chrome *b*, and two explanations for his result are considered.

5. The cross-over point for the effect of addition of adenosine diphosphate upon the steady-state oxidation-reduction level of the cytochromes lies between *b* and *c*. This result is consistent with observations on mitochondrial preparations and indicates the similarity of the electron-transfer and phosphorylation mechanisms in heart sarcosomes and in liver mitochondria.

6. The observations suggest that the response of the oxidation-reduction levels of the components of the respiratory chain to increases and decreases of concentration of adenosine diphosphate is a basic characteristic of the electron-transfer and phosphorylation mechanism and is to be found in sarcosomes and mitochondria of many cell types.

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Light-Scattering and Absorption Effects Caused by Addition of Adenosine Diphosphate to Rat-Heart-Muscle Sarcosomes

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Holton (1955) has applied the Uvispek spectrophotometer to the measurement of light-absorption changes in suspensions of rat-heart-muscle sarcosomes. Although his recordings of the extinction changes caused by complete reduction of the cytochrome components are in agreement with those obtained in this Laboratory, the spectroscopic changes caused by the addition of adenosine diphosphate (ADP) to the aerobic sarcosome suspension differ sharply. Possible causes for this discrepancy have been discussed by Slater (1955

and by Chance & Baltscheffsky (1958). The experiment described in this paper appears to explain the discrepancy completely.

EXPERIMENTAL

Materials. The reagents used here were similar to those used by Chance & Baltscheffsky (1958). The rat-heart-sarcosome preparation was based upon the method of Cleland & Slater (1953a).

Methods. In the previous paper, a spectroscopic method especially designed to be insensitive to light-scattering

changes was used. This consists of a double-beam spectrophotometer in which the difference in extinction between two closely spaced wavelengths was recorded. This method can, in addition, be used to measure simultaneously the extinction change at either one of the wavelengths used in the double-beam method (Chance, 1951). We have therefore selected one of the two wavelengths to be suitable for measuring non-specific light-absorption effects caused by changes in light-scattering of the sarcosomal suspension ($443\text{ m}\mu$) and the other wavelength for measuring the oxidation and reduction of cytochrome *b* ($430\text{ m}\mu$). Since these wavelengths differ by only $13\text{ m}\mu$, the difference between the extinctions at these two wavelengths will be relatively insensitive to the non-specific effects. The data given in Fig. 8A of Chance & Baltscheffsky (1957) show that negligible specific spectroscopic changes occur at $443\text{ m}\mu$ upon addition of ADP to the aerobic sarcosome suspension.

RESULTS AND DISCUSSION

In the top trace of Fig. 1, the phosphorylative and respiratory activities of the sarcosomal suspension are recorded by the vibrating platinum microelectrode. Immediately after the addition of $1.2\text{ }\mu\text{moles}$ of α -oxoglutarate to give a final concentration of 0.8 mM , the respiration rate remains slow

at a value of $0.2\text{ }\mu\text{M-O}_2/\text{sec}$. Upon the addition of $1.2\text{ }\mu\text{moles}$ of ADP to give a final concentration of $480\text{ }\mu\text{M}$, the respiration accelerates 5.5-fold and remains so until the oxygen concentration has decreased by $72\text{ }\mu\text{M}$. Thereupon the respiratory rate drops by a factor of 7. The addition of $0.02\text{ }\mu\text{mole}$ of Dicumarol [$3:3'$ -methylenebis-(4-hydroxycoumarin)] to give a final concentration of $8\text{ }\mu\text{M}$ then causes a sixfold increase of respiration.

The double-beam spectroscopic record shows, immediately after the addition of α -oxoglutarate, an upward deflexion of the trace corresponding to an increase of extinction at $430\text{ m}\mu$ compared with that of $443\text{ m}\mu$. This is attributed to the reduction of cytochrome *b*. The addition of ADP causes a downward deflexion of the trace that represents the characteristic decrease of extinction at $430\text{ m}\mu$. This is attributed to the oxidation of cytochrome *b*. The oxidation level of cytochrome *b* decreases somewhat while the ADP is being phosphorylated and then falls sharply while the ADP concentration falls towards zero. Addition of Dicumarol ($0.02\text{ }\mu\text{mole}$ to give a final concentration of $8\text{ }\mu\text{M}$) causes an abrupt reoxidation of cytochrome *b*, and a steady state obtains until the end of the record.

Fig. 1. Mirror-galvanometer recordings of respiratory activity (platinum microelectrode trace), single-beam changes of extinction (trace labelled $443\text{ m}\mu$) and double-beam changes of extinction (trace labelled $430-443\text{ m}\mu$). Rat-heart-muscle sarcosomes were prepared according to Cleland & Slater (1953a). The reaction mixture contained 4 mg. of protein/ml., 0.3 M -sucrose, $\text{mm-ethylenediaminetetra-acetic acid}$, 17.5 mM -phosphate, 8.8 mM -KCl, pH 7.5. Cuvette volume 2.5 ml. with a 1 cm. optical path. The molar concentrations of the reagents added are indicated on the figure and are converted into μmoles by dividing by 400. Additions: $1.2\text{ }\mu\text{moles}$ of ADP (0.01 ml. of 0.12 M -ADP solution), $2\text{ }\mu\text{moles}$ of α -oxoglutarate, $0.02\text{ }\mu\text{moles}$ of Dicumarol. These additions cause negligible changes in tonicity. Respiratory rates ($\mu\text{M-O}_2/\text{sec.}$) and oxygen concentration changes during phosphorylation are indicated below the platinum microelectrode trace. The sensitivities of the recordings at 443 and at $430-443\text{ m}\mu$ are indicated. An increase of absorption at $443\text{ m}\mu$ is indicated by a downward deflexion of both traces. An upward deflexion of the $430-443\text{ m}\mu$ trace indicates an increase of absorption at $430\text{ m}\mu$. Since the absorption band of cytochrome *b* disappears on oxidation, this is registered by a net downward deflexion of the $430-443\text{ m}\mu$ trace. Temperature, 26° .

Non-specific light-absorption, measured at $443\text{ m}\mu$ after the addition of α -oxoglutarate, shows an upward deflexion of the trace corresponding to a decrease of extinction. Such an extinction change is attributed to a swelling of the sarcosomes (Cleland & Slater, 1953*b*). Upon addition of ADP there is a large increase of extinction at $443\text{ m}\mu$ indicative of a shrinking of the sarcosomes. However, this reaction is slow compared with the spectroscopic effect measured at 430 and $443\text{ m}\mu$. After $\frac{1}{2}$ min. the extinction of the suspension becomes stable. When the added ADP is exhausted, as indicated by the slackening of respiration, the extinction at $443\text{ m}\mu$ begins to decrease and continues to do so in the absence of ADP. Addition of Dicumarol stops this decrease and causes a slight increase of extinction.

The single- and double-beam recordings differ from each other in three ways. First, the single-beam recording has no critical wavelength dependency as shown by other experiments, whereas the double-beam recording shows a sharp wavelength dependency with a peak at $430\text{ m}\mu$ [see Fig. 8 of Chance & Baltscheffsky (1958)]. Secondly, the kinetic effects differ: the double-beam recording shows a rapid response to the addition of ADP, whereas the single-beam recording responds slowly. (This is not an instrumental effect since both galvanometers have adequately rapid response to reproduce faithfully these reaction kinetics.) Conversely, the double-beam recording shows no change after the added ADP has been exhausted, and the single-beam recording shows a rapid decrease of extinction. Thirdly, the sign and magnitude of the effects are different. The addition of ADP causes a decrease of absorption at $430\text{ m}\mu$ measured with respect to $443\text{ m}\mu$, whereas the single-beam record shows an increase of absorption at $443\text{ m}\mu$. Thirty seconds after the addition of ADP the latter record indicates an increase of extinction 3.7 times the decrease measured by the double-beam system. The major findings given here have been reproduced in a large number of experiments.

It is now possible to compare these results with those of Holton (1955), who has recorded the effect of ADP addition to heart-muscle sarcosomes with a single-beam technique at $433\text{ m}\mu$. From our data, about 75% of the spectroscopic change recorded with such a technique is a non-specific increase of absorption. Holton also finds an increase of absorption upon ADP addition, and at the end of about 2 min. he finds a slow decrease of absorption which corresponds closely to that shown in our Fig. 1, provided that we accept the very likely assumption that the $110\text{ }\mu\text{M}$ -ADP used in his experiment was expended at the end of 2 min.

Thus the single-beam recordings of the effect of ADP obtained by Holton and by us are in good agreement. However, it is not justifiable to attribute the change of extinction caused by ADP addition to a specific spectroscopic effect. Thus his interpretation that cytochrome *b* is more reduced in the presence of ADP than in its absence under his experimental conditions appears to be incorrect.

These records permit us to correlate in a preliminary fashion the metabolic state of the sarcosomes and light-scattering changes. In the presence of α -oxoglutarate and in the absence of ADP (state 4), the sarcosomes appear to swell; in the presence of both α -oxoglutarate and ADP (state 3), the sarcosomes shrink rapidly at first and then become comparatively stable. It appears that the sarcosomes are less stable in quiescent state 4 than in the active state 3. Since adenosine triphosphate (ATP) is present in both states, it is suggested that 'high-energy' intermediates formed during oxidative phosphorylation are more useful in maintaining the sarcosomes in an unswollen state than is ATP itself.

SUMMARY

1. Simultaneous single-beam and double-beam recordings of extinction changes in a suspension of heart-muscle sarcosomes show that addition of adenosine diphosphate causes a non-specific increase of absorption (measured at $443\text{ m}\mu$) and a specific decrease of absorption at $430\text{ m}\mu$ measured with respect to $443\text{ m}\mu$. The non-specific change is about four times as large as the specific one.

2. A single-beam spectrophotometric technique is not suitable for the measurement of spectroscopic effects in suspensions of sarcosomes and mitochondria that show simultaneous non-specific changes of light-absorption.

3. If it is assumed that the non-specific light-absorption properties of the sarcosomes are indicative of the ion-transport processes, these results suggest that intermediates formed during oxidative phosphorylation are more effective in preventing swelling of the sarcosomes than is adenosine triphosphate itself.

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