Inhibition of Electron and Energy Transfer in Mitochondria

I. EFFECTS OF AMYTAL, THIOPENTAL, ROTENONE, PROGESTERONE, AND Methylene Glycol*

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The inhibition by oxybarbiturates of diphosphopyridine nucleotide (DPN)-linked oxidations in mitochondria was observed some time ago (1-4). Their effect on the associated phosphorylation was subsequently reported (5, 6), but it is now apparent that oxybarbiturates do not uncouple oxidative phosphorylation (7) nor do they inhibit dinitrophenol-activated adenosine triphosphatase (7). Amytal inhibition of DPNH oxidation has been stated to be insensitive to dinitrophenol (3, 7), and Amytal appears to have no effect on succinoxidase activity (7). Oligomycin (8, 9) and guanidine (10) exhibit some effects similar to those of Amytal, but these are reversible by dinitrophenol (8, 10, 11). This difference in sensitivity to uncoupling agents is found to be a matter of degree and may not represent a significant distinction between sites of action of Amytal and guanidine.

In nonphosphorylating preparations in which electron transfer proceeds through the components of the respiratory chain, and not through the Amytal and antimycin insensitive bypass (19), the site of Amytal inhibition was shown to be on the oxygen side of flavoprotein (12, 13) and has since been localized between flavoprotein and ubiquinone (14). A related site involved in succinate oxidation is here found to be sensitive to high concentrations of methylene glycol and of Amytal (11, 15, 16); a similar effect of Amytal has been reported for α-glycerophosphate (17) and choline oxidation (18).

In phosphorylating mitochondria, spectroscopic studies show a crossover point for Amytal between flavin and DPNH: Amytal concentrations required for half-maximal inhibition of both respiratory rate and steady state oxidation levels of the respiratory components have been determined. Somewhat higher concentrations of methylene glycol have similar effects on DPNH oxidation.

The object of this paper is to present quantitative data on the inhibition of respiratory activity by Amytal in oxidation not only of DPN-linked substrates but of succinate as well. That the effectiveness of Amytal is less in the presence of uncoupling agents than in the presence of adenosine diphosphate (ADP) and phosphate indicates an inhibition of energy transfer in addition to an inhibition of electron transfer. The spectroscopic effects of Amytal are studied with particular emphasis upon the responses of cytochrome b and pyridine nucleotide, which are especially useful in comparisons between the effects of Amytal and thiopental. A mechanism for the effects of Amytal is presented. Related effects of progesterone (19) and methylene glycol are included. Preliminary reports have been presented elsewhere (4, 11, 16).

**EXPERIMENTAL PROCEDURE**

The mitochondrial preparations, reaction media, and physical techniques are identical with those described previously (20). It should be noted that in this series, as in the previous one (20), the mitochondrial cytochrome c or pyridine nucleotide is assayed (see figure legends) and is considered to be a more relevant quantity than the protein concentration or the "milligram equivalents of rat liver" because not all mitochondrial proteins are involved in electron transfer.

Sodium Amytal (Eli Lilly and Company) was dissolved in water to give solutions at a concentration of 0.1 to 0.2 mM, and suitable volumes were added to the reaction mixture. Fresh solutions were usually made up each day. Since the Amytal solutions are not neutralized, special care was taken to monitor pH after adding high concentrations of Amytal. Changes of no more than a few tenths of a pH unit occurred.

Progesterone was dissolved in 50% propylene glycol; the solvent was not inhibitory at the levels employed. The hexetidine (8) was kindly donated by Drs. B. Hagihara and H. A. Lardy.

The experimental procedures used in this series of papers are described in order to give a better understanding of the operations performed during the experiments. There are two general types of diagrams. (a) Spectroscopic observations are made on a slow time scale in which the recordings of one component are plotted on curvilinear coordinates characteristic of the Esterline Angus direct writing recorders. (b) For multiple recording of a number of quantities, an oscillograph camera is used. A typical record is exemplified in Fig. 1. Here, several galvanometers throw light beams onto moving paper, and simultaneous recordings on rectangular coordinates can be made. In such recordings the traces are often complicated by the fact that full scale is used for all three light beams; hence, the curves may cross. Full scale is used in the interests of accuracy in recording. In such diagrams the traces are identified by lettering and by their different widths or photographic densities.

In all traces, time increases from left to right, and the record usually begins a few seconds after the mitochondria have been diluted in the reaction medium. In some cases, supplementation of the mitochondria with several substances may have occurred (for example, in Fig. 8, glutamate and Amytal have been added).
Thus, the various states of the mitochondria are identified as States 1 to 5 (see Table 1 below), and the particular chemicals present are also identified. The additions of various constituents during the time course of the experiment are indicated, and the final concentrations (moles per liter) are given. The times at which additions were made are indicated by arrows and often are accompanied by deflections of the traces, due either to disturbances caused by the stirring rod or to the rapid initiation of the chemical reaction. In some cases the recorder traces are driven off scale, when, for example, the stirring rod interrupts the light beam, and in such circumstances a portion of the trace may be deleted in order to avoid confusion with the other traces.

The time scale is given on each one of the diagrams, and since the time taken as the origin may be arbitrarily chosen, usually only the increment of time corresponding to a given movement of the paper is indicated.

For oxygen recordings, the calibration is made from the initial condition, in which the reaction medium is air-saturated, to the final condition of anaerobiosis caused by oxygen utilization by the mitochondrial suspension. Either the scale of the oxygen trace or actual rates in micromoles per liter per second are included. Since the polarographic technique employs an open cuvette, there is some oxygen diffusion into the solution at very low rates of respiration (11, 21).

In the spectrophotometric traces, the wave lengths used appear directly on the diagram. In addition, the sensitivity is calibrated in terms of the increment in absorbancy corresponding to a given deflection on the recorder paper. Although we have arbitrarily given sensitivities in absorbancy units, these values can readily be converted to percentages of change by dividing by 0.00434. The sense of the spectrophotometric recording has been consistently arranged in this series of papers so that an upward deflection corresponds to an oxidation of the component involved and a downward deflection to a reduction. This usage is similar to the convention used for plotting oxidation-reduction potentials.

This series of papers reports measurements of five of the components of the respiratory chain at appropriately selected pairs of wave lengths. Reduced pyridine nucleotide (essentially DPNH in guinea pig kidney mitochondria) is measured in terms of the difference between absorbancies at 340 and 374 mμ. Cytochrome b is measured in terms of the difference between absorbancies at 430 and 410 mμ; flavoprotein, in terms of the difference at 455 and 500 or 465 and 510 mμ; cytochromes c + c 1, at 550 and 540 mμ; cytochrome a, at 605 and 630 mμ; the α-band of cytochrome b, at 562 and 549 mμ. The extent to which a given component is uniquely measured at one of these wave lengths depends upon the experimental conditions. Fortunately, the Amytal-dinitrophenol-guanidine treatments of the mitochondria affect largely pyridine nucleotide, cytochrome b, and flavoprotein; cytochromes c and a are only slightly affected by these treatments.

For this reason, the reduction of pyridine nucleotide is measured with no known interference from other components. In measurements of the Soret band of cytochrome b, 410 mμ is very near the isosbestic point between the oxidized and reduced forms of cytochrome c, and the peak of the reduced cytochrome b band is at 430 mμ. Difference spectra for the effects of guanidine and Amytal presented in this series of papers support the view that change of cytochrome b dominates the Soret region after guanidine or Amytal treatment. Although measurements of flavoprotein at 455 mμ can be affected by changes in cytochromes a + a 3 that may occur in aerobic-anaerobic transitions, very little change in these components occurs in the presence of Amytal and guanidine. Cytochrome a 1 is measured simultaneously with cytochrome c at 550 mμ; however, its contribution to cytochrome c is small, less than one-third. Nevertheless, when the cytochrome designation, cytochrome c, is used alone in these papers it will imply a simultaneous measurement of cytochrome c 1. Cytochrome b is measured uniquely at its α-band (562 mμ). Cytochrome a 3 causes some interference at 605 mμ, but its steady state changes are less than those of cytochrome a under the conditions of these observations.

In some cases, measurements of cytochrome b and flavoprotein are made in the region of the Soret band, together with simultaneous fluorescence measurements. Interference of the fluorescence emission with the spectrophotometric changes is avoided by using steady light for the former and chopped light for the latter. There is the possibility, however, that the absorption measurements will interfere with the fluorescence measurements.

With the double beam method, reference wave lengths (410 or
510 mp) can be chosen so that they lie outside the response curve for the filter used in measuring fluorescence (peak at 450 mp, half-width, 15 mp). Thus, only the flashes of light at 430 mp and, to some extent, those at 465 mp will cause interference. We have therefore interposed a switch in the measurement of the fluorescence changes so that there is electronic circuit response of the fluorometer only when the noninterfering flash of the reference wave length (410 or 510 mp) is illuminating the sample. Although the details of this arrangement are outside the scope of this publication, the explanation at least indicates the physical possibility of making simultaneous measurements of pyridine nucleotide fluorescence and cytochrome b or flavin kinetics in the region of the Soret band.

Additions of all reagents are made in such a small volume that dilution artifacts are negligible.

In a number of cases the experimental record may be continued further than seems necessary for the demonstration of the particular phenomenon under study. For example, in Fig. 8 the interest of the experiment centers about the moment of addition of hexetidine. However, the preceding and succeeding portions of the traces give important calibrations, for example, of the oxidation-reduction levels of cytochrome b at States 4, 3, and 5 (see Fig. 1; these are summarized in Table I). Chemical changes may cause a transition from one state to another, denoted here by “State 3 to 4 transition,” for example.

RESULTS

Amytal Inhibition of Oxidation of DPN-linked Substrate—A representative experimental illustrative of the experimental method emphasizes the responses of respiratory activity, cytochrome b, and pyridine nucleotide to the effects of Amytal. This record also serves as an essential control upon the accuracy and stability of the physical apparatus; although similar data are not put forward in connection with the succeeding experiments, Fig. 1 can be considered representative with respect to both technique and accuracy. The experimental procedure involves serial additions of a wide variety of reagents to the same sample of mitochondria, in contrast to the more conventional procedure, in which a number of samples are studied simultaneously, each one observed under slightly different experimental conditions. The approach used here includes essential controls on the sample being tested and does not depend upon the assumption of a duplication of conditions in different samples.

Rat liver mitochondria are suspended in the aerated reaction medium containing phosphate but no substrate (State 1, see Table I). Oxidation of endogenous substrate is scarcely accelerated by successive additions of malate, glutamate, and malonate, the latter being added in order to ensure that the endogenous succinate, or the succinate produced from glutamate oxidation, does not interfere with Amytal inhibition. Upon addition of the DPN-linked substrates, pyridine nucleotide reduction increases abruptly and that of cytochrome b at a slower rate. Addition of malonate causes no change in the level of cytochrome b but does induce an oxidation of reduced pyridine nucleotide due to the inhibition of the succinate-linked reaction (20). The additions up to this point give a State 4 level, characteristic of the oxidation of a DPN-linked substrate. Upon addition of 000 μM ADP, the State 4 to 3 transition occurs; respiration is activated 7-fold and oxidation of cytochrome b and pyridine nucleotide occur. The inhibitory effects of Amytal are studied by a sequence of additions beginning with 160 μM. This concentration decreases the respiration rate more than 3-fold and causes oxidation of cytochrome b and reduction of pyridine nucleotide. Further additions of Amytal cause increasing oxidation of cytochrome b and increasing reduction of pyridine nucleotide; the final value of reduction of pyridine nucleotide somewhat exceeds the State 4 level (this point is discussed below). The respiratory rate is reduced below the rate at which accurate measurements can be obtained (less than 0.05 μM oxygen per second).

On the basis of the absorbancy calibrations and of an independent spectrophotometric calibration of the fluorometer, the concentration changes of cytochrome b and pyridine nucleotide can be calculated and are plotted together with data on the respiratory activity in Fig. 2. Half-maximal inhibition of respiratory activity is obtained with 120 μM Amytal, whereas half maximal effects on cytochrome b and pyridine nucleotide occur at 450 and

<table>
<thead>
<tr>
<th>State</th>
<th>Oxygen</th>
<th>Substrate</th>
<th>Uncoupler</th>
<th>Inhibitor</th>
<th>Phosphatase Potential ATP/(ADP + P_i)</th>
<th>Respiration Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Excess</td>
<td>Endogenous</td>
<td>None</td>
<td>None</td>
<td>Endogenous</td>
<td>Slow</td>
</tr>
<tr>
<td>2</td>
<td>Excess</td>
<td>Approaches zero</td>
<td>None</td>
<td>None</td>
<td>Excess</td>
<td>Very slow</td>
</tr>
<tr>
<td>2*</td>
<td>Excess</td>
<td>Approaches zero</td>
<td>Excess</td>
<td>None</td>
<td>Low</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>Excess</td>
<td>Excess</td>
<td>None</td>
<td>None</td>
<td>Excess</td>
<td>Medium</td>
</tr>
<tr>
<td>3*</td>
<td>Excess</td>
<td>Excess</td>
<td>None</td>
<td>Excess</td>
<td>Low</td>
<td>Fast</td>
</tr>
<tr>
<td>4</td>
<td>Excess</td>
<td>Excess</td>
<td>None</td>
<td>Excess</td>
<td>Very high</td>
<td>Fast</td>
</tr>
<tr>
<td>4*</td>
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<td>None</td>
<td>Amytal</td>
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<td>Slow</td>
</tr>
<tr>
<td>5</td>
<td>None</td>
<td>Excess</td>
<td>None</td>
<td>Excess</td>
<td>Low</td>
<td>Very slow</td>
</tr>
<tr>
<td>5*</td>
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<td>Excess</td>
<td>None</td>
<td>Cyanide</td>
<td>Low</td>
<td>None</td>
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</tbody>
</table>

* Subscripts refer to sub-states induced by general effects (u = uncoupled) or by specific substances (a = Amytal, cn = cyanide).
320 μM Amytal, respectively (Table II). The value for respiratory inhibition is considerably smaller than the 0.6 mM reported by Ernster et al. (3); at their value, it is possible that succinate was present in the experiments with the mitochondria. Thus the malonate supplement to the mitochondria is important for obtaining our high sensitivities to Amytal. Aldridge and Parker, using pyruvate as a substrate, obtained a value of 210 PM (23, 24). In an experiment similar to that of Fig. 2, half-maximal inhibition of β-hydroxybutyrate was obtained with 190 PM Amytal, provided that malonate was present. In the absence of malonate, 350 PM Amytal was required. In titrations of suspensions of intact ascites tumor cells, Amytal concentrations of 0.8 mM were required for half-maximal inhibition (22). Here again, it was very likely that endogenous succinate was present.

In the absence of malonate, it has been observed that low concentrations of Amytal cause no immediate inhibition of respiration, since only pyridine nucleotide oxidation is inhibited and oxidation of endogenous succinate continues. Higher concentrations of Amytal inhibit through exhaustion of succinate or by inhibition of oxidation of succinate itself (see below). Thus, previous experiments, in which the possibility of oxidation of endogenous succinate or of that produced from the citric acid cycle was not controlled, give incorrect values for the inhibition constants for pyridine nucleotide oxidation in mitochondria.

The titration curves for both cytochrome b and pyridine nucleotide appear to deviate considerably from a rectangular hyperbola (see Fig. 2) and to approach a linear relationship. The possibility that this relationship is due to a stoichiometric titration was tested by varying the mitochondrial concentration. The same concentrations of Amytal were required for half-maximal effects for mitochondrial concentrations that differed by a factor of more than 2. It is probable that the linear relationship between concentration and effect is due to a side reaction of Amytal, possibly some small uncoupling activity—a phenomenon that is discussed below in connection with inhibition of oxidation of succinate.

The effect of pH on the Amytal concentration required for half-maximal oxidation of cytochrome b in guinea pig kidney mitochondria was tested. There was no measurable change between pH 6.8 and 7.5, but a 50 to 100% increase in the required Amytal concentration was observed at pH 8.5.

**Effects of Progesterone**—According to the work of Yielding

![Graph](image_url)

**Table II**

<table>
<thead>
<tr>
<th>Source</th>
<th>Data*</th>
<th>Inhibitor</th>
<th>Substrate (with malonate)</th>
<th>Acceptor</th>
<th>Respiration</th>
<th>Pyridine nucleotide</th>
<th>Cytochrome b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>413</td>
<td>Amytal</td>
<td>β-Hydroxybutyrate</td>
<td>ADP + P₁</td>
<td>190</td>
<td>220</td>
<td>400</td>
</tr>
<tr>
<td>Rat liver</td>
<td>413</td>
<td>Amytal</td>
<td>β-Hydroxybutyrate</td>
<td>ADP + P₁</td>
<td>350</td>
<td>220</td>
<td>400</td>
</tr>
<tr>
<td>Rat liver</td>
<td>413</td>
<td>Amytal</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>120</td>
<td>320</td>
<td>450</td>
</tr>
<tr>
<td>Rat liver</td>
<td>413</td>
<td>Amytal</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>330</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Pigeon heart</td>
<td>416</td>
<td>Amytal</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>2000</td>
<td>3000</td>
<td>3500</td>
</tr>
<tr>
<td>Rat liver</td>
<td>413</td>
<td>Thiopental</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>360</td>
<td>Uncouples</td>
<td>280</td>
</tr>
<tr>
<td>Rat liver</td>
<td>414</td>
<td>Methylene glycol</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>2300</td>
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<td>2600</td>
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<tr>
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<td>416</td>
<td>Methylene glycol</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>1500</td>
<td>Uncouples</td>
<td>5000</td>
</tr>
<tr>
<td>Rat liver</td>
<td>415</td>
<td>Amytal</td>
<td>Succinate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>7000</td>
<td>2000</td>
<td>6000</td>
</tr>
<tr>
<td>Rat liver</td>
<td>415</td>
<td>Methylene glycol</td>
<td>Succinate (with malonate)</td>
<td>ADP + P₁</td>
<td>4000</td>
<td>2000</td>
<td>3500</td>
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<tr>
<td>Pigeon heart</td>
<td>474</td>
<td>Progesterone</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>300</td>
<td>500</td>
<td>350</td>
</tr>
<tr>
<td>Rat liver</td>
<td>580</td>
<td>Rotenone</td>
<td>Malate-glutamate (with malonate)</td>
<td>ADP + P₁</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Protocol numbers, as in figure legends.
mitochondria are used instead of rat liver mitochondria. Increasing concentrations of progesterone cause respiratory inhibition, half-maximal effects being obtained at 300 \(\mu M\), an amount 30-fold greater than that required for the nonphosphorylating preparations (19) but comparable to that required for ascites tumor cells (25). The effects upon pyridine nucleotide and cytochrome b are identical to those observed in the presence of Amytal and suggest a similar mechanism of action for the two inhibitors.

**Progesterone**—Very low concentrations of this inhibitor, as of Amytal and progesterone, cause respiratory inhibition (26, 27), cytochrome b oxidation, and pyridine nucleotide reduction (Azzone, Estabrook, and Hommes, in preparation). Table II includes concentrations of rotenone giving half-maximal effects.

**Amytal Inhibition in Presence of Uncoupling Agent**—Although Amytal is known to inhibit electron transport in nonphosphorylating preparations (13), there are important differences between the effects of Amytal upon phosphorylating mitochondria in which the respiration has been activated by ADP and phosphate rather than by an uncoupling agent, DIBz (11). The results of Fig. 4 were obtained in a manner similar to that used for Figs. 1 and 2 and show half maximal respiratory inhibition in the presence of 330 \(\mu M\) Amytal instead of 120 \(\mu M\). Superficially, the response of the respiratory carriers appears to be similar. However, cytochrome b is half-maximally oxidized at a lower concentration of Amytal than that shown in Fig. 2 (300 compared with 450 \(\mu M\)). The amount of pyridine nucleotide reduced during Amytal inhibition in the presence of DIB is only half that reduced in Fig. 2; at the highest concentration of Amytal, the curve shows an upward trend. These facts suggest a diminution rather than an increase of pyridine nucleotide reduction. It is apparent from this figure that there are quantitative differences between Amytal inhibition of respiration activated by ADP and phosphate and that activated by uncoupling agents.

**Thiobarbiturates**—Thiobarbiturates are recognized to combine an ATPase activation with an inhibition of respiration (24). The response of pyridine nucleotide in thiopental inhibition of respiration under the conditions of Figs. 1 and 2 is especially revealing in this respect (Fig. 5). Although the inhibition of respiration follows a course similar to that of Figs. 1 and 2, higher concentrations of thiopental than of Amytal are required, and the inhibition does not reach completion at concentrations approaching 1 mm. Cytochrome b oxidation reaches a plateau at concentrations of thiopental considerably less than those that would give maximal inhibition of respiration. Pyridine nucleotide, instead of showing a reduction, as in the case of Amytal inhibition, shows a slight oxidation above the State 3 level. It is apparent that thiopental combines an Amytal-like inhibition of respiration with an uncoupling effect which exceeds even that obtained in the experiment of Fig. 4, in which the Amytal titration was carried out in the presence of DIB. Thus oxybarbiturates and thiobarbiturates may be readily distinguished by their completely different effects upon the steady state level of reduced pyridine nucleotide.

**Amytal Inhibition of Succinate Oxidation**—Experimental

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*The abbreviations used are: \(\beta\) HB, \(\beta\)-hydroxybutyrate; FP, flavoprotein associated with succinate dehydrogenase; Q cytochrome b, ubiquinone-coenzyme Q-cytochrome b; DIB, n-butyl 3,5-diido-4-hydroxybenzoate. The latter was supplied by Dr. H. A. Lardy.*
A related feature of Amytal inhibition can be demonstrated by the use of hexetidine (8); the experiment is illustrated in detail by Fig. 8. In this case, pigeon heart mitochondria supplemented with glutamate-succinate are in an inhibited state of respiration due to the presence of 1.0 mM Amytal. The addition of 170 µM ADP has no effect upon pyridine nucleotide whatsoever but does cause an oxidation of cytochrome b and an acceleration of respiration from 0.64 to between 0.70 and 0.89 µM O₂ per second. Addition of 40 µl of hexetidine largely removes the inhibitory effects of Amytal; the respiratory rate is stimulated to 1.4 µM O₂ per second as compared with 0.89 in Fig. 7A. It is apparent that Amytal is more than twice as effective in inhibiting respiration stimulated by ADP and phosphate as in inhibiting respiration stimulated by Dicumarol.

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nucleotide is highly oxidized. Activated respiration continues until the dissolved oxygen is exhausted and cytochrome c becomes abruptly reduced. Although the mechanism of action of hexetidine is beyond the scope of this paper, its detergent-like properties are probably responsible for the reversal of inhibition of respiration, a view proposed by Pumphrey and Redfearn (15), who used freezing and thawing, deoxycholate, and CaCl₂ to disrupt the mitochondria. It is significant that we observe here a reactivation of both respiration and DPNH oxidation on addition of hexetidine to the Amytal-inhibited system.

Figs. 4 and 7 have indicated a diminution, caused by uncoupling agents, of respiratory inhibition by Amytal. Amytal inhibition clearly prevents the State 4 to 3 transition due to ADP and phosphate; for example, Fig. 8 shows no oxidation of pyridine nucleotide caused by the addition of ADP and phosphate to Amytal-treated mitochondria in State 4. Whereas ADP and phosphate are completely ineffective in this reaction, uncoupling agents can cause pyridine nucleotide oxidation in spite of Amytal inhibition of electron transfer. Figs. 9A and B illustrate two experiments, one with rat liver mitochondria (9A) and one with pigeon heart mitochondria (9B); both kinds of mitochondria are supplemented with malate and glutamate. In the case of the rat liver mitochondria, a control experiment in which no Amytal is present indicates the degree of pyridine nucleotide oxidation caused by increasing concentrations of DIB. The experiment repeated in the presence of 0.7 mM Amytal shows considerable oxidation of pyridine nucleotide, even though previous respiratory activation under these conditions (compare with Fig. 2) was negligible. In Fig. 9B, 2 mM Amytal has only a small inhibitory effect upon the oxidation of reduced pyridine nucleotide in the presence of the uncoupling agent. Table II indicates that a 50% reduction is caused by 2 mM Amytal. It is apparent from these experiments that the ability of Amytal to cause pyridine nucleotide reduction (illustrated by Figs. 1 and 2) is lost in the presence of sufficiently high concentrations of uncoupling agent (Fig. 5). The inhibition of reduced pyridine nucleotide oxidation caused by progesterone and by rotenone is similarly reversed by uncoupling agents. These results confirm the views expressed above regarding the multiple effects of thiopental.

Response of Flavoprotein to Amytal Inhibition—Up to this point, the responses of cytochrome b and pyridine nucleotide indicate that the site of Amytal action lies between these two components. Whereas Amytal inhibits electron transfer between flavoprotein and cytochrome b in nonphosphorylating preparations (13) (flavoprotein is reduced and cytochrome b is oxidized), a different type of response is observed in the phosphorylating mitochondria (Fig. 10): flavoprotein responds in the same way as cytochrome b, both becoming oxidized as Amytal inhibits the oxidation of a DPN-linked substrate. In Fig. 10A rat liver mitochondria in State 1 are supplemented with 10 mM β-HB with resulting reduction of flavoprotein (State 4). Addition of 2 mM Amytal causes oxidation of flavoprotein (State 4A). Observations of cytochrome b and pyridine nucleotide under these conditions reveal a considerable oxidation of cytochrome b and a small reduction of pyridine nucleotide. Addition of succinate to the Amytal-treated mitochondria causes a large reduction of flavo-
inhibits electron transfer to cytochrome c so that the reduction by Amytal and the speed of action of this inhibitor is indicated in Fig. 11B. Rat liver mitochondria in State 1 are treated with transition. However, at the end of the second cycle, the oxygen Amytal causes oxidation of cytochromes c + cl above the State 4). ADP addition causes the cyclic oxidation and reduction of this component (State 4 to 3 to 4 transition). Addition of 2 mM Amytal causes oxidation of cytochromes c + cl above the State 1 and 3 levels, as was observed in the case of flavoprotein and cytochrome b. Addition of succinate to the Amytal-inhibited system causes reduction of cytochrome c, as in the case of flavoprotein. Two further additions of 170 PM Amytal cause cyclic responses of cytochromes c + cl due to the State 4 to 3 to 4 transition. However, at the end of the second cycle, the oxygen is exhausted and State 5 is obtained, a condition that affords a calibration of the total amount of cytochromes c + cl present. 

Some idea of the completeness of inhibition of DPNH oxidation by Amytal and the speed of action of this inhibitor is indicated in Fig. 11B. Rat liver mitochondria in State 1 are treated with sufficient azide (480 PM) to cause nearly complete reduction of cytochromes c + cl. However, the addition of 2.4 mM Amytal inhibits electron transfer to cytochrome c so that the reduction is reversed, and the oxidation proceeds even beyond the State 1 level. The half-time of the reaction is approximately 3 seconds. Again, electron flow to cytochrome c can be activated by succinate addition, which leads to reduction of cytochrome c almost to the level obtaining before addition of Amytal. The succession of reagents used here is extremely useful for obtaining oxidation and reduction of mitochondrial components without exhaustion of oxygen and can be applied to the study of suspensions of whole cells such as ascites tumor cells. 

**Difference Spectra for Amytal Inhibition**—Up to this point, no information has been put forward to indicate that specific cytochromes are measured at the pairs of wave lengths selected in the various experiments. The difference spectra of Fig. 12 verify the fact that pyridine nucleotide, cytochromes b and c + cl, and flavoprotein are measured at the wave lengths employed in the preceding experiments. The spectrum of Fig. 10A represents the difference of absorption between Amytal-treated and untreated mitochondria in State 3. The large absorption band at 340 m\u00b4 identifies reduced pyridine nucleotide, whereas the decrease at 430 m\u00b4 identifies cytochrome b. It is important to note that 430 and 410 m\u00b4 are appropriate pairs of wave lengths for the observation of cytochrome b without interference from cytochromes c + cl or a3 + a (28). Fig. 12A does not clearly show the responses of flavoprotein and cytochromes c + cl, and for this reason Fig. 12B, which covers the visible region at considerably higher magnification than Fig. 12A, is provided. Absorbancy decreases at 563 and 550 m\u00b4 identify Amytal-induced oxidations of cytochrome b and of cytochromes c + cl. Below 520 m\u00b4 the absorbancy increase identifies flavoprotein oxidation.

The reversal of the Amytal effects caused by succinate addition, as in Figs. 10 and 11, shows reduction of cytochromes c and b and of flavoprotein with no increased absorption at 340 m\u00b4 (pyridine nucleotide is already highly reduced in the presence of Amytal and a DPN-linked substrate). However, the 315 m\u00b4 band, considered in detail elsewhere (29, 30), appears under these conditions.

**Methylene Glycol**—Since experimental results on methylene glycol have been presented only cursorily in the past (16), we introduce the consideration of its action as an inhibitor with a demonstration of the qualitative aspects of the reaction it initiates. The nature of the inhibition depends upon the state of the mitochondria and the nature of the substrate. In the experiment of Fig. 12, rat liver mitochondria are supplemented with \( \beta \)-HB in the presence of phosphate (State 4). Addition of 500 m\u00b4 ADP shows a stimulation of respiration (State 3) to 1.4 m\u00b4 O2 per second, and 3 m\u00b4 methylene glycol almost completely inhibits respiration (0.1 m\u00b4 O2 per second).

* Methylene glycol, formaldehyde hydrate, \( \text{H}_2\text{C(OH)}_2 \).
Inhibition of Electron and Energy Transfer. I

Fig. 12. Spectra representing the difference in absorption between a suspension of rat liver mitochondria treated with β-OHB and one treated with β-HB and Amytal. For Curve A, 3 mg of protein per ml of sucrose-phosphate medium, fluoride-free, were present; for Curve B, the concentration was 7 mg per ml. Temperature, 25°. (504B, 3, 522A).

Fig. 13. Inhibition of respiration by additions of methylene glycol to a suspension of rat liver mitochondria supplemented with β-hydroxybutyrate and ADP (State 3). The sucrose-phosphate medium (fluoride-free) contained 3 mg of protein per ml; 10 μM DPN was reduced in the State 3 to 4 transition. Temperature, 26°. (5500).

Fig. 14. Inhibition of DPNH oxidation by Amytal and methylene glycol in nonphosphorylating heart muscle preparations. Heart muscle mitochondria were suspended in 0.01 M phosphate buffer, pH 7.4, at a concentration of 2.0 mg of protein per ml. After addition of 58 μM DPNH, respiration rates were measured polarographically at 26°, pH 7.4. (469A).

Concentrations of methylene glycol that inhibit the oxidation of a DPN-linked substrate cause little inhibition of succinoxidase activity, as indicated in the graph of Fig. 13, where a 14-fold activation of respiration is observed with the supplement of 50 mM succinate. Higher concentrations of methylene glycol are nevertheless effective in inhibiting succinate oxidation, as indicated by the successive decreases of respiration rate with increasing concentrations of methylene glycol.

Homologues of methylene glycol appear to be inactive, for example, propylene glycol. It is unlikely, however, that the aldehyde function is of significance in the inhibitory action; 5 mM acetaldehyde produces no observable effects.

The fact that the addition of succinate reactivated respiration of methylene glycol-inhibited DPNH oxidation suggests that a nonspecific effect is not involved and that the effect of low concentrations of methylene glycol is similar to those of Amytal. Data presented below show, in addition, that the increased reduction of pyridine nucleotide caused by addition of methylene glycol is reversed by uncoupling agents.

Effect of Methylene Glycol on Nonphosphorylating Preparations—It is of considerable importance to determine whether methylene glycol, like Amytal, inhibits electron transfer in nonphosphorylating preparations. Fig. 14 provides a comparison of the effects of Amytal and methylene glycol on the oxidation of DPNH in a Keilin and Hartree heart muscle preparation (kindly provided by Dr. C. P. Lee). It is apparent that methylene glycol is inhibitory at concentrations approximately 10 times those required for inhibition by Amytal; half-maximal effects are obtained at 0.27 mM Amytal and at 2.0 mM methylene glycol. At higher concentrations of methylene glycol (approximately 10 mM), some inhibition of succinate oxidation can be observed.

Response of Cytochrome b and Pyridine Nucleotide of Phosphorylating Mitochondria to Methylene Glycol Inhibition—In the experiment of Fig. 15, rat liver mitochondria are supplemented with malate and glutamate and, in addition, with malonate, which prevents interference of endogenous succinate. Addition of 600 μM ADP causes the State 4 to 3 transition and is accompa-
ned by oxidation of cytochrome b and of pyridine nucleotide. The steady state respiration rate is 1.0 μM O₂ per second. Successive additions of methylene glycol cause respiratory inhibition, reduction of pyridine nucleotide, and oxidation of cytochrome b.

These data are graphed in Fig. 16. It is significant that pyridine nucleotide is affected at lower concentrations of methylene glycol than are required for cytochrome b. The values for half-maximal effects on pyridine nucleotide, respiratory activity, and cytochrome b are, respectively, 2.0, 2.3, and 2.6 mM methylene glycol.

The responses of the respiration and of cytochrome b in pigeon heart mitochondria are similar to those observed with the rat liver material (Table II). Pyridine nucleotide reduction is much less extensive, however, and at higher concentrations of methylene glycol, reduction of the reduced pyridine nucleotide was observed. This effect was similar to, although less marked than, that observed in the presence of thiopental. Thus it is apparent that higher concentrations of methylene glycol can lead to some uncoupling effects.

Detailed Studies of Response of Respiratory Carriers to Methylene Glycol Inhibition—1. Cytochrome b: Fig. 17 compares the effects of methylene glycol on the oxidation of a DPNH-linked substrate and on succinate. The experiment, carried out with rat liver mitochondria supplemented with β-HIB and succinate, indicates the State 4 to 3 transition caused by the addition of 400 μM ADP. Additions of 2.1 and 4.2 mM methylene glycol cause oxidation of cytochrome b beyond the State 3 level, as in Fig. 15, and are accompanied by a high degree of inhibition of respiration. Addition of succinate at this point causes reactivation of respiration (compare Fig. 13) and a large reduction of cytochrome b. These data suggest that the site of inhibition in both DPNH and succinate oxidation is on the substrate side of cytochrome b. Since pyridine nucleotide is simultaneously reduced (Fig. 15), the site of inhibition is bracketed by these two components.

The effect of methylene glycol apparently differs according to whether the mitochondria are in State 1 or State 4. Figure 17B shows that two additions of 1.1 mM methylene glycol to mitochondria in State 4 (β-HIB) require about 3.5 minutes for maximal effect as compared with approximately 10 seconds in Fig. 17A.

Flavoprotein: Since the previous experiment suggests that the site of action of methylene glycol is bracketed, on the one hand, by the oxidation of cytochrome b and, on the other hand, by the reduction of pyridine nucleotide, the response of flavoprotein is of interest. Fig. 18 illustrates the response of rat liver mitochondria in State 4 (β-HIB) to a supplement of 500 μM ADP, which causes the transition from State 4 to State 3 and leads to an oxidation of flavoprotein. Additions of 6.2 mM methylene glycol cause further oxidations of cytochrome b and flavoprotein beyond the State 3 level, a fact in accordance with observations of flavoprotein in the presence of Amytal.

Effect of Uncouplers on DPNH Reduction in Presence of Methylene Glycol: As in the case of Amytal inhibition, the high degree of DPNH reduction obtained by treatment with methylene glycol can be reversed by additions of uncoupling agents. In Fig. 19 rat liver mitochondria are observed in the presence of malate, glutamate, and malonate. Additions of dibromomethanol in the control experiment (no methylene glycol) cause progressive oxidation of reduced pyridine nucleotide. In the presence of 3.4 mM methylene glycol, a distinct oxidation is still obtainable.

FIG. 15. Effect of methylene glycol on the respiration of rat liver mitochondria and on the steady state levels of cytochrome b and pyridine nucleotide (PN). Experimental procedures were identical with those of Fig 1. Protein, 3.2 mg per ml of medium, was present. (414, 5).

FIG. 16. Correlation of respiratory rate, cytochrome b oxidation, and pyridine nucleotide reduction as a function of the concentration of added methylene glycol, which inhibits the oxidation of DPNH-linked substrates in rat liver mitochondria. (414, 5).

FIG. 17. A, Effects of methylene glycol on the steady state level of cytochrome b in the presence of β-hydroxybutyrate and succinate. In this experiment the changes are observed in State 3. Rat liver mitochondria were suspended in a sucrose-phosphate medium (fluoride-free) at a concentration of 3 mg of protein per ml. (55OC, 2). B, Effect of methylene glycol on the oxidation-reduction level of cytochrome b in State 4. Conditions were identical to those of Fig. 17A. (55C).
Inhibition of Electron and Energy Transfer.

**Discussion**

Spectroscopic observations of intact mitochondria show that inhibition by Amytal, methylene glycol, and progesterone of DPN-linked electron transfer causes oxidation of cytochrome $b$ and reduction of DPN. In the case of Amytal and methylene glycol, a site of inhibition between flavoprotein and pyridine nucleotide has been identified. However, studies of nonphosphorylating preparations show a reduction of flavoprotein and an oxidation of ubiquinone and cytochrome $b$ in the Amytal-inhibited system. The survey of this series of papers suggests that the class of inhibitors we are concerned with are not highly specific in their effects on phosphorylating mitochondria and that three principal types of inhibition may be inextricably intermingled: (a) inhibition of nonphosphorylating electron transfer; (b) inhibition of energy transfer; and (c) uncoupling effects. The latter not only reverse some types of energy transfer inhibition but may themselves be inhibitory.

It seems remarkable that certain inhibitors of the same class (Amytal, methylene glycol, and thiopental) might both inhibit energy transfer and at higher concentrations act as uncoupling agents. It is possible that enzymatic sites involved in the three functions referred to above are sufficiently similar to permit all to combine with inhibitors of this class, or that a single complex enzymatic site is involved in all three functions. A study likely to be fruitful would be an examination in detail of the configurations of the inhibitors that are optimally suited for interaction with these three apparently diverse and distinct functions of the respiratory chain of phosphorylating mitochondria.

Considering the first property of the inhibitors to be inhibition of electron transfer, inhibition at a site between the DPNH dehydrogenase flavoprotein and the ubiquinone-cytochrome $b$ region would account for the inhibitory effects observed on nonphosphorylating electron transfer.

Superimposed upon inhibition of electron transfer may be additional effects due to inhibition of energy transfer pathways; these are here defined as inhibitory effects that are reversible by addition of uncoupling agents. Two types of effects of uncoupling agents are observed here; the more obvious is a reactivation of respiration. Only partial reactivation of respiration is obtained with Amytal inhibition, although nearly complete reactivation can be obtained with guanidine-inhibited electron transfer. In all cases (Amytal, progesterone, and methylene glycol), however, the increased reduction of DPN observed in the presence of these substances at inhibitory concentrations is reversed by the addition of uncoupling agents. This observation suggests that inhibition at one of the sites is reversed by the uncoupling agent whereas inhibition at another site is unaffected. The site sensitive to uncoupling agents is identified with inhibition of energy transfer, and the site insensitive to uncoupling agents is identified with inhibition of electron transfer.

These views are clarified by reference to Fig. 20, which illustrates sites of action of inhibitors that are insensitive to uncoupling agents (electron transfer inhibitors, cyanide, carbon monoxide, antimycin $A$, malonate, and others). In addition, the site called "oxybarbiturates" denotes the uncoupler-insensitive property of this group of substances, together with that of methylene glycol and progesterone. Inhibition of energy...
transfer at the DPNH-flavin site is indicated by the series of dots. Such inhibitory action prevents the activation of DPNH oxidation by ADP and phosphate and results in the high degree of pyridine nucleotide reduction characteristically observed with this type of inhibitor. Such inhibition also slows the transfer of energy to the energy-requiring DPN reduction. The energy source may be ATP itself or internally generated, high energy intermediates (energy transferase mechanisms (62)). Additions of uncoupling agents reverse the inhibition of energy transfer and hydrolyze high energy intermediates. Thus energy-linked pyridine nucleotides can no longer be maintained in the reduced state.

It is desirable to explain the response of flavoprotein to inhibition of electron transfer (a reduction) and to inhibition of electron and energy transfer (an oxidation). Here, reference to a more detailed diagram of the respiratory chain is desirable (Fig. 21). Inhibition of electron transfer between Fp, (Fig. 21) and Q cytochrome b would cause reduction of Fp, and oxidation of Q cytochrome b as indeed is observed in nonphosphorylating preparations. Simultaneously, the inhibition of energy transfer reactions to ADP and phosphate occurs. This inhibition effectively changes the energy state of the system, with respect to energy-linked pyridine nucleotide reduction, from a State 3 to a State 4 level; energy transfer inhibition increases the concentration of high energy intermediates, X ~ I, and causes Fp, oxidation and DPN reduction. In many ways this change resembles the response obtained when ATP is added to terminally inhibited mitochondria in that an oxidation of flavoprotein and a reduction of pyridine nucleotide is observed (33). Thus, the mechanism proposed involves a reduction of Fp, due to an electron transfer inhibition and a simultaneous oxidation of a flavoprotein (Fp,) due to energy transfer inhibition. The effects upon ubiquinone and cytochrome b are similar, and both these components become more oxidized.

Inhibition of succinate oxidation at high concentrations of Amytal depends upon the integrity of the mitochondrial structures as indicated by the effects of hexetidine, but has not yet been shown to be reversed by uncoupling agents (15). However, the change of electron transfer pathway found to be caused by disruption of the mitochondria (34, 35) complicates the interpretation of these results. Since, however, we have oligomyein and atractylate (8, 36) as models of energy transfer inhibitors that block succinate oxidation in a manner reversible by uncoupling agents, there is no reason why energy transfer inhibition should not be involved to some extent in the inhibition of succinate oxidation in the phosphorylating mitochondria.

**Reaction Mechanisms**—The experimental data on which formulations of the reaction mechanisms may be based are essentially kinetic in nature, since steady state levels in the respiratory chain are the consequence of at least two opposing reactions. Such kinetics impose upon reaction mechanisms one or more rate-limiting steps. In order to translate such a kinetic result to a chemical mechanism, it is necessary to postulate intermediate compounds that have chemical reality in the sense that they represent at least transition states of the components and may, in addition, be sufficiently stable ultimately to be isolated and purified.

Such reaction mechanisms involving hypothetical intermediates are useful in criticism of the hypothesis on which they are based and in the design of further experiments. In addition, the hypothesis that intermediate compounds play a part in reaction mechanisms suggests to biochemists the conditions under which the compounds may reach a maximal concentration and may have a relatively high stability. Such indeed is the case with the postulated inhibited form of reduced diphenolphosphate nucleotide (35).

The theory providing for an energy transfer component that is site-specific (I) and one that is not site-specific (X) appears to provide the most convenient framework for a consideration of the experimental data (35, 37). It is represented schematically in Fig. 22.

An inhibition of the energy transfer reaction gives changes of the oxidation-reduction states of the carriers identical with those observed when ADP or phosphate is exhausted or when ATP is added in reversed electron transfer (33). Two choices of an inhibitory site in the energy transfer reactions are to be considered.

\[
\text{Carrier } I + X \rightarrow X \sim I + \text{Carrier} \quad (1)
\]

\[
X \sim I + P, \rightarrow X \sim P, + I \quad (2)
\]

\[
X \sim P + ADP \rightarrow ATP + X \quad (3)
\]

First, a binding of X by Amytal or related inhibitors would prevent respiratory activation. Such a binding of X would cause inhibition of the oxidation of both DPNH and succinate at the same concentrations of the inhibitors, since X is by definition common to all sites. Phosphate would also be expected to be involved in the inhibition reaction, although at the present time there is no evidence that it is. Since DPNH and succinate oxidation is inhibited at quite different concentrations of these inhibitors, we consider much more likely a hypothesis in which the inhibitor binds the energy transfer compound characteristic of the DPNH-flavin site (I,) (38). Since it is found experimentally that inhibition by this group of substances is characterized by an accumulation of high energy intermediates, as evidenced by increased pyridine nucleotide reduction and sensitivity of the inhibitor to uncoupling agents, a configuration of I, that combines most readily with the inhibitor is postulated to be its high energy form (X ~ I,). Thus, the general equation for the action of this class of inhibitors is

\[
X + \sim I_0 + \text{Inhibitor} \rightarrow X \sim I_0 \cdot \text{Inhibitor} \quad (4)
\]

This hypothesis has all the desirable characteristics of the others and in addition allows respiration and phosphorylation to proceed uninhibited through the succinate oxidase pathway, since, according to it, the inhibitor interacts with the substance.
bound to pyridine nucleotide ($I_D$) and not with one bound to the other carriers involved in succinate oxidation ($I_B$ or $I_C$). One of the possible explanations of the inhibition of succinate oxidation at higher concentrations of Amytal and methylene glycol is a binding to the intermediates $I_C$ or $I_D$, but at the present time the experimental data in favor of this hypothesis are inconclusive. However, the reactivation of the Amytal-inhibited oxidation of succinate by addition of hexadiazide is suggestive.

**Effect of Uncoupling Agents**—The experiments of Fig. 9 show that the high level of reduced pyridine nucleotide observed in the presence of these inhibitors can no longer be maintained in the presence of uncoupling agents. This result immediately suggests that a high level of pyridine nucleotide reduction requires a high energy intermediate that is dissipated in the presence of the uncoupling agent. The fact that pyridine nucleotide reduction obtained with supplements of DPN-linked substrates is sensitive to uncoupling agents, as is that obtained with succinate, suggests that there may be an energy requirement for reduction of pyridine nucleotide not only with succinate, but also with malate and glutamate, as the electron donor. Although a complete discussion of this property of phosphorylating mitochondria is beyond the scope of this paper, a consideration of such a possibility is essential to an understanding of these experimental observations.

In this context, the effect of the uncoupling agent upon Equation 5 satisfies the experimental observations by a dissipation of the high energy configuration.

$$X \sim 1 \cdot \text{Inhibitor} \rightarrow X + I + \text{Inhibitor} \quad (5)$$

Thus, the addition of uncoupling agents to mitochondria inhibited by Amytal, methylene glycol, or progesterone causes a decrease of the energy content and an oxidation of the reduced pyridine nucleotide.

It is observed that Amytal is less effective in inhibiting respiration activated by uncoupling agents than that activated by ADP and phosphate. This disparity can similarly be explained in terms of Equation 5, which would, in the absence of other effects, serve to reverse completely the inhibitory effects. The simultaneous inhibition of electron transfer (observed in non-phosphorylating preparations) explains the failure of uncoupling agents to reactivate completely respiration inhibited by high concentrations of Amytal.

Last, it is necessary to consider whether the reoxidation of pyridine nucleotide caused by addition of uncoupling agents to inhibited mitochondria is due to Equation 5 exclusively or to an increase of electron flow to oxygen as well. This question is readily answered by the fact that an appreciable degree of pyridine nucleotide reduction can be obtained with only partial blocking of respiration (see Fig. 1, addition of 180 $\mu$M Amytal). Nevertheless, under the conditions of Fig. 9A the respiratory rate (less than 3% of the rate of State 3) was not measurable even though pyridine nucleotide was extensively oxidized.

**Relation to ATPase Activity**—Since it is postulated that uncoupling agents reverse the effect of this class of inhibitors upon energy transfer reactions, the inhibition of dinitrophenol-stimulated ATPase activity is not to be expected. Actually very little inhibition of ATPase activity is caused by concentrations of Amytal less than 1 mM; only 23% inhibition is observed with 2 mM Amytal (39). If only one of the three sites of energy transfer were inhibited, then inhibition of ATPase activity would scarcely be expected.

**SUMMARY**

1. The inhibitory effects of Amytal, progesterone, methylene glycol, and rotenone upon oxidation of diphosphopyridine nucleotide (DPN)-linked substrates in phosphorylating mitochondria are characterized by an oxidation of cytochrome $b$ and a reduction of pyridine nucleotide.
2. Methylene glycol inhibits electron transfer in nonphosphorylating DPNH oxidase preparations.
3. High concentrations of Amytal and methylene glycol are also found to inhibit oxidation of succinate.
4. Inhibition by this class of agents is characterized by a high degree of pyridine nucleotide reduction. In the case of Amytal, progesterone, rotenone, and methylene glycol, the high degree of pyridine nucleotide reduction is sensitive to uncoupling agents.
5. Thiopental is found to represent the properties of Amytal plus those of an uncoupling agent, in accordance with previous observations of its property as an adenosine triphosphatase activator (7).
6. This class of inhibitors combines an inhibition of electron transfer between flavin and cytochrome $b$ with an inhibition of energy transfer at the DPNH-flavin site. The inhibition of energy transfer causes increased pyridine nucleotide reduction and leads to increased flavoprotein oxidation in phosphorylating preparations.
7. The property of this class of inhibitors to affect both electron and energy transfer, and to uncouple as well, suggests that similar chemical configurations like that of the inhibitors may be involved in these superficially independent chemical reactivities.

**REFERENCES**

Inhibition of Electron and Energy Transfer in Mitochondria: I. EFFECTS OF AMYTAL, THIOPENTAL, ROTENONE, PROGESTERONE, AND METHYLENE GLYCOL
Britton Chance and Gunnar Hollunger


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