by causing transport of calcium into the cells and that the antiserotonin activity of the epinephrines may be because they combine with the serotonin receptor and block it as an antimetabolite would do. The antagonism of histamine seemed less direct.

* Supported in part by a grant from the United States Public Health Service.

† With the technical assistance of N. K. Campbell.

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*A HIGH-ENERGY INTERMEDIATE OF OXIDATIVE PHOSPHORYLATION**

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Communicated by David M. Bonner, May 2, 1960

Previous reports from this laboratory have described an enzyme system from the bacterium *Alcaligenes faecalis* which couples the formation of ATP† to the oxidation of DPNH. The complex contains a particulate DPNH oxidase, a soluble heat labile fraction, and a polynucleotide of the RNA type. One of the functions of this polynucleotide, in conjunction with magnesium, is to bind the soluble heat labile factor to the particles.[1–5]

The purpose of this communication is to show that the enzyme system is dissociated into particulate and soluble components by incubation with DPNH, but not with DPN. The soluble fraction thus eluted produces net ATP synthesis when incubated with Pi and ADP. It is therefore proposed that this compound is a high energy intermediate of oxidative phosphorylation. Two techniques were used for measuring phosphorylation by this intermediate. The first used P^{32} incorporation into organic phosphate, and the second measured ATP formed with the firefly assay system.

Methods.—The bacteria were grown, washed, and sonically disrupted as pre-

viously described.[1, 2] The media was fortified with salts[6] and 2 mg/L of calcium pantothenate. The oxidative phosphorylation complex was precipitated by centrifugation in the presence of $MgCl_2$ at 100,000 $\times$ g for 20 min at approximately 0°. The pellet was then resuspended and reprecipitated to yield washed phosphorylating particles.[5] Hexokinase was made by the method of Colowick and was twice recrystallized.[7]

Experimental.—Dissociation of enzyme complex with DPNH: Washed phosphorylating particles containing approximately 10–15 mg of protein and 8–10 per cent nucleic acid were incubated in a total volume of 2.5 ml at 24° for 3–5 min in the standard preincubation mixture containing 12 μmoles of $MgCl_2$, 5.9 μmoles of KCl, and either no pyridine nucleotide or 3 μmoles of DPN or DPNH as indicated. The reaction mixtures were then rechilled and recentrifuged. The supernatant solutions were saved and the pellets were resuspended in 0.01 M KCl. The pellets were then tested for oxidative phosphorylation with DPNH as substrate, and the supernatant solutions were tested for their ability to esterify Pi without added substrate.

The pellets were tested for oxidative phosphorylation in a mixture of glycylglycine and phosphate buffers pH 7.4 with the final Pi concentration 1 μmole/ml, $MgCl_2$, ATP or ADP, hexokinase, glucose, and P^{32}. DPNH was used as substrate and a control vessel contained an equal amount of DPN.[2] The difference in phosphorylation observed between the experimental and control vessels is the value reported as oxidative phosphorylation. Incorporation of P^{32} into the organic fraction was estimated by a modification of the method of Berenblum and Chain,[8–10] which depends on extracting the molybdic acid complex of Pi with isobutanol benzene. All samples were timed with a thin window gas flow counter for 1,000 counts and were corrected for background.

Incorporation of phosphate by the supernatant solutions was measured in a similar medium, except that the glycylglycine and phosphate concentrations were just one-half the concentrations used in testing the pellets, and in most cases ADP was the acceptor without added hexokinase or glucose. No substrate was added to the supernatant solutions as the experiments were designed to examine the question of whether or not a high energy intermediate exists in the supernatant solution obtained from the preincubation with DPNH. If it does, then P^{32} would be incorporated into the organic fraction without the addition of an oxidizable substrate. The supernatant solutions from preincubation with DPNH and DPN will be referred to as S^{DPNH} and S^{DPN}.

Effect of preincubation with DPNH: Results of typical experiments are shown in Table 1. Preincubation with DPNH, but not with DPN, caused a marked inhibition of oxidative phosphorylation in the pellets as seen in lines 1 and 3. It is also evident that S^{DPNH} catalyzed esterification of a large amount of Pi in comparison with the control supernatant solutions from preincubations with DPN or no pyridine nucleotide. This is shown in lines 2 and 4. When Pi, hexokinase, ADP, and glucose are added to the standard preincubation mixture, the inhibition of oxidative phosphorylation in the pellets caused by preincubation with DPNH is much reduced. Associated with this, there is virtually no phosphorylation catalyzed by the supernatant solution. In other experiments inclusion of dinitrophenol (2×10^{-3} M) and Pi, or of arsenate (1×10^{-3} M) in the preincubation

mixture, produced similar though less consistent effects in reducing the inhibition of oxidative phosphorylation in the pellets preincubated with DPNH.

The second half of Table 1 shows that the inhibition caused by preincubation with DPNH on oxidative phosphorylation catalyzed by the pellet can be largely reversed by adding S^{DPNH} back to the pellet several minutes before testing.

These results are compatible with the hypothesis that, during oxidation of DPNH by the enzyme system, a soluble component is dissociated in the high energy state. This factor is required for oxidative phosphorylation, but cannot become reassociated with the particles until the high energy bond is cleaved by phosphate. Dinitrophenol and arsenate presumably play similar roles by accelerating hydrolysis of the high energy intermediate, thus freeing it for recombination with particles. There are, of course, a number of other plausible explanations for the observations which will be discussed below.

Relationship to oxidative phosphorylation: During the preincubation of the enzyme complex with DPNH there is a marked shift to an alkaline pH since the system is weakly buffered. Addition of glycylglycine at pH 6.0, 7.4, and 8.0 either prevents or markedly decreases the effect of preincubation with DPNH. This suggests that the effect observed may be a pH effect alone and not specifically related to oxidative phosphorylation. This does not seem to be the case for two reasons. First, S^{DPNH} has a marked stimulatory effect on oxidative phosphorylation catalyzed by particles preincubated with DPNH as shown in the second part of Table 1. In addition S^{DPNH} has been shown to replace the heat labile factor in the ammonium sulfate separated system and conversely heat labile factor from the ammonium sulfate fractionation restores phosphorylation to particles preincubated with DPNH. Secondly, preliminary experiments by Dr. G. Hovenkamp have shown that there is a consistent difference in phosphorylation by S^{DPNH} and S^{DPN} in strongly buffered systems at higher pHs.

TABLE 1

THE EFFECT OF PREINCUBATION WITH DPNH ON AN ENZYME COMPLEX WHICH CATALYZES OXIDATIVE PHOSPHORYLATION

Expt.	Additions to standard pre-incubation mixture	Fraction tested	Phosphorylation in millimicromoles after preincubation with: No pyr. nucl.	DPN	DPNH	% Inhib. due to DPNH
1	None	Pellet	111	91	15	86
1	None	Supern. Sol.	1.7	2.0	13.4	..
2	None	Pellet	161	110	37	77
2	None	Supern. Sol.	1.0	1.6	7.8	..
2	Pi, Hexo, ADP, Gluc.	Pellet	119	115	72	40
2	Pi, Hexo, ADP, Gluc.	Supern. Sol.	1.0	0.9	1.8	..
3	None	Pellet	...	87	5	94
3	Pi 0.001 *M*	Pellet	...	69	19	72
3	Pi, Hexo, ADP, Gluc.	Pellet	...	50	24	52

Reconstitution of Pellet Activity

	Additions to pellet	No pyr. nucl.	DPN	DPNH	% Inhib. due to DPNH
4	None	101	80	27	73
4	Sup. of no pyr. nucl.	...	..	37	63
4	Sup. of DPNH	...	..	73	27

Perhaps the most obvious alternative possibility to the hypothesis outlined is that an enzyme that catalyzes a Pi-ATP exchange reaction, has been eluted from the particles.[11-14] Polynucleotide phosphorylase could also lead to P^{32} uptake

if it was present in S^{DPNH}.[15] Finally, soluble oxidative phosphorylation remains a remote possibility since S^{DPNH} contains significant amounts of unoxidized DPNH. The experiments described below were designed to evaluate the hypothesis of a high energy intermediate in S^{DPNH} and the alternative explanations outlined.

Proportionality: If a finite amount of a high energy compound exists in the S^{DPNH}, then the amount of phosphorylation observed at completion of the reaction should be proportional to the amount of S^{DPNH} used, while if the findings are to be explained on the basis of a Pi-ATP exchange reaction, the final amount of P^{32}

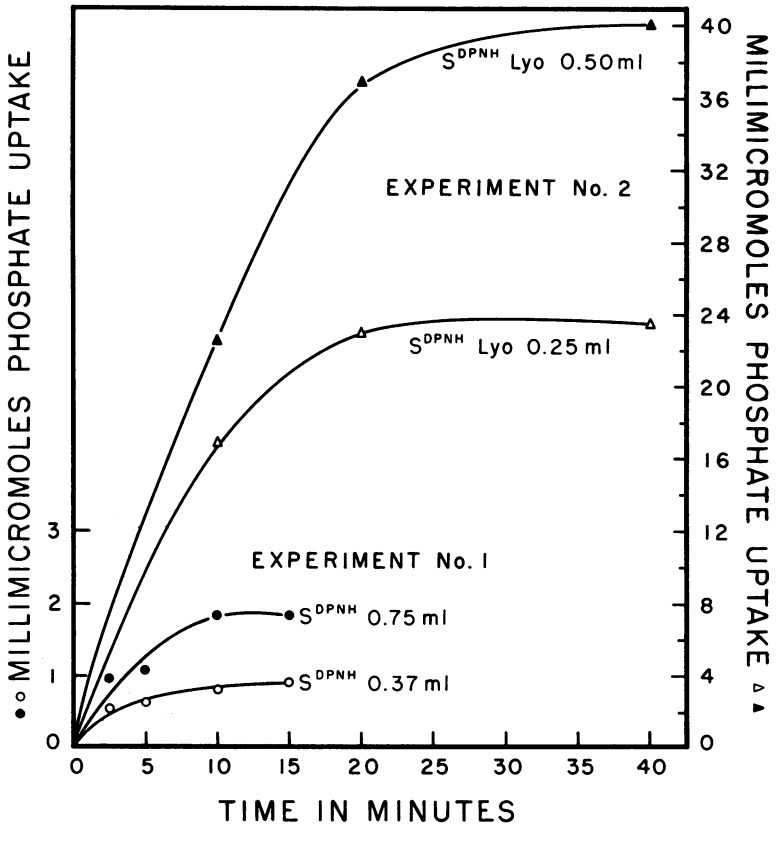

FIG. 1.—Proportionality between amount of S^{DPNH} used and amount of phosphorylation observed. For experiment 1 use left scale and for experiment 2 use right scale for phosphorylation.

incorporated should be independent of the amount of S^{DPNH} used. Figure 1 shows the results of two experiments bearing on this point. In the first experiment fresh S^{DPNH} was used, and the figures plotted represent the phosphorylations observed with the boiled blanks subtracted. In experiment 2, S^{DPNH} was concentrated by lyophilization and the reaction was run in the presence of 0.05 *M* NaF, which has a marked stimulatory effect on the phosphorylation observed from S^{DPNH} (*vide infra*). These results indicate a reasonable proportionality between the amount of phosphate esterified at completion of the reaction and the amount of S^{DPNH} used.

TABLE 2

THE EFFECT OF PREINCUBATION OF S^{DPNH} WITH SALTS BEFORE P^{32} ADDITION (PHOSPHATE ESTERIFICATION IN MILLIμMOLES)

Preincubation with			Phosphorylation by S^{DPNH}	Phosphorylation S^{DPN} S^{DPNH} boiled No Preincubation	
Glycylglycine pH 7.4, 2.5×10^{-2} *M*,	$MgCl_2$	6 μM/ml	7.24	0.2	0.4
KCl 1×10^{-3} *M*,	"	"	5.80	...	...
$KHPO_4$ pH 7.4, 5×10^{-4} *M*,	"	"	1.07	...	...
$KHPO_4$ pH 7.4, 5×10^{-5} *M*,	"	"	2.77	...	...
$KHPO_4$ pH 7.4, 5×10^{-6} *M*,	"	"	5.51	...	...
Na_2HAsO_4 pH 7.4, 5×10^{-4} *M*,	"	"	2.12	...	...
Na_2HAsO_4 pH 7.4, 5×10^{-5} *M*,	"	"	4.48	...	...
Na_2SO_4 pH 7.4, 5×10^{-4} *M*,	"	"	4.37	...	...

Discharge of intermediate: If a high energy intermediate exists in S^{DPNH}, then it should be phosphorylized by preincubation with cold phosphate and, on later addition of P^{32} and ADP, little or no P^{32} should be taken up since all the high energy intermediate would be combined with cold phosphate and none would be left for combination with P^{32}. S^{DPNH} phosphorylized with cold Pi is referred to as Pi-discharged S^{DPNH}. Similarly, preincubation with arsenate would be expected to interfere with subsequent phosphorylation. To examine this point, fresh S^{DPNH} was incubated in the presence of $MgCl_2$ with various concentrations of cold phosphate, arsenate and, as controls with sulfate, glycylglycine, or KCl, for 5 min at room temperature. These preincubation mixtures were then poured into tubes containing P^{32}, ADP, and appropriate additions to make the final glycylglycine concentration 0.025 *M*, and the phosphate 0.0005 *M*. Table 2 shows an experiment representative of many similar ones. Preincubation of S^{DPNH} with glycylglycine (shown to have little effect in previous experiments) or KCl produces comparatively small effects on the amount of phosphorylation, while phosphate and arsenate do produce more marked effects dependent on their concentrations. Sulfate produces a comparatively small effect. These results are again compatible with the hypothesis of a high energy intermediate and provide further evidence against a Pi-ATP exchange.

Specificity of nucleotide acceptor: In order to get more definite information about a possible Pi-ATP exchange reaction, the effectiveness of ATP and AMP were compared with ADP as acceptors of phosphate in the presence of S^{DPNH}. These

TABLE 3

THE EFFECT OF PHOSPHATE, ADP, AMP, AND ATP ON PHOSPHORYLATION BY S^{DPNH} (PHOSPHORYLATION IN MILLIμMOLES)

Acceptor	Phosphorylation after preincubation with No pyridine nucleotide	DPN	DPNH
ADP 0.10 μM no hexokinase, glucose	1.74	1.46	6.16
ADP 0.50 μMole	...	...	4.98
ADP 0.05 μMole	...	...	3.79
ADP 0.005 μMole	...	...	2.23
ATP 0.50 μMole	...	...	2.47
ATP 0.05 μMole	...	...	2.37
ATP 0.005 μMole	...	...	2.32
AMP 0.10 μMole	...	...	2.31
ADP 0.1 μMole, hexokinase glucose	1.37	1.46	9.25
No glycylglycine	...	...	9.05
No PO_4	...	...	0.014
No hexokinase	...	...	9.10
NO ADP	...	...	2.57

experiments were run in the absence of hexokinase and glucose. The results in Table 3 are representative of several similar experiments and show that ADP is a more effective acceptor from S^{DPNH} than either ATP or AMP. The second experiment (shown below the line in the table) indicates that some phosphorylation takes place without added acceptor, and that the phosphorylation with added ATP or AMP is not significantly above this basal level which is presumably the result of endogenous acceptor. These facts do not fit a Pi-ATP exchange hypothesis or any synthetic reaction requiring ATP which might simulate such an exchange. The very marked stimulation of phosphorylation produced by addition of ADP would rule out the possibility that the P^{32} incorporation was dependent on phosphorolysis of a polynucleotide, although the possibility might exist that there was an exchange going on with polynucleotide synthesis and breakdown dependent

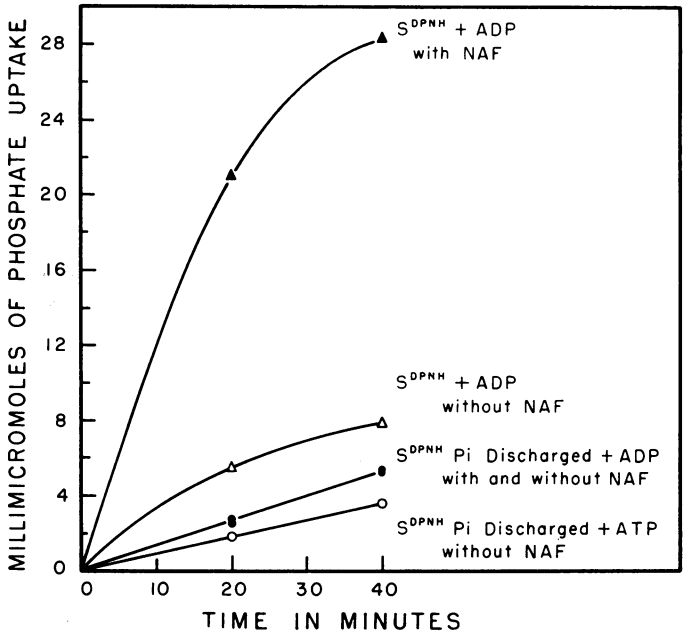

FIG. 2.—The effect of NaF on phosphorylation by S^{DPNH}.

on mononucleotide and a polynucleotide primer. This possible role of polynucleotide phosphorylase will be discussed later.

Effect of inhibitors: The rather unlikely possibility that S^{DPNH} contains a soluble oxidative phosphorylation system is made even more remote by the effects of inhibitors. Dinitrophenol in a concentration of 2×10^{-3} *M* causes an inhibition of from 60 to 90 per cent in phosphorylation linked to DPNH oxidation in the particulate system, but produced only 3–5 per cent inhibition in phosphorylation with S^{DPNH}. Similarly, 0.05 *M* NaF caused an 87 per cent inhibition in oxidative phosphorylation catalyzed by the particulate system, while it produced a marked stimulation in the amount of phosphorylation from S^{DPNH} as shown in Figure 2. It has no effect on the comparatively slow rate at which P^{32} is incorporated by S^{DPNH} which has been exposed to cold phosphate to cleave the high energy inter-

mediate before the addition of P^{32} and ADP. This suggests that these are two unrelated reactions. The stimulatory effect of NaF on phosphorylation with S^{DPNH} is presumably due in part at least to its effect on ATPase since there is a weak ATPase in S^{DPNH} (100 milliμmoles ATP hydrolyzed/ml/hr) which is partially inhibited by NaF. This was determined with hexokinase and glucose-6-phosphate dehydrogenase and TPN. Figure 2 also shows that the rate of phosphorylation using discharged S^{DPNH} is slower when ATP is used as acceptor than with ADP. This again provides clear evidence against the exchange reaction hypothesis.

Effect of polynucleotide, coenzyme concentrate, and boiled S^{DPNH}: If the esterification of P^{32} by S^{DPNH} were dependent on a polynucleotide primer in the S^{DPNH}, then addition of polynucleotide or boiled S^{DPNH} to Pi-discharged S^{DPNH} would be

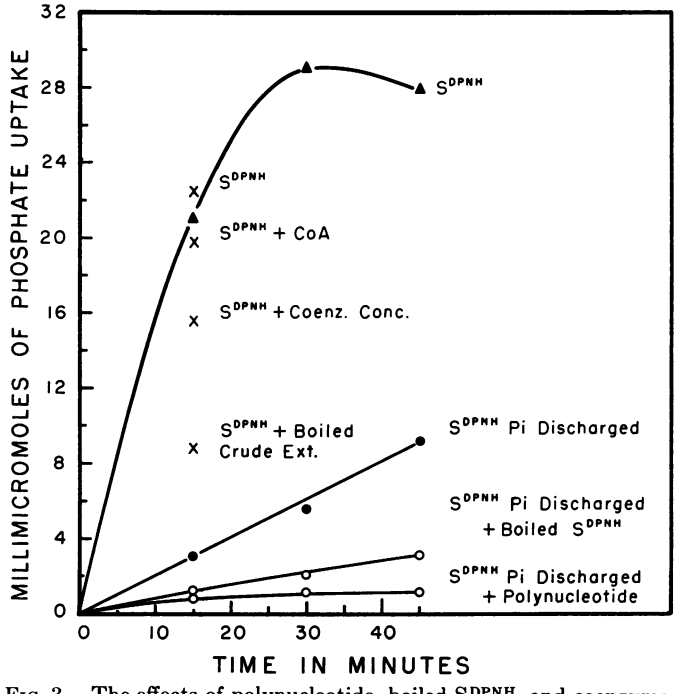

FIG. 3.—The effects of polynucleotide, boiled S^{DPNH}, and coenzymes on phosphorylation by S^{DPNH}.

expected to produce a stimulation of Pi esterification. Polynucleotide made from *Alcaligenes faecalis* washed particles,[2] which is required for oxidative phosphorylation by the complete system, produced a marked inhibition of P^{32} esterification when added to phosphate-discharged S^{DPNH}. Boiled S^{DPNH} had the same effect as shown in Figure 3. A crude liver coenzyme concentrate and boiled crude *Alcaligenes faecalis* extract, the latter also known to be active in oxidative phosphorylation, were both inhibitory when added to undischarged S^{DPNH} in a separate experiment shown by crosses in Figure 3. Coenzyme A was slightly inhibitory. These experiments provide clear evidence that the incorporation of P^{32} into the organic fraction by S^{DPNH} cannot be explained by the action of polynucleotide phosphorylase in the supernatant solution.

In order to demonstrate conclusively that S^{DPNH} contains a high energy intermediate, it was necessary to show net synthesis of ATP from ADP, Pi, and S^{DPNH}. The ATP assay with the firefly system seemed to be ideal for this since it is specific for ATP and is very sensitive. Dr. W. D. McElroy and Mrs. L. Geiger very kindly ran these assays.[16] It had already been found that S^{DPNH} contained small amounts of adenylate kinase as shown in a test using hexokinase, glucose-6-phosphate dehydrogenase, and TPN to assay for ATP with ADP as substrate. Using the firefly system to assay for ATP formed, experimental vessels contained Pi, ADP, and S^{DPNH}. The control vessels had no Pi added and thus measured ATP formation from ADP catalyzed by adenylate kinase. This value was subtracted from the value found in the vessel containing Pi to give net ATP synthesis corrected for adenylate kinase. These values were compared with the values obtained with the same S^{DPNH} solutions by the P^{32} assay with the results shown in Table 4. It will

TABLE 4

PHOSPHORYLATION BY S^{DPNH}
COMPARISON OF P^{32} UPTAKE AND ATP FORMATION

		30 sec			3 min		
Expt.	Assay	Total	Control	Δ	Total	Control	Δ
1	P^{32}	13.0	1.0	12.0	30	3.5	26.5
1	Firefly	14.7	3.7	11.0	2.9	1.5	1.4
1 repeat	Firefly	13.2	8.1	5.1	7.4	3.7	3.7
2	P^{32}	6.4	0.9	5.5	19.0	1.7	17.3
2	Firefly	7.2	2.2	5.0	2.4	...	...
2 repeat	Firefly	6.5	3.5	3.0	...	...	...
2 No NaF	Firefly	11.6	8.1	3.5	...	...	...
3	P^{32}	1.8	0.4	1.4	3.9	0.4	3.5
3	Firefly	1.1	0.5	0.6	...	...	...

Results are expressed as millimicromoles of Pi uptake or ATP formation with the total figure observed, followed by the control to be subtracted and under Δ the difference. In the P^{32} assay the control is phosphorylation by the Pi-discharged S^{DPNH}, and in the ATP assay it is phosphorylation without added Pi. All experiments run in presence of 0.05 *M* NaF except as indicated. Experiments 1 and 2 used lyophilized S^{DPNH} while in Experiment 3 dialyzed lyophilized S^{DPNH} was used.

be seen that there is good agreement between the results obtained in the two different ways in 3 separate groups of experiments at 30 sec. After this time the P^{32} assay continues to show an increase while the ATP value determined by firefly assay falls. This means that ATP is being converted to something else by some unspecified pathway of ATP utilization. There is a good reason why the ATP values are somewhat lower than the P^{32} figures even at 30 sec. This is explained by the fact that the lyophilized S^{DPNH} used contains some Pi. In the second group of experiments the actual phosphate concentration was measured in the reaction mixtures. The experimental vessel contained 0.616 μmole of Pi/ml while the control contained 0.153 μmole/ml. Thus, the adenylate kinase control vessels probably measured some ATP formation from the intermediate and the corrected values are therefore somewhat low.

Discussion.—These experiments are divided into two groups by method. In the first group, P^{32} incorporation into the organic fraction was used as the assay method, and in the second group, net synthesis of ATP was demonstrated using the firefly assay system. In order to show the accumulation of a high energy intermediate in the supernatant solution from the DPNH preincubation using P^{32} as an assay system, one must rule out other known reactions by which P^{32} can be incorporated into the organic phase. In this case these consist in P^{32}-ATP exchange

reactions, polynucleotide phosphorylase, and the unlikely one of soluble oxidative phosphorylation. The exchange reactions have been ruled out by showing that at completion of the reaction P^{32} incorporation is proportional to the amount of supernatant solutions used, that cold phosphate incubation before addition of P^{32} causes a marked inhibition of P^{32} uptake, and that ADP is more active than ATP. The lack of activity of ATP may be somewhat puzzling when it is remembered that there is ATPase present in the supernatant solution from DPNH preincubation of the enzyme complex. The rate of ATP splitting is so slow, however, that at the end of the reaction some 25 milliμmoles of ADP would be formed, while 50 milliμmoles of ADP added at the beginning of the experiment causes only a small increase in phosphorylation over the basal level as shown in Table 3.

The possibility that polynucleotide phosphorylase action might explain the P^{32} incorporation is ruled out by the fact that ADP causes a marked stimulation in P^{32} incorporation and that polynucleotide made from *Alcaligenes faecalis* is strongly inhibitory. Soluble oxidative phosphorylation has been ruled out on the basis of studies with inhibitors.

While these results with P^{32} incorporation are strongly suggestive that incubation of the enzyme system with DPNH produces an accumulation of an energy-rich intermediate of oxidative phosphorylation in the supernatant solution, they are not in themselves entirely conclusive, since they do not demonstrate net ATP synthesis. The experiments using the firefly assay demonstrate this clearly and, furthermore, are in close agreement at 30 sec with the results obtained with the P^{32} assay. The subsequent decrease in ATP with time, and the continued rise in P^{32} incorporation into the organic fraction, can only mean that the ATP formed by the reaction of Pi and ADP with the intermediate is being used by one or more of the many reactions by which the terminal phosphate of ATP is converted into other organic phosphate compounds in cells.

Summary.—(1) A soluble high energy intermediate of oxidative phosphorylation has been separated from phosphorylating particles during oxidation of DPNH.

(2) The intermediate has been assayed using P^{32} as a measure of inorganic phosphate uptake and by direct measurement of ATP formation.

(3) Alternative explanations of the observations have been examined and rejected.

Grant number E 2443-Cl from the National Institutes of Health and number G 7649 from the National Science Foundation made this work possible. The skillful technical assistance of Mr. Matthew Hormanski is gratefully acknowledged. It is a pleasure to acknowledge my debt to Dr. W. D. McElroy and Dr. C. A. Thomas for many helpful discussions and suggestions, as well as to Dr. E. Racker for similar though less extensive labors.

* Contribution Number 277 from McCollum-Pratt Institute and the Department of Biology, Johns Hopkins University, Baltimore, Maryland.

† The following abbreviations will be used: ATP, ADP, and AMP for adenosine tri, di, and monophosphates, DPNH and DPN for reduced and oxidized diphosphopyridine nucleotide, Pi for inorganic phosphate, S^{DPNH} for the supernatant solution recovered by centrifugation after preincubation of bacterial particles with DPNH, Hexo for hexokinase, Gluc. for glucose, DNP for 2,4 dinitrophenol.

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DIFFERENT ENZYMIC EXPRESSIONS OF MUTANTS OF HUMAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Communicated by Herman M. Kalckar, May 18, 1960

The recent advances in understanding the specificity of genes on metabolic pathways[1] and the action of genes on molecular structure of protein[2] provide an impetus for the study of more complex interactions of genes at the level of biochemical differentiation and quantitative regulation of enzymes. These latter features are especially apparent in mutants of higher forms of life, where the deletion of an enzymic activity may be incomplete in regard to quantity, anatomical distribution, or timing.[3–5]

Particularly adaptable to such inquiries [4, 6, 7] has been the hereditary condition of humans, primaquine-sensitive hemolytic anemia,[8, 9] a disorder characterized by an 85–95 per cent reduction in activity of glucose-6-phosphate dehydrogenase (G-6-PD*)

$$\text{G-6-P} + \text{TPN} \rightleftharpoons \text{6-PG} + \text{TPNH}$$

in erythrocytes,[10] and by a tendency for these erythrocytes to undergo destruction when an affected individual takes primaquine or certain aromatic drugs.

Previous reports have dealt with a comparison of the lability, chromatographic behavior, and catalytic parameters of partially purified G-6-PD from normal (N) and primaquine-sensitive (S) erythrocytes.[4, 11, 12] No significant difference was found between the G-6-PD from both sources. Those findings are here supplemented with the studies of the erythrocytic G-6-PD from a Caucasian male infant with congenital non-spherocytic hemolytic anemia (H), a chronic hemolytic disorder which recently has been noticed also to be associated, in some pedigrees, with an almost complete absence in activity of erythrocytic G-6-PD.[13, 14] Because of the different racial stock from which such patients come and because of the more severe