a second order constant) decreased at the highest concentrations.
At room temperature (28°), however, the rate of change of \( a'' \) expressed as a first order constant (which is the quantity actually measured) was over \( 2 \times 10^4 \) sec\(^{-1} \) when equal volumes of enzyme and oxygen-equilibrated buffer were used, and it seemed possible that the rate of photodecomposition of the enzyme-CO compound might be limiting the rate of the over-all process. The dissociation has therefore been observed directly, and with the highest flash energies available it half completed in 3.5 microseconds, suggesting that rate constants considerably greater than \( 2 \times 10^4 \) sec\(^{-1} \) might be measured. This expectation has been confirmed experimentally with nitric oxide which reacts rapidly with reduced cytochrome oxidase (1). The second order rate constant at 28° is approximately \( 8 \times 10^7 \) M\(^{-1}\) sec\(^{-1}\), and if buffer equilibrated with 1 atmosphere of NO (concentration in solution, \( 2 \times 10^{-3} \) M) is mixed with an equal volume of enzyme a first order rate constant of \( 8 \times 10^4 \) sec\(^{-1}\) would be expected. The rate actually measured was \( 6 \times 10^4 \) sec\(^{-1}\), or between 2 and 3 times faster than the most rapid rate observed for the \( a''-02 \)-oxygen reaction. The limitation of the observed rate of the O\(_2\) reaction cannot, therefore, be attributed to the photosensitive decomposition of the enzyme-CO compound. The dissociation has therefore been observed directly, and with the highest flash energies available it half completed in 3.5 microseconds, suggesting that rate constants considerably greater than \( 2 \times 10^4 \) sec\(^{-1} \) might be measured. This expectation has been confirmed experimentally with nitric oxide which reacts rapidly with reduced cytochrome oxidase (1). The second order rate constant at 28° is approximately \( 8 \times 10^7 \) M\(^{-1}\) sec\(^{-1}\), and if buffer equilibrated with 1 atmosphere of NO (concentration in solution, \( 2 \times 10^{-3} \) M) is mixed with an equal volume of enzyme a first order rate constant of \( 8 \times 10^4 \) sec\(^{-1}\) would be expected. The rate actually measured was \( 6 \times 10^4 \) sec\(^{-1}\), or between 2 and 3 times faster than the most rapid rate observed for the \( a''-02 \)-oxygen reaction. The limitation of the observed rate of the O\(_2\) reaction cannot, therefore, be attributed to the photochemical step. Further, when a series of NO concentrations was examined, it was observed that with the highest values the maximal concentration of reduced oxidase present in the solutions during and after the flash decreased. This effect would be expected to occur when the rate of combination of NO with reduced enzyme became competitive with the rate of dissociation of CO by the photolysis flash. With oxygen, on the other hand, there was no measurable change in the peak concentration of reduced enzyme, as observed spectrophotometrically at 444 nm, even with the highest oxygen concentrations. This finding is consistent with the low value of the observed first order constant for the oxygen reaction.

Clearer results for the oxygen reaction were obtained by lowering the temperature to 2° which increased the solubility of oxygen 1.7-fold as compared with 28°. The change in the rate of the reaction of the reduced enzyme with oxygen, expressed as a second order constant, is shown in Fig. 2, which covers an 8-fold range of oxygen concentration. In other experiments at 2° the buffer solutions were usually (a) equilibrated with oxygen by bubbling the gas through a cylinder of buffer surrounded by ice giving a concentration of \( 2.1 \times 10^{-3} \) M, and (b) equilibrated with air at room temperature (28°), giving a concentration of \( 2.5 \times 10^{-4} \) M. The measured first order constants for the rapid phase of the reaction (\( a'' + 02 \)) in experiments with four different preparations of the enzyme were: with oxygen-equilibrated buffer, \( 1.7 \times 10^4 \) sec\(^{-1}\) (range, 1.4 to \( 2.1 \times 10^4 \)), and with air-equilibrated buffer, \( 0.6 \times 10^4 \) sec\(^{-1}\) (range, 0.5 to \( 0.78 \times 10^4 \)). The ratio of the rates was thus 2.8:1, while the ratio of the concentrations was 8.4:1. This effect may be explained by postulating an intermediate step in the reaction which becomes rate-limiting at the highest oxygen concentrations. One obvious possibility is the formation of an O\(_2\)-heme \( a_3 \) compound analogous to oxyhemoglobin, but this is perhaps unlikely because examination of reaction records made at different wave lengths suggests that the intermediate may have an absorption spectrum more like that of reduced \( a_3 \) than that of either \( a^{3+} \)-CO or \( a'' \)-NO. In particular, observations made at 434 nm (isosbestic for \( a'' \) and \( a'' \)) show no changes which can be attributed to a postulated intermediate. An alternative is that the reduced enzyme exists in two electronically similar but configurationally different forms, only one of which is oxidized by molecular O\(_2\). In order to settle the spectrum of an intermediate, which cannot have a lifetime greater than about 30 microseconds at 2°, it will be necessary to construct apparatus able to work at pressures substantially greater than 1 atmosphere, and perhaps to bring about a more rapid photochemical breakdown of the oxidase with a Q-switched laser. The existing flash apparatus, however, should suffice to make a systematic examination of the effects of pH and ionic strength on the reaction of \( a_3 \) with oxygen, and to determine directly the contributions of \( a_3 \) and \( a \) to the difference spectrum seen on reaction of oxygen with the reduced enzyme.

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Evidence for Histidine at the Active Site of Myosin A*

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The possibility of a histidyl residue being present at the adenosine triphosphatase active site of myosin A has been suggested previously from photooxidation studies of myosin A in the presence of methylene blue (1). Because of the complexity of the change in ATPase activity with photooxidation time, no definitive conclusions concerning the role of histidyl residues in the catalytic functioning of myosin A could be reached. During the photooxidation reaction an initial increase in ATPase was

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observed at the early stages of photooxidation followed by a
decrease in activity. These effects were similar to others in
which low concentrations of sulphydryl reagents caused an
initial activation of myosin ATPase followed by inactivation
with higher concentrations of the same reagents (2). Since
both the sulphydryl and the histidyl residues were oxidized at
similar rates, it was difficult to decide if the destruction of
specific sulphydryl or histidyl residues or both was involved in
the observed changes in ATPase activity.

It has been shown recently that when myosin A is treated with
bis-β-carboxyethyl disulfide (3), 14 of the 15 sulphydryl residues
per 200,000 molecular weight (4) will undergo a disulfide-sulphy-
dryl interchange reaction with the reagent and only a single
sulphydryl residue remains nonexchangeable. By reversing
the exchange with β-mercaptoethanol, complete restoration of
ATPase occurs. If, however, the remaining sulphydryl residue
is carboxymethylated with iodoacetamide, regeneration of
ATPase does not occur. The fact that the presence of adenosine
triphosphate protected this sulphydryl residue from reacting
with iodoacetamide led to the conclusion that this hard-to-ex-
change sulphydryl residue was located at or near the active site of
the myosin A molecule.

The availability of disulfide-exchanged myosin afforded us
the opportunity of studying the photooxidation of a derivative
in which the number of potentially oxidizable groups had been
decreased, and which, therefore, would decrease the complexity
of the interpretations. Only a single sulphydryl group should
now be available for oxidation whereas the histidyl residues as
well as the methionyl residues would still be completely sus-
ceptible to the photooxidation reaction.

Disulfide-exchanged myosin was prepared as previously de-
scribed (3). The photooxidation procedure used has also been
reported earlier and was essentially similar to that described by
Ray and Koshland (5). At various time intervals aliquots from
the photooxidation vessel were removed and set aside in the dark
for analysis. One sample was used for regeneration of ATPase
activity with β-mercaptoethanol (3). The ATPase activities
were measured at pH 7.5 in the presence of Ca++ (3). Another
sample was allowed to react with 0.1 M iodoacetamide-14C (Tra-
cerlab, 0.76 mc per mmole) for 20 minutes at 25°, pH 8.5. This
sample was precipitated several times by dilution to remove
excess iodoacetamide and methylene blue. It was then divided
into two parts, one for hydrolysis in 6 N HCl at 110° in a sealed,
evacuated tube; the second part was dissolved in a saturated
solution of Ba(OH)₂ for hydrolysis at 110° for 24 hours in order
to detect methionine and tryptophan (5). By use of a liquid
flow scintillation counter (Nuclear-Chicago, model 8350) at-
tached to an automatic Phoenix amino acid analyzer (6), it was
possible to analyze for 14C-labeled carboxymethyllysteyline as well
as for histidine on the same hydrolysate. Methionine and
tryptophan were analyzed from samples taken from the Ba(OH)₂
hydrolysate.

Fig. 1 demonstrates the rate of loss of ATPase activity of a
typical sample of photooxidized DEM.1 The reaction is first
order with a rate constant equal to 0.099 min⁻¹. Several samples
of DEM gave rate constants which varied from 0.098 to 0.102
min⁻¹.

1 The abbreviation used is: DEM, disulfide-exchanged myosin.
Fig. 2 shows the rate of photooxidation of the histidyl residues in DEM. If the linear portion of the curve is extrapolated to zero time, a log difference plot can be obtained, and this difference plot gives a straight line with a first order rate constant equal to 0.007 min⁻¹ (Fig. 2). The histidyl residues in DEM, therefore, are photooxidized at two different rates, a fast and a slow rate. The first order rate constant for the destruction of the fast histidyl residues can be seen to be equal to the first order rate constant for the loss in ATPase activity. On the assumption that 25 histidyl residues are present in myosin A per 200,000 molecular weight² (4), 8 are photooxidized in the fast portion of the curve. Of these 8, the destruction of only 1 is apparently involved in the catalytic functioning of the enzyme since the rate constants for both processes are equivalent.

A significant feature of the photooxidation of DEM is that the single, nonexchanged sulfhydryl residue is only very slowly photooxidized. In 20 minutes, when over 90% of the ATPase activity has been lost, only 12% of the single sulfhydryl residue has been photooxidized, as determined by the isolation of 14C-labeled residual ATPase activity. Of these 8, the destruction of only 1 is apparently involved in the catalytically functioning of the enzyme since the rate constants for both processes are equivalent.

The results presented appear to support the conclusion that a single histidyl residue in myosin A may be involved at or near the ATPase active center of the enzyme. That a sulfhydryl residue may also be involved is supported from our earlier results (3) and from the data of Sekine, Barnett, and Kielley (4). Indeed, two sulfhydryl residues have been postulated as being part of the ATPase active center of myosin A (3, 4, 7). It may well be that the sulfhydryl groups could function as binding sites for ATP whereas the histidyl residue may act as the catalytic group involved in the cleavage of the terminal phosphate residue of ATP.

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The Biosynthesis of Starch in Spinach Chloroplasts*

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The biosynthesis of starch in plants takes place by the transfer of the glucosyl moiety of either adenosine diphosphate glucose or uridine diphosphate glucose to a starch primer (1–3). Leaves of higher plants are known to accumulate starch during photosynthesis. Murata and Akazawa (4) have recently shown that in leaves, starch is formed from ADP-glucose only. All of these ADP-glucose:starch transglucosylases were associated with starch granules and therefore particulate. Frydman and Cardini (5) obtained a soluble preparation from sweet corn which transferred glucose from ADP-glucose to phytoaglycogen. They have also recently (6) shown that soluble extracts prepared from tobacco leaves or from potato tubers transferred glucose from ADP-glucose to an α-1,4-glucan primer. The present communication reports the presence of a soluble ADP-glucose:starch transglucosylase in chloroplasts. Evidence will also be provided for a possible regulation of starch biosynthesis during photosynthesis.

The chloroplasts were isolated from fresh spinach which was obtained from the local supermarkets (7). The washed leaves were homogenized with a solution of 0.5 M sucrose containing 0.1 M phosphate buffer, pH 7.4, 0.01 M EDTA, and 0.005 M GSH. The nuclear debris and unbroken cells were removed by centrifuging at 300 × g for 1 minute, and the chloroplasts were then isolated by centrifuging at 1,000 × g for 10 minutes. The chloroplasts were washed with a solution of 0.5 M sucrose containing 0.01 M phosphate, pH 7.4, 0.002 M EDTA, and 0.005 M GSH. The washed chloroplasts were suspended in 0.1 M Tris-succinate buffer, pH 7.0, containing 0.005 M GSH and 0.002 M EDTA, and disrupted in a French press at 20,000 p.s.i. The broken chloroplasts were centrifuged at 105,000 × g for 90 minutes, and the clear supernatant fraction was used as the enzyme source. Fractionation with ammonium sulfate yielded two fractions: one precipitating between 0 and 40% saturation, contained most of the ADP-glucose:starch glucosyltransferase activity, and the other precipitating at 40 to 60% saturation, contained most of the ADP-glucose pyrophosphorylase activity.

The ADP-glucose:starch glucosyltransferase was further purified by adsorption on calcium phosphate gel and elution by 0.05 M potassium phosphate buffer, pH 7.5, containing 0.006 M GSH. It was reprecipitated with ammonium sulfate and then dialyzed overnight against a buffer containing 0.01 M Tris-succinate, 0.005 M GSH, and 0.002 M EDTA, pH 7.0. This fraction transferred 3.6 μmoles of glucose per hour per mg of protein and was purified 9-fold over the 105,000 × g supernatant fluid.

The ADP-glucose:starch glucosyltransferase was assayed by the incorporation of the glucose-¹⁴C from ADP-glucose into amylose. The reaction mixture, which contained 38 μmoles of ADP-glucose¹⁴C (7.5 × 10⁶ e.p.m. per μmole), 10 μmoles of glycine-NaOH buffer, pH 8.5, 5 μmoles of KCl, 2 μmoles of GSH, 1

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