuous synthesis of an energy supply is required throughout the mitotic period.

These three hypotheses are thus (1) an energy reservoir, (2) an activated mitotic apparatus, and (3) a continuous energy supply. The experiments described below were designed to decide which hypothesis is tenable by determining which experimental finding is correct.

MATERIALS AND METHODS

The cell type used in these experiments was synchronously dividing eggs of the sea urchin. The advantage of these eggs is their obligate aerobic

metabolism (Krahl, 1950), which allows use of specific respiratory inhibitors, such as CO, as opposed to non-specific glycolytic inhibitors, such as fluoride and iodoacetate.

The eggs were those of *Strongylocentrotus purpuratus*, which were prepared and fertilized according to the usual procedure of this laboratory (Mazia and Zimmerman, 1958). The method of ATP analysis was the luciferin-luciferase assay (Strehler and Totter, 1953), modified for ATP specificity by a method similar to that of Wahl and Kozloff (1962). The inhibitor was a CO/air mixture, identical to that used by Swann (1953), composed of 97 per cent CO/3 per cent air. The advantage of this particular gas mixture is that respiration is normal in the light, but is inhibited in the dark (Krahl, 1950).

Ten minutes before the inhibition was to begin the eggs were placed in a 50 x 160 mm cylindrical gas bubbling tube, and illuminated by white light (12,800 foot candles/sq. ft.). In the dark experiments the inhibition was begun by turning the light off. In the green light experiments, an interference filter was placed in front of the illuminated gassing tube (537 m μ Baird-Atomic; 523 m μ Balzars). The CO/air mixture was bubbled through a 0.6 mm capillary at the bottom of this tube at a constant rate of 250 cc/minute. This bubbling rate was sufficient to keep the suspension stirred. The gas was vented through a length of 3 mm tubing, inserted into a gas-tight hypodermic syringe, above the level of the egg suspension. Samples were taken by simply pushing the syringe, and thus the piece of tubing, below the water level. The gas pressure forced the desired sample of eggs out of the tube, which was then used for analysis. One part of this sample was used for ATP analysis. The remaining eggs were fixed in Carnoy's (3 alcohol: 1 acetic acid) and transferred to 45 per cent acetic acid, viewed with phase microscopy, and the rate of progress of mitosis determined.

The rate of mitosis was determined by the following operations. The average time intervals between the various phases of mitosis at 15.0°C were determined. These time intervals are expressed as cumulative minutes of mitosis from the beginning of prophase. As seen from the ordinate in Fig. 1, a cell in metaphase has progressed through 25 minutes of mitosis, whereas a cell in early anaphase has gone through 29.5 minutes of mitosis. If a cell population is 50 per cent in metaphase and 50 per cent in early anaphase, then this population will have progressed a total of (0.50 x 25) plus (0.50 x 29.5), or 27.25 minutes of mitosis. The plot of cumulative minutes of mitosis against actual time will thus give a line whose slope will be unity for the average cell population. If the rate of progress through mitosis is faster or slower than normal, the slope will be greater or smaller than one. The ratio of the slopes of the progress of

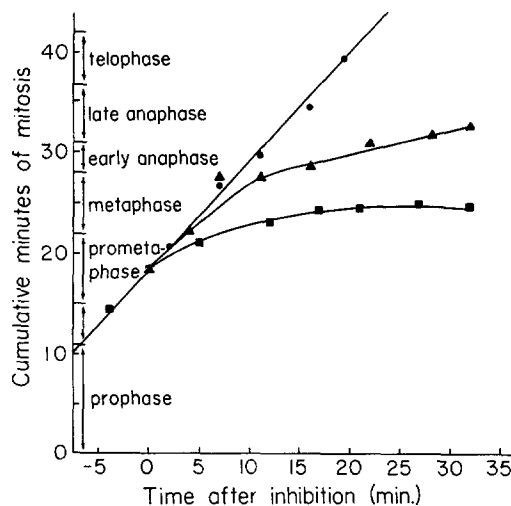


FIGURE 1

Relative progress of mitosis in various types of CO inhibition. ● control, ▲ CO in 537 m μ green light (intensity of 330 foot candles), ■ CO in total darkness. The per cent distribution of mitosis at the beginning of inhibition was 13 per cent late prophase, 55 per cent prometaphase, 24 per cent metaphase, and 8 per cent early anaphase. After 30 minutes' inhibition the controls were all 2-cells. In green light, 28 per cent were metaphase, 3 per cent early anaphase, 41 per cent late anaphase, 25 per cent telophase, and 3 per cent interphase (2-cell). In darkness, 38 per cent were prometaphase, 26 per cent metaphase, and 36 per cent late anaphase.

the experimental and control cells, times 100, thus gives the per cent difference in rate of mitosis due to the experimental conditions.¹

RESULTS

Effects of CO in Darkness on the Progress of Mitosis

The effect of CO inhibition on the progress of mitosis is depicted in the lower curve of Fig. 1. As seen, CO in the dark results in an asymptotic decrease in the rate of mitosis, which reaches zero rate after 22 minutes of inhibition.

The results of another experiment, shown in Table I, demonstrate that cells can be arrested at any stage of mitosis, and that this arrest lasts as long as the inhibitor is present. Line 1 of this table

¹The author is indebted to Dr. John Amoore for suggesting the method of determining minutes of mitosis of a cell population.

represents the percentage distribution of the various mitotic stages at the beginning of the inhibition. Line 2 demonstrates the slight progress of mitosis that occurs during the first few minutes of inhibition. The remainder of the table shows that the cells which are found in every stage of mitosis after 30 minutes' inhibition remain arrested in that stage, since the distribution of the mitotic stages does not change. (The figures seem to indicate that some late prophases progress into prometaphase. However, this is not considered significant since the late prophase nuclear membrane becomes difficult to distinguish during the course of inhibition).

Like CO in the dark, every inhibitor of ATP synthesis tested (amytal, anoxia, Antimycin A,

in the progress of mitosis shown in the lower curve of Fig. 1. When mitosis is completely arrested after 22 minutes inhibition, it is seen that the ATP level is 50 per cent of normal.

Effects of CO in Green Light on Mitosis and ATP Levels

Although the CO mixture used in these experiments is identical to the one used by Swann (1953) in his experiments on sea urchin eggs, the results are different. Swann had interpreted his results as showing that mitosis was insensitive to CO inhibition. The significant difference in experimental conditions was that CO inhibition was applied in 525 m μ green light, whereas in the present experi-

TABLE I
Per Cent of Cells in Various Phases of Mitosis During Prolonged Inhibition
Eggs were fertilized asynchronously so that some cells would be in every stage of mitosis at the beginning of the inhibition. Samples were taken at the times indicated.

Minutes after inhibition	Early prophase	Late prophase	Prometaphase-metaphase	Early anaphase	Late anaphase	Number of cells counted
0	45	35	17	3	0	100
30	30.5	16.5	37.9	0.5	15.9	500
55	31.5	9.9	46.8	1.8	10.3	500
80	28.8	10.2	45.2	1.8	13.8	500
Controls, 80 minutes	Just beginning cleavage of 2nd division					

azide, and 2,4-dinitrophenol) has been found to arrest mitosis. Except with lipid-soluble Antimycin A, the arrest of mitosis is completely reversible. For example, cells arrested during mitosis for 4 hours by anoxia all recovered, and 70 per cent of these developed into normal blastula.

Effects of CO in Darkness on ATP Levels

Measurements of the ATP level during the normal division cycle show no significant changes (interphase, 27 measurements, 0.357×10^{-6} gm ATP/1000 eggs, standard deviation of 0.018; various phases of mitosis, 19 measurements, 0.347×10^{-6} gm ATP/1000 eggs, standard deviation of 0.027). During CO inhibition in the dark the ATP level decreases, as seen in the lower curve of Fig. 2. This decrease coincides with the decrease

ments CO inhibition was applied in complete darkness. Swann's experiments required light since photomicrographs were being taken. Green light was chosen as it was assumed to be outside the absorption bands of the light reversible CO-cytochrome oxidase complex.

The significance of green light was, therefore, determined by comparing CO inhibition of mitosis and ATP synthesis in 537 m μ green light and in darkness. The results of this comparison, shown in Figs. 1 and 2, demonstrate that inhibition in green light has less effect on both mitosis and ATP synthesis than inhibition in darkness. Essentially identical results have also been obtained with 523 m μ light. The effect of green light varies with light intensity, which suggests a photochemical reaction. This reaction is probably the photodissociation of the CO-cytochrome oxidase complex,

which has several absorption peaks in the green region (Castor and Chance, 1955; Warburg, 1949).

The comparison of rates of mitosis in the inhibited cells shows a relationship to the ATP level. In Fig. 1, the average rate of mitosis for the first few minutes in green light is 80 per cent of the normal rate. After about 13 minutes of inhibition the rate has changed to 30 per cent of the normal rate, and then remains constant. These changes in

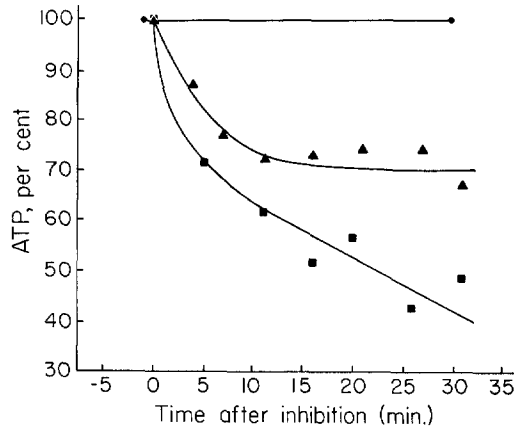


FIGURE 2

ATP levels during various types of CO inhibition (same conditions as Fig. 1). ● control, ▲ CO inhibition in green light, ■ CO inhibition in total darkness. The eggs used for this comparison were taken from one female. 100 per cent ATP is 0.347×10^{-9} gm/egg.

rate correspond to the changes in ATP level shown in Fig. 2. It is seen that in the first 12 minutes of inhibition the ATP level drops to 70 per cent of normal, and then remains at a constant level. Fig. 1 shows that in darkness the rate of mitosis asymptotically approaches zero. As seen in Fig. 2, this approach to zero rate parallels the approach of the ATP to the 50 per cent level. These results indicate that between 50 and 100 per cent of the normal ATP level there is a correlation between the rate of mitosis and ATP level.

DISCUSSION

The results of these experiments show that specific inhibition of ATP synthesis by CO in the dark can arrest mitosis at any stage. This finding means that the energy reservoir and activated mitotic apparatus hypotheses are no longer tenable, since

their basic premise is that all or certain phases of mitosis are insensitive to inhibition of ATP synthesis.

The alternative hypothesis, that all phases of mitosis require the continuous synthesis of ATP, is supported by three of the above findings. The first is the arrest of mitosis by CO inhibition when the ATP falls below 50 per cent of the normal level. The second is shown in the green light experiments, in which a correlation between rate of mitosis and ATP level was found. The third finding is that, although respiratory inhibition can arrest mitosis (*eg.*, CO, amytal, anoxia, Antimycin A, and azide), arrest can also occur when respiration is stimulated by low concentrations (5×10^{-5} M) of the respiratory uncoupler of ATP synthesis, 2,4-dinitrophenol. This would suggest that ATP is the important respiratory product. Furthermore, analyses of ATP levels and rates of mitosis in the presence of these other inhibitors (which shall be published in detail in the future) show that mitosis stops when the ATP reaches the same 50 per cent level.

Why mitosis cannot continue when the ATP level falls below 50 per cent is not clear, but must await elucidation of the meaning of "ATP level." One explanation could be that when the ATP level reaches 50 per cent, it is simply not available for mitosis. Possible reasons for this could be related to cell size and diffusion of ATP from the mitochondria, localization of ATP in cell compartments, or even reversible changes in the properties of the enzymes which utilize ATP caused by the ADP and AMP produced during inhibition. Another explanation is that the ATP level reflects the rate of ATP synthesis and turnover. Thus, cellular processes such as mitosis require a certain critical rate of turnover, which happens to be mirrored in the ATP level.

The dependence of mitosis on ATP is strengthened by, and also imparts physiological significance to, the recently discovered ATPase of the isolated mitotic apparatus (Mazia *et al.*, 1961) and the reports that ATP can cause anaphase movement in glycerated models of tissue culture fibroblasts (Hoffman-Berling, 1959). The experiments also agree empirically with recent reports that the rate of mitosis in pea root tips (Amoore, 1962) and eye cornea (Utkin, 1961) is decreased in the presence of respiratory inhibitors.

These results emphasize the necessity of deter-

mining the true extent of inhibition of ATP synthesis in inhibitor studies. This determination should indicate how much of the ATP synthesis is actually inhibited, and the time course of this inhibition. The importance of the quantitative aspects of inhibition is shown by the comparison of CO inhibition in darkness with CO inhibition in green light. The importance of the time factor is indicated by the continued progress of mitosis until the ATP level reaches a critical point.

It is probable that the many reports that have been interpreted as showing that all or part of the mitotic processes are insensitive to inhibition of

ATP synthesis may be the result of not taking these factors into account.

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