CHEMICAL PACEMAKERS

III. ACTIVATION ENERGIES OF SOME RATE-LIMITING
COMPONENTS OF RESPIRATORY SYSTEMS*

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INTRODUCTION

In a previous paper Hadidian and Hoagland (1939–40) studied activation energies, μ values as calculated from the Arrhenius equation, for the two major components of the crude beef heart extract obtained by the method of Stotz and Hastings (1937). In this system succinic acid loses hydrogen in the presence of the extract’s succino-dehydrogenase and becomes fumaric acid. The hydrogen then combines with oxygen in the presence of the extract’s cytochrome-cytochrome oxidase. The consumption of oxygen in Warburg vessels can thus serve as a measure of the total reaction’s velocity.

Hadidian and Hoagland found that (1) the respiratory enzyme system extracted from the beef heart, and presumably containing two major enzyme components, yielded a μ value of 11,200 ± 200 calories; (2) this μ value shifts abruptly to 16,000 ± 200 calories when the enzyme system is poisoned with a critical amount of NaCN, thus suggesting that the former value is characteristic of the dehydrogenase activity and the latter of oxidase activity,—since cyanide, by reducing the availability of the oxidase, would, at a critical concentration, make this the limiting slow step.

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The Arrhenius equation, \( V = Ze^{-\mu RT} \), describes the speed of a variety of chemical reactions as a function of temperature, where \( V \) is chemical velocity, \( e \) is the base of natural logarithms, \( T \) is the absolute temperature, \( Z \) is a constant, \( R \) is the gas constant, equal to 1.99 or 2 cal./mol, and \( \mu \) is the critical thermal increment or energy of activation; i.e., the amount of energy per mol above the average energy of the system required to render the particular molecules reactive. Taking logarithms on both sides of the equation, we obtain, \( \log V = C - \mu/2.3 RT \), and, if the data fit the equation, a plot of \( \log V \) against \( 1/T \) should give a straight line, with intercept \( C \) and negative slope \( \mu/4.6 \). From the slope of the line the \( \mu \) value in calories per mol may be calculated.
or chemical pacemaker in the chain; (3) this view is further confirmed by the fact that the μ value shifts back to 11,200 calories if a critical amount of selenite, shown by Stotz and Hastings to be a specific poison for the dehydrogenase, is added to the enzyme system already poisoned with sufficient cyanide to yield a μ of 16,000 calories. It was thought desirable to study further the various components involved in this reaction system to determine whether these same activation energies, or μ values, could be obtained from the isolated components. If 11,200 and 16,000 calories are respectively characteristic \textit{per se} of succino-dehydrogenase and cytochrome-cytochrome oxidase, then we would expect to obtain these same values in the study of the two components independently. If, on the other hand, these values depend on the reactions (i.e., all the reactants involved in a given step in the reaction), then different values may be obtained in the study of the isolated component enzymes reacting under varying conditions. This latter view is to be expected since the energy of activation refers to energy relationships between particular linkages of enzyme and substrate and these may vary not only from one substrate to another, but different parts of the enzyme molecule may also be active under varying physical and chemical conditions. In our previous paper (Hadidian and Hoagland, 1939-40) we were careful, for example, to point out that the μ value of 16,000 calories obtained under the conditions of our experiments was not necessarily always to be found associated with cytochrome-cytochrome oxidase. The fact that the respective values of 11,200 and 16,000 calories obtained from the enzyme extract agree with values obtained from experiments \textit{in vivo} was regarded as especially significant. The μ value may be the same for a given enzyme in the presence of a variety of substrates, as Gould and Sizer (1938) have shown; it may also be independent of pH and other variables over a wide range (Sizer, 1937), thus indicating that the same enzyme processes are involved in the splitting of essentially identical substrate linkages from one substrate to another. The temperature method of analysis may thus serve to help unravel the problem of enzyme specificity, both with regard to substrate specificity and to specificity of its own active centers.

To extend the study the following experiments were undertaken: (1) temperature studies of the oxidation of \textit{p}-phenylenediamene catalyzed by the beef heart extract, a reaction presumably involving only the oxidase and not the dehydrogenase component of the system; (2) temperature studies of the succinate oxidation by pyrophosphate-poisoned enzyme; and (3) temperature studies of the succinate oxidation when a dye was substituted for the cytochrome-cytochrome oxidase component after this component had been inactivated by cyanide.
To amplify the reasons for undertaking these three plans of investigation it may be pointed out that the oxidation of \( p \)-phenylenediamine involves the oxidase component but not the dehydrogenase component, thus affording an opportunity for the independent study of the cytochrome-cytochrome oxidase activity. Pyrophosphate, according to Stotz and Hastings (1937), does not inhibit the dehydrogenase activity, but \( 6 \times 10^{-8} \) M of it does inhibit to about 38 per cent the oxidase activity. Thus we might find that the pyrophosphate-partially-poisoned enzyme would yield the same result as the cyanide-partially-poisoned enzyme \((i.e., 16,000 \text{ calories})\), since both might presumably make the oxidase step the slow link or pacemaker. Addition of sufficient cyanide to the enzyme system stops the oxidation of succinate completely by poisoning the oxidase. If to this system a reversible oxidation-reduction dye with the proper potential is added as substitute for the oxidase, the oxidation of succinate is restored (Stotz and Hastings, 1937). By suitable choice of dye and of its concentration this reaction may be made to serve as a measure of the activity of the succino-dehydrogenase component.

**Procedure**

Methods employed in the preparation of the enzyme, the measurement of oxygen consumption, the measurement of reaction velocities, and the calculation of \( \mu \) values are described in our previous paper (Hadidian and Hoagland, 1939-40). In all the \( p \)-phenylenediamine experiments fresh solutions of the \( p \)-phenylenediamine were prepared for each reaction. It was noted that addition of high concentrations of pyrophosphate to the reaction mixture caused a considerable rise in the pH of the resulting mixture. In all of these experiments the reaction mixture was buffered to pH 7.4 by the addition of KH\(_2\)PO\(_4\). When high concentrations of cyanide were used, the pH was adjusted in a similar manner.

Stotz and Hastings (1937) found cresyl blue to be the most efficient dye in replacing the oxidase component. We found experimentally that a mixture containing 0.5 ml. enzyme, \( 1.0 \times 10^{-8} \) M cyanide, and \( 5.0 \times 10^{-6} \) M cresyl blue in an atmosphere of pure oxygen gave oxygen consumption most nearly approximating that of the unpoisoned, freshly extracted, system.

Our reaction velocities were measured along the approximately linear portions of the reaction curves immediately following the "initial lag." All experiments were repeated at least once and the results found to check within the limits of experimental error.

**RESULTS**

**Oxidation of \( p \)-Phenylenediamine**

According to Stotz *et al.* (1938) the oxidation of \( p \)-phenylenediamine involves not only cytochrome \( c \)-cytochrome oxidase but also the autooxidizable relatively non-cyanide-sensitive cytochrome \( b \). In the preparation they
used, about 20 per cent of the oxidation was apparently due to this latter factor functioning concurrently with cytochrome c. If such were the case with the preparations used in our experiments, then the Arrhenius equation plot of temperature and velocity of this reaction would be expected to yield not a straight line but a curve concave upwards (Crozier, 1924–25).

The fact that our temperature curves are rectilinear, as will be seen, suggests that if both cytochromes are appreciably involved they act sequentially and not concurrently unless the $\mu$ values are identical or nearly so. Our linear Arrhenius plots thus indicate either that (a) the two cytochrome steps are sequential or (b) they act concurrently with activation energies which are of the same order of magnitude. We have, as yet, no satisfactory evidence to enable us to resolve these alternatives.

Fig. 1 shows the oxygen consumption curves at different temperatures for the oxidation of $p$-phenylenediamine. The same enzyme preparation was used on 2 consecutive days. Change in the activity of the enzyme was slight, so that the results of 2 days’ experiments could be plotted together. Fig. 2 shows the Arrhenius equation plot obtained from the data of Fig. 1. It is a straight line with a $\mu$ value of 9,500. If the concentration of $p$-phenylenediamine is reduced to 1/10 of the concentration used in these experiments, the velocity of the reaction is reduced by more than 60 per cent but the $\mu$ value remains the same. It is clearly not 16,000 calories which we had previously obtained when the oxidase component was the slow step in a sequence involving succinate and succino-dehydrogenase.

**Enzyme Poisoned with Pyrophosphate**

According to Stotz and Hastings (1937) addition of $6.0 \times 10^{-5}$ M of pyrophosphate does not inhibit the dehydrogenase component, but does inhibit, by 38 per cent, the oxidase component as measured by $p$-phenylenediamine oxidation. It was found that addition of this concentration of pyrophosphate directly to the reaction mixture containing 1.5 ml. of M/15 phosphate buffer (pH 7.4) causes a considerable rise in the pH of the resulting mixture. If the mixture is buffered to pH 7.4 by the addition of $\text{KH}_2\text{PO}_4$, there is no inhibition of the $p$-phenylenediamine oxidation by concentrations of pyrophosphate as high as $6.0 \times 10^{-4}$ M, yet lower concentrations than this at pH 7.4 may cause 90 per cent inhibition in the oxidation of succinate by the enzyme extracts (Fig. 3). The dehydrogenase has been shown not to be poisoned by pyrophosphate (Stotz and Hastings, 1937) and the lack of inhibition of the $p$-phenylenediamine reaction indicates that pyrophosphate does not poison the oxidase component. We
thus have evidence for a step other than the two which involve succinodihydrogenase and cytochrome c-cytochrome oxidase in the oxidation of succinate which is acted upon by pyrophosphate. This step apparently is

![Graph](image)

**Fig. 1.** Oxygen consumption curves for oxidation of \( \rho \)-phenylenediamine at different temperatures. \( 1 \times 10^{-4} M \ \rho \)-phenylenediamine and 0.5 ml. enzyme. \( O \) = determinations made the day of the preparation of enzyme. \( \triangle \) = determinations made the following day.

The temperature in °C. is given with each curve.

![Graph](image)

**Fig. 2.** Arrhenius equation plot of data given in Fig. 1. \( \mu = 9,500 \)

one of the sequence of steps involved in the oxidation of succinate as shown by the high degree of inhibition produced by relatively low concentrations of pyrophosphate.

Temperature studies of pyrophosphate-poisoned reactions yield further support to this view. Fig. 4 shows the Arrhenius equation plot of such
Fig. 3. Effect of varying concentrations of pyrophosphate on the succinate and $p$-phenylenediamine reactions. $p$-phenylenediamine reaction: Temperature 37°C., pH 7.4, $5.0 \times 10^{-4}$ M $p$-phenylenediamine, 0.5 ml. enzyme. Succinate reaction: Temperature 37°C., pH 7.4, $6.0 \times 10^{-4}$ M succinate, 0.5 ml. enzyme.

Identical symbols indicate experiments done simultaneously with the same enzyme preparation.

Per cent inhibition = \[
\frac{\text{Velocity of normal reaction} - \text{velocity of poisoned reaction}}{\text{Velocity of normal reaction}}
\]

Fig. 4. Arrhenius equation plot of an experiment with pyrophosphate-poisoned enzyme. $6.0 \times 10^{-4}$ M succinate, $1 \times 10^{-6}$ M pyrophosphate. 0.5 ml. enzyme. pH 7.4. $\mu = 17,500$. 
data. A $\mu$ of 17,500 calories is obtained which differs from the two other values (11,200 and 16,000) obtained from this system.\(^2\)

**Oxidation of Succinate by Enzyme in Which a Dye Is Substituted for the Oxidase**

It was thought possible that by poisoning the oxidase component completely and replacing it by a sufficient amount of a suitable dye the normal activity of the enzyme system might be restored. Such a system would afford an opportunity for the study of the succino-dehydrogenase without the cytochrome-cytochrome oxidase component.

It was found experimentally that the addition of $1.0 \times 10^{-4}$ M cyanide to 0.5 ml. enzyme would stop the oxidation of succinate almost completely (for such a system the $O_2$ consumption is 2–3 c. mm. for the first 15 minutes). Stotz and Hastings (1937) found cresyl blue to be the most efficient dye in replacing the oxidase component. $5.0 \times 10^{-6}$ M of this dye added to the completely poisoned system restores the oxygen consumption to normal in the presence of an atmosphere of oxygen (Fig. 5). Addition of this concentration of cresyl blue to the normal reaction mixture causes no inhibition. Varying the concentration of the succinate within large limits does not change the initial velocity of this reaction (Table I). If, however, air is used instead of pure oxygen, the velocity is reduced about 20 per cent.

Fig. 6 shows a series of oxygen consumption curves for this reaction at various temperatures. An Arrhenius equation plot of these data yields a $\mu$ value of 18,500 (Fig. 7, curve I). Lowering the concentration of the enzyme from 0.5 to 0.05 ml., while slowing the reaction, does not change the $\mu$ value (Fig. 7, curve II), thus indicating that the dehydrogenase-catalyzed step is pacemaker and that its critical increment of 18,500 calories is different in this reaction than that of 11,200 obtained when the chain is that of the "normal" extracted system (Hadidian and Hoagland, 1939-40). Lowering the concentration of the cresyl blue from $5.0 \times 10^{-4}$ to $5.0 \times 10^{-7}$ M changes the $\mu$ value to 22,000 calories (Fig. 8, curve I). Further lowering of the cresyl blue concentration (by one-half) causes no further significant change in the $\mu$ value (Fig. 8, curve II). We thus see that the

\(^2\) By itself this difference in $\mu$ would not constitute evidence for another step since it might result from modification of the dehydrogenase by the pyrophosphate. However, taken in conjunction with Stotz and Hastings' evidence that pyrophosphate does not poison the dehydrogenase, and our data shown in Fig. 3, the postulation of an additional step seems necessary.
activation energy of this particular dye-catalyzed step is 22,000 calories and that it is made pacemaker by lowering the dye concentration.

![Graph showing oxygen consumption curves](image)

**Fig. 5.** Oxygen consumption curves for the normal and the dye-substituted reactions. 
○ = normal: 6.0 × 10^{-6} M succinate, 0.5 ml. enzyme. △ = dye-substituted: 5.0 × 10^{-6} M cresyl blue, 1.0 × 10^{-5} M cyanide, 6.0 × 10^{-4} M succinate, 0.5 ml. enzyme.

**TABLE I**

<table>
<thead>
<tr>
<th>Temperature 37°C</th>
<th>pH 7.4</th>
<th>0.5 ml. enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial velocity taken at 2-5 min. after beginning of reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration of succinate $\times 10^{-6}$/3 cc.</td>
<td>Initial velocity c. mm. O₂/min.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>19.6</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>20.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17.6</td>
<td></td>
</tr>
</tbody>
</table>

**Doubly Washed Enzyme**

Stotz and Hastings (1937) have reported that by doubling the number of washings a preparation is obtained which, in spite of high oxidase and dehydrogenase activity, showed a lowering of the rate of oxidation of succinate. Such a preparation we found showed a 40 per cent decrease in
Fig. 6. Oxygen consumption curves for an experiment with dye-substituted enzyme at different temperatures. $5 \times 10^{-4} \text{M}$ cresyl blue, $1.0 \times 10^{-5} \text{M}$ cyanide, $6.0 \times 10^{-5} \text{M}$ succinate, 0.5 ml. enzyme. The temperature in °C. is given with each curve.

Fig. 7. I. Arrhenius equation plot of data given in Fig. 6. $\mu = 18,500$. II. Arrhenius equation plot of an experiment using one-tenth as much enzyme. $6.0 \times 10^{-5} \text{M}$ succinate, $0.1 \times 10^{-5} \text{M}$ cyanide, 0.05 ml. enzyme, $5.0 \times 10^{-5} \text{M}$ cresyl blue. $\mu = 18,600$. 
the rate of succinate oxidation with the enzyme preparation as obtained after 16 washings (instead of the normal 8), 41 per cent inhibition in the rate of the reaction with $5.0 \times 10^{-6}$ M cresyl blue substituted for the oxidase component (measure of dehydrogenase activity), and 7 per cent decrease in the rate of oxidation of $p$-phenylenediamine (measure of cytochrome c-cytochrome oxidase activity). Decrease in the activity of the enzyme is shown to parallel the decrease in the activity of the dehydrogenase component. The use of this extract might thus yield a $\mu$ value characteristic of succino-dehydrogenase, since more of the dehydrogenase activity is removed by the excessive washing than of the oxidase activity and this should make it the slow step or chemical pacemaker.

The two $\mu$ values we have found associated with succino-dehydrogenase are 11,200 calories (Hadidian and Hoagland, 1939-40) when it acts in a sequence with cytochrome c-cytochrome oxidase, and 18,500 calories when the dehydrogenase reacts in a sequence in which cresyl blue has been substituted for the cytochrome oxidase after the latter has been inactivated by cyanide. We should thus expect a $\mu$ of 11,200 to occur with the doubly washed extract and one of 18,500 calories when the doubly washed enzyme is used with cresyl blue substituted for the oxidase.

**Fig. 8.** Arrhenius equation plots of experiments using low concentrations of cresyl blue. I. $6.0 \times 10^{-5}$ M succinate, $1.0 \times 10^{-5}$ M cyanide, 0.5 ml. enzyme, $5.0 \times 10^{-7}$ M cresyl blue. $\mu = 22,000$. II. $6.0 \times 10^{-5}$ M succinate, $1.0 \times 10^{-5}$ M cyanide, 0.5 ml. enzyme, $2.5 \times 10^{-7}$ M cresyl blue. $\mu = 22,300$.  

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Experiments involving the doubly washed enzyme without cyanide and without cresyl blue yield a $\mu$ of 11,300 calories, as was expected (Fig. 9, curve II). However, experiments in which the doubly washed enzyme is used with cresyl blue after complete cyanide inhibition of its oxidase also yield 11,300 calories (Fig. 9, curve I) and not 18,500. In the first series of experiments the oxygen consumption at a given temperature is approximately linear with time for a considerable period (over 60 minutes at 25°C.). In the second series with cyanide and cresyl blue the curves fall off after about 10 minutes and the rates were calculated from only the first three points at each of the temperatures where the curves of oxygen consumption and time are linear. The value of 11,300 calories in this second case (Fig. 9, curve I) may be a coincidence. Certainly the data are by no means as reliable as those involved in our other $\mu$ plots where the reactions are of zero order over many observations.

Why removal of dehydrogenase activity by washing should yield a different $\mu$ from that obtained by reducing the concentration of the enzyme, when in both cases the cytochrome oxidase is completely blocked by cyanide and cresyl blue is substituted in its place, we do not know. If we assume that the data of curve I are reliable, in some unknown way the double washing produces a system in which the $\mu$ value for the dehydrogenase reacting with cresyl blue is the same as that encountered for the system reacting with active cytochrome-cytochrome oxidase.
DISCUSSION

In a previous paper (Hadidian and Hoagland, 1939-40) it was concluded that 11,200 calories was the energy of activation associated with succino-dehydrogenase activity and 16,000 calories, the energy of activation associated with the cytochrome-cytochrome oxidase activity. In the present study of the two components under different conditions neither of these two values was obtained (except in the case of the doubly washed enzyme). Therefore it is quite evident that these values cannot be associated with these enzymes under all circumstances, but that they characterize the particular step involving them in the reaction as a whole.

The fact that varying the concentration of p-phenylenediamine by a factor of 10 does not change the energy of activation indicates that the reduction of cytochrome by p-phenylenediamine cannot be the limiting factor in the reaction. This is in agreement with the findings of Stotz et al. (1938) that p-phenylenediamine reduces cytochrome c rapidly.

In the dye-substituted reactions with high concentration of cresyl blue there appears a situation in which the dehydrogenase concentration is the limiting factor. The evidence for this is furnished by the following: (1) the μ value (18,500) does not change with decreasing concentrations of

![Graph](image-url)
the enzyme; (2) the velocity of the reaction is a linear function of enzyme concentrations up to 0.5 ml. of enzyme (Fig. 10). With low concentration of the cresyl blue the velocity of the reaction is a linear function of the concentration of the dye. The $\mu$ value in this case changes to 22,000 calories.

Since pyrophosphate does not inhibit the dye-substituted reaction (Stotz and Hastings, 1937) nor the oxidation of $p$-phenylenediamine (Fig. 4), it must act on something besides succino-dehydrogenase or cytochrome $c$-cytochrome oxidase. Exactly where it acts to furnish a $\mu$ of 17,500 we do not know. It may possibly be on cytochrome $b$ or $a$ or on some other possible carrier in the sequential chain of reactions.

**SUMMARY**

1. In a previous paper it was found that 11,200 calories is obtained for the energy of activation in the oxidation of succinate to fumarate in the presence of crude beef heart extract when succino-dehydrogenase was made the limiting factor. 16,000 calories was obtained with this preparation when cytochrome-cytochrome oxidase was made the limiting factor. In the present paper activation energies of the components of this enzyme system are further studied.

2. Oxidation of $p$-phenylenediamine catalyzed by the extract and known not to involve the dehydrogenase component yields Arrhenius equation plots indicating a pacemaker reaction with a $\mu$ of 9,500 calories.

3. An activation energy of 17,500 calories is obtained for the oxidation of succinate to fumarate in the presence of the beef heart extract partially poisoned by pyrophosphate. Evidence is presented that this value corresponds to a link in the respiratory chain other than that of succino-dehydrogenase or cytochrome $c$-cytochrome oxidase.

4. Addition of a suitable amount of cresyl blue to a beef heart extract reaction mixture, completely inhibited by cyanide, restores the oxidation of succinate to normal in the presence of pure oxygen. In this system, in which the dye is substituted for the oxidase, when the enzyme extract (dehydrogenase) is made the limiting factor, a $\mu$ of 18,500 calories is obtained; when cresyl blue is made the limiting factor, the $\mu$ value is 22,000 calories.

5. Results of these experiments indicate that energies of activation are associated not with the enzyme as such, but with the particular reaction steps involving them as catalysts.
CITATIONS