of α -chymotrypsin has shown that such dependencies are dependent upon the structures and stereochemistry of the substrates. These observations have been interpreted in terms of variation of the conformation of the active site of the enzyme with hydrogen ion concentration.

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OXIDATIVE PHOSPHORYLATION IN FRACTIONATED BACTERIAL SYSTEMS, XIII. EFFECTS OF PHOTOOXIDATION ON THE SOLUBLE COUPLING FACTORS*

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Loss of enzymatic activity upon photooxidation of proteins by methylene blue, visible light, and oxygen has been correlated with the destruction of peptide-linked histidine residues within the protein without destruction of peptide bonds.¹ Although other amino acid residues can be destroyed during photooxidation,^{2, 3} this technique suggested a means of obtaining insight into the role of protein-bound histidine in oxidative phosphorylation. It was of further interest to determine the effects of photooxidation on the exchange reactions associated with the coupling process. The photooxidation technique was therefore applied to the supernatant fraction of *Mycobacterium phlei*, since this fraction contains protein coupling factors and exhibits the exchange reactions. Preliminary results indicate that this treatment affects certain exchange reactions but has no effect on the coupling activity elicited by the soluble fraction with certain substrates as electron donors.

Materials and Methods.—*Preparations:* *Mycobacterium phlei*, ATCC 354, was grown, and cell-free homogenates were prepared by methods previously described.⁴ The crude supernatant fluid obtained by centrifugation of the homogenate at 140,000 $\times g$ for 90 min was adjusted with *M* Tris-HCl buffer to pH 8.2. Photooxidation of the supernatant fraction was accomplished by the method of Yamagata *et al.*⁵ Methylene blue was added to a final concentration of 0.5 mg/ml supernatant fluid, and the pH was readjusted to 8.2. The material was irradiated with visible light at 3°C for various periods of time up to 4 hr. Controls containing methylene blue were kept in the dark. Following irradiation the supernatant fluid was fractionated with ammonium sulfate,⁶ and the precipitate obtained at 30–60% saturation was removed and dialyzed against distilled water at 3° for 48 hr. The

dialyzed material was treated with 1–2% charcoal to remove the methylene blue and the material used directly or concentrated by lyophilization.

Enzyme assays: The coupling factors of the fractionated supernatant preparations were assayed for their ability to restore oxidative phosphorylation to the untreated washed particulate fraction. Oxygen uptake was measured by the conventional manometric procedures at 30°. Substrates utilizing different electron transport pathways were used in the assay system. Phosphate disappearance was measured by the method of Fiske and Subba-Row.⁷ Malate-vitamin K reductase and β -hydroxybutyrate dehydrogenase were assayed spectrophotometrically.⁸ The P^{32} -ATP and ADP- C^{14} -ATP exchange reactions were assayed at 30° by the methods of Cooper and Kulka⁹ following chromatographic separation of the nucleotides (Dowex 1-X2, chloride form). ATPase was measured at pH 8.2 by following the liberation of inorganic orthophosphate, whereas ATP formation catalyzed by adenylate kinase was determined spectrophotometrically with hexokinase, glucose, TPN, and glucose-6-phosphate dehydrogenase.

Results.—Coupled phosphorylation with the fractionated system from *M. phlei* requires the addition of a particulate and a supernatant fraction (Table 1). The

TABLE 1
THE EFFECT OF PHOTOOXIDATION ON COUPLED PHOSPHORYLATION

	— β -Hydroxybutyrate—			—DPNH—		
	Oxygen uptake (μ atoms)	ΔPi (μ moles)	P:O	Oxygen uptake (μ atoms)	ΔPi (μ moles)	P:O
P	0.0	0.0	—	1.4	0.7	0.50
P+S	7.6	3.7	0.75	4.0	2.5	0.62
P+S (Mb-dark)	8.0	4.3	0.54	4.1	3.0	0.73
P+S (Mb-light)	6.3	3.7	0.59	3.0	2.5	0.83
				1.4*	1.9	1.36

* Results obtained with a different photooxidized preparation.

The system consisted of 0.2 ml of the washed particulate fraction (P) containing 22.5 mg protein per ml, 0.4 ml of fractionated supernatant material adjusted to 20.5 mg protein per ml (S, untreated material; S (Mb-dark), exposed to methylene blue for 3 hr in the dark; S (Mb-light), exposed to visible light and methylene blue for 3 hr). In addition, the vessels contained 10 μ moles of inorganic phosphate, 2.5 μ moles of ADP, 20 μ moles of glucose, 15 μ moles of MgCl_2 , 25 μ moles of KF , 100 μg of DPN, 60 μ moles of β -hydroxybutyrate, and water to a final volume of 1.3 ml. DPNH generation was also accomplished with crystalline yeast alcohol dehydrogenase (0.6 mg), 60 μ moles of ethanol, and 200 μg of DPN. The activity was followed for 10 min at 30° following the addition of substrate.

soluble protein components can be resolved by column chromatography into a number of different factors which are required for restoration of coupled activity with different segments of the respiratory chain.^{6, 10} Nevertheless, it has not been possible to separate the different soluble oxidative components from the coupling factors associated with them. Since the particles alone exhibit low levels of coupled phosphorylation with certain substrates (DPNH or succinate), it was important to distinguish between the effect of photooxidation on the soluble oxidative components and the effect on the soluble coupling factors. The effects of photooxidation on the coupling factors were therefore ascertained by studying the ability of the photooxidized preparation to restore phosphorylation with different substrates which have different requirements with respect to the oxidative factors.

Restoration of coupled phosphorylation with β -hydroxybutyrate as substrate was observed with the methylene blue photooxidized supernatant fraction. Some inactivation (17–48%) of β -hydroxybutyrate dehydrogenase occurred during photooxidation; however, the extent of restoration of phosphorylation as measured by the P:O ratio was similar to that obtained with the control system (methylene

blue dark-treated fraction). Although methylene blue is an effective uncoupling agent in the *M. phlei* system,⁴ treatment with charcoal appears to be effective in the removal of the dye.

The particulate fraction alone exhibits some phosphorylation with a DPNH generating system. The ability to stimulate coupled phosphorylation by the supernatant fraction was not affected by photooxidation (Table 1). Unlike β -hydroxybutyrate as an electron donor, with the DPNH generating system, restoration of phosphorylation did not parallel the capacity to restore oxidation. Restoration of phosphorylation occurred to the same degree as observed with the untreated or methylene blue dark-treated control system, whereas the degree of restoration of oxidation with the photooxidized preparations was considerably lower. In some experiments complete inactivation of the supernatant factor which stimulates DPNH oxidation had occurred during photooxidation. Although the endogenous oxidation of the particulate fraction was not further stimulated by the addition of the photooxidized preparation, phosphorylation was stimulated over 50 per cent.

Restoration of activity did not occur with malate as substrate since photooxidation of the supernatant fraction destroys its ability to restore oxidation with this compound and the particles alone are inactive with malate. In agreement with this result, malate-vitamin K reductase, the soluble enzyme responsible for initiating malate oxidation in the *M. phlei* system,¹⁰ was destroyed during treatment. Attempts to protect this activity from inactivation by the addition of substrate or cofactors during photooxidation have been unsuccessful.

It was also of interest to measure the effects of photooxidation on several of the exchange reactions which are associated with phosphorylation and the effect on adenylate kinase. The bulk of the exchange activities has been shown to reside in the supernatant fraction.¹⁰ In contrast to the insensitivity of the soluble components necessary to restore coupled activity, certain of the exchange activities were substantially decreased by photooxidation. Thus, the Pi^{32} -ATP exchange reaction, ATPase, and adenylate kinase activities were decreased 72, 82, and 46 per cent, respectively, by photooxidation (Table 2). The ADP- C^{14} -ATP exchange reaction was insensitive to prolonged treatment (4 hr) with light and methylene

TABLE 2
EFFECTS OF PHOTOOXIDATION ON THE EXCHANGE ACTIVITIES

	Activity (mmoles/min/mg protein)		
	Pi^{32} -ATP	ATPase	ADP- C^{14} -ATP
S	3.43	28.3	99
S (Mb-dark)	3.39	21.6	103
S (Mb-light)	0.96	5.0	140

The reactions were carried out at room temperature by the method described by Cooper and Kulka.⁹

blue. In most preparations stimulation of the ADP- C^{14} -ATP exchange reaction was observed with the photooxidized preparation. This stimulation was greater than could be accounted for by the loss of ATPase.

The particulate fraction from *M. phlei* also exhibited the exchange reactions, but the level of these activities was only 20–30 per cent of that found in the supernatant fraction. Some stimulation (35%) of the particulate Pi^{32} -ATP exchange activity was observed with the untreated supernatant fraction; however, neither the methylene blue dark nor the light-treated preparation showed stimulation of

TABLE 3
 Pi^{32} -ATP AND ATPase ACTIVITIES OF THE RECONSTITUTED
 AND TREATED SYSTEMS

	Activity ($\mu\text{moles/min}$)	
	$\text{Pi}^{32}\text{-ATP}$	ATPase
Particles (P)	0.87	16.7
Supernatant fraction (S)	4.0	53.7
P+S	7.5	64.5
S (Mb-dark)	3.4	35.0
P+S (Mb-dark)	3.1	50.5
S (Mb-light)	1.4	9.0
P+S (Mb-light)	2.2	25.0

The Pi^{32} -ATP assay system consisted of 0.2 ml of washed particles, 0.25 ml of untreated, methylene blue dark-treated, and methylene blue light-treated supernatant fractions containing 20.5, 16.5, and 13.3 mg of protein per ml, respectively. In addition, the system contained 4.5×10^{-3} M MgCl_2 and KCl, 0.04 M Tris buffer pH 7.2, 5 μmoles of ATP, 10 μmoles of ADP, 5 μmoles of inorganic phosphate containing P^{32} (3.4×10^6 cpm/ μmole), and water to a final volume of 1.35 ml. The ATPase assay contained 0.1 ml of washed particles (28.3 mg protein per ml), the supernatant fractions as indicated above, 5.0 μmoles of ATP, 0.02 M MgCl_2 , 0.1 M Tris buffer pH 8.2, and water to a final volume of 0.7 ml.

this activity when added to the particles (Table 3). Stimulation of ATPase and the $\text{ADP-C}^{14}\text{-ATP}$ exchange¹⁰ was not observed following recombination of the particulate and the supernatant fractions. The system from *Micrococcus lysodeikticus* as described by Ishikawa and Lehninger¹¹ exhibits a much higher degree of stimulation of the Pi^{32} -ATP exchange in the reconstituted system than does the *M. phlei* system.

Amino acid analysis¹² after acid hydrolysis of the methylene blue light-treated material and of the methylene blue material kept in the dark revealed significant loss of histidine and methionine residues in the light-treated preparation. Both amino acids were present at less than half the control level. Some discrepancy in the levels of serine, proline, and tyrosine was found in the methylene blue light-treated preparation. The levels of these amino acids were found to be higher than those of the control samples. Among the inert amino acids the levels compared well, the ratios of light-treated to control amounts for valine and alanine being 0.91 and 1.01, respectively. No analysis for tryptophan was obtained.

Discussion.—Boyer and co-workers^{13, 14} have demonstrated the formation of protein-bound phosphohistidine in mitochondria under conditions of oxidative phosphorylation. The limitations of the photooxidation technique are such as not to permit a definite test of the role of phosphohistidine in the bacterial system at present. Nevertheless, it would be of interest to determine whether protein-bound histidine or phosphohistidine is involved at the active site of the enzymes catalyzing the exchange reactions which are lost upon photooxidation. Barnard and Stein³ have suggested a role for imidazole in such reactions. Problems inherent in the photooxidation method are multiple; for example, destruction of histidine residues may change the tertiary configuration of the enzyme leading to indirect inactivation. Further, it is known that photooxidation leads to destruction of other amino acids, not all of which may be detected by amino acid analysis following acid hydrolysis. Methods which exhibit greater specificity for histidine residues will be utilized.

The effect of photooxidation on the soluble coupling factors of *M. phlei* can only be assessed with preparations in which the soluble oxidative factors are either partially or completely inactivated. Complete inactivation of the oxidative factor(s) requires the utilization of substrates like DPNH which exhibit oxidation

with the particles alone and only slight phosphorylation. A more direct approach to determine the effects of photooxidation on the coupling activity would be to examine the fractionated system from *M. lysodeikticus*, since the supernatant fraction obtained following centrifugation is required for restoration of phosphorylation only.

The level of the Pi^{32} -ATP exchange reaction and the level of ATPase following fractionation of the supernatant fraction from *M. phlei* was found to be low when compared to the over-all rate of phosphorylation observed with the reconstituted system, and thus appears to differ from that observed with the mitochondrial system.¹⁵ The loss of these activities following photooxidation had little or no effect on the capacity of the supernatant fraction to restore oxidative phosphorylation.

Inactivation of ATPase and the Pi^{32} -ATP exchange activities of the supernatant fraction during photooxidation may be similar to that observed with mammalian mitochondria by Beyer¹⁶ and Cooper and Kulka⁹ with near ultraviolet light. The loss of histidine residues in protein by irradiation with ultraviolet light has recently been shown.¹⁷ Treatment with UV light was also shown to inactivate the ADP- C^{14} -ATP exchange reaction in mitochondrial preparations;⁶ however, this exchange activity was not affected in the bacterial system following photooxidation with methylene blue and visible light. The rate of the ADP- C^{14} -ATP exchange reaction of the untreated and treated bacterial supernatant fraction was of the same magnitude as that of the over-all rate of coupled phosphorylation observed with the reconstituted particulate and supernatant system.

Summary.—Evidence has been presented that methylene blue catalyzed photooxidation of soluble bacterial coupling factors from *Mycobacterium phlei* may be capable of selectively affecting phosphate transfer and exchange reactions, without affecting oxidative phosphorylation. Photooxidation resulted in inactivation of ATPase, the Pi^{32} -ATP exchange reaction, and adenylate kinase, but had no effect on the ADP- C^{14} -ATP exchange reaction.

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TEMPLATES FOR THE FIRST PROTEINS OF EMBRYONIC DEVELOPMENT*

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The gathering strength of the messenger hypothesis,¹ and accumulating evidence in favor of polyribosomes as the sites of protein synthesis,² are exerting strong influence on current research in chemical embryology. The scheme now generally accepted for microorganisms and certain mammalian cells^{1, 2} provides a number of specific mechanisms by which cellular differentiation could be initiated and controlled. Their essential feature is a direct genomic regulation of the spectrum of proteins made in different cells of the developing organism.

Sea urchin eggs, with a long history of use in experimental embryology,³ are a particularly favorable material, because they can be obtained in quantity, develop with excellent synchrony, and are reasonably permeable to labeled precursors. During the past two years, a conflict has arisen from experiments on macromolecule synthesis during early development in these forms. Some of the data were available earlier, but the conflict itself stems from the requirements of the current scheme of protein synthesis, according to which the sequence information is carried by more-or-less unstable messenger RNA's. Protein synthesis is either greatly stimulated or actually switched on at fertilization. RNA synthesis is negligible or absent before fertilization, and even after fertilization, is either very slow⁴⁻⁶ or absent.⁷ Brachet *et al.*⁷ and Gross and Cousineau⁸ have expressed doubt that the postfertilization synthesis of messenger RNA could be sufficient to account for the observed stimulation of protein synthesis. Nemer⁵ and Wilt,⁶ among others, consider that postfertilization RNA synthesis does supply missing templates, and that this gives competence to previously inactive ribosomes and may therefore switch on protein synthesis. The inactivity of ribosome preparations from unfertilized eggs, their activation upon fertilization, and their responsiveness to poly-U^{9, 10, 12, 24} have been used in support of the second hypothesis. Consideration of the behavior of parthenogenetic merogones and experiments with actinomycin D^{4, 11} have, however, led Gross and Cousineau⁸ to support the idea that templates for the early proteins may pre-exist in the unfertilized egg. Tyler¹² has reported experiments on egg fragments and homogenates thereof whose results are consistent with such an idea.

The experiments reported here do not prove that templates pre-exist in the unfertilized egg; but if templates are a requirement for all protein synthesis, they make such a conclusion reasonable. They suggest strongly that the early acceleration of protein synthesis following fertilization cannot depend upon new messenger RNA.