

THE RELATION OF A SOLUBLE FACTOR TO
PHOSPHORYLATION DURING THE OXIDATION OF REDUCED
NICOTINAMIDE ADENINE DINUCLEOTIDES

by

KARIN ANN SMITH

A THESIS

submitted to


OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF ARTS


June 1963

APPROVED:



Professor of Chemistry


In Charge of Major



Head of Department Chemistry



Chairman of School Graduate Committee



Dean of Graduate School

Date thesis is presented July 16, 1962

Typed by Jolene Wuest

ACKNOWLEDGMENT

The author wishes to express her gratitude to her professors, especially Dr. R. W. Newburgh and Dr. Vernon H. Cheldelin, for their invaluable help and guidance.

In addition, I wish to thank my family, fellow students, and colleagues of the Science Research Institute for their encouragement and helpful discussions.

Finally, I wish to express my appreciation to the Science Research Institute itself for making available the facilities necessary for the completion of this study.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
HISTORICAL BACKGROUND	3
METHODS AND MATERIALS	13
A. Methods	13
B. Materials	16
RESULTS	17
DISCUSSION	25
SUMMARY	29
BIBLIOGRAPHY	30
APPENDIX	35

THE RELATION OF A SOLUBLE FACTOR TO PHOSPHORYLATION DURING THE OXIDATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDES

INTRODUCTION

Previous studies in this laboratory have shown that the pentose cycle enzymes, the activity of which results in the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH_2)^{/1}, occur in the soluble fraction of kidney, liver, or heart tissue homogenates. (28, p. 89-96). Both cell-fractionation studies (17, p. 1-18) and studies of intact cells (5, p. 3044-3055) have shown that the enzymes of glycolysis, the activity of which results in the formation of reduced nicotinamide adenine dinucleotide (NADH_2), are also located in the soluble extramitochondrial portion of the cytoplasm.

-
- [1] Abbreviations used in this thesis are the following:
NAD--nicotinamide adenine dinucleotide
 NADH_2 --reduced nicotinamide adenine dinucleotide
NADP--nicotinamide adenine dinucleotide phosphate
 NADPH_2 --reduced nicotinamide adenine dinucleotide phosphate
ADP--adenosine diphosphate
ATP--adenosine triphosphate
DCPIP--dichlorophenolindophenol
ATPase--adenosine triphosphatase
Tris--2-amino-2-hydroxymethyl propane-1, 3 diol
RNA--ribonucleic acid
TCA--trichloroacetic acid
RLM--rat liver mitochondria
HMP--Keilin-Hartree respiratory particle preparation
DS--dialyzed soluble supernatant fraction from beef heart
BHM--beef heart mitochondria
EDTA--ethylenediaminetetraacetic acid

Although molecular oxygen is the terminal acceptor in the oxidation of metabolic intermediates, it has been repeatedly demonstrated that carefully isolated mitochondria from numerous sources are incapable of oxidizing exogenous NADH_2 . (20, p. 176). In the same manner, exogenous NADPH_2 appears to be excluded by the mitochondria. (7, p. 26).

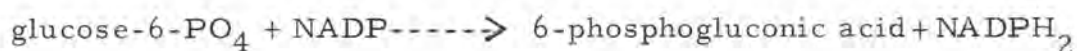
It has been shown in this laboratory, however, that addition of a soluble fraction permits the oxidative phosphorylation of the reduced dinucleotides by isolated beef heart mitochondria. (19, p. 771-779).

As the mechanism of action of the soluble fraction on the permeability of the mitochondria had not been elucidated, it seemed desirable to investigate this subject more completely. In particular, it was of interest to try to establish whether the dinucleotide actually traversed the mitochondrial boundary or reduced some component of the soluble fraction itself which was then capable of acting as a substrate for oxidative phosphorylation via the electron transport system.

HISTORICAL BACKGROUND

In glycolysis, the oxidation of glyceraldehyde-3-phosphate to 1, 3-diphosphoglyceric acid involves the formation of reduced nicotinamide adenine dinucleotide. Since this cofactor is present in only catalytic amounts, some mechanism for the regeneration of the oxidized dinucleotide must exist.

Similarly, the pentose cycle involves the formation of reduced nicotinamide adenine dinucleotide phosphate in the reactions:



Again, the dinucleotides, present only in catalytic quantities, must be regenerated in the oxidized form.

The enzymes responsible for these reactions, namely (1. 21. 12) glyceraldehyde phosphate dehydrogenase, (1. 11. 43) glucose-6-phosphate dehydrogenase, and (1. 11. 38) 6-phosphogluconate dehydrogenase, as well as the other enzymes of glycolysis and the pentose cycle have been shown to be located in the extramitochondrial portion of the cell cytoplasm. (28, p. 89-96) and (17, p. 1-18).

Several authors have reported the existence of a cytoplasmic mitochondrial barrier, which is defined as an inability of the enzymes of the intact mitochondria to act on specific substrates in the

in the cytoplasm. (14, p. 275-286). In particular, the existence of a barrier to NAD and NADH_2 has been repeatedly established, though at times some questions have been raised with regard to NADP and NADPH_2 . (1, p. 303-315). However, most authors consider the mitochondria to exhibit a similar barrier for all four dinucleotides. Mitochondria from various tissue sources, including heart, liver, kidney, and various tumor cells, have been shown to exhibit such a barrier. (2, p. 1499). These results have been reported not only in cell fractionation studies, but also in studies of intact cells. (5, p. 3044-3050). The function of such a barrier is open to speculation, and it has at various times been mentioned as a possible control mechanism. Little or no evidence is available, however, as to the true function.

It has been repeatedly emphasized that the electrons from the reduced dinucleotide may be carried indirectly to the electron transport system, rather than by transport of the dinucleotide itself. Theoretically, any extramitochondrial substance which can be reduced by an NAD-linked reaction to yield a product utilizable as a substrate for electron transport can serve to mediate electron transfer. At least three such systems have been reported.

Bücher, et al., (4, p. 552-570), Sacktor (33, p. 138-152), and others have shown the existence of a glycerophosphate "shuttle".

Thus, dihydroxyacetone phosphate is reduced extramitochondrially to α -glycerophosphate with concomitant oxidation of NADH_2 . The α -glycerophosphate, permeable to the mitochondrial barrier, serves as a substrate for mitochondrial electron transport, being oxidized to dihydroxyacetone phosphate. This " α -glycerophosphate pathway" may be of special importance in insect flight muscle.

Similarly, Devlin and Bedell (10, p. 564-566) have reported a β -hydroxybutyrate "shuttle". The pyridine nucleotide-linked reduction of acetoacetate to β -hydroxybutyrate provides the necessary substrate for electron transport that can cross the mitochondrial barrier.

More recently, Schäfer and Lamprecht (35, p. 259-287) have reported another such "shuttle". Their system involves the extramitochondrial NADH_2 -linked reduction of glyoxylate to glycolate. Glycolate then may serve as a substrate for electron transport and is reconverted to glyoxylate within the mitochondria by their purified enzyme, 2-hydroxyacid dehydrogenase.

Several other pathways for the oxidation of cytoplasmic (extramitochondrial) NADH_2 also have been suggested. Kaplan et al. (20, p. 1-16) reported the occurrence of a NAD--NAD transhydrogenase in cell homogenates from several sources and suggested that it might

mediate the oxidation of extramitochondrial NADH_2 . However, Stein, Kaplan, and Ciotti (38, p. 979-986) reported that the amount of NAD--NAD transhydrogenase was higher in digitonin particles, indicating that it is probably an intramitochondrial enzyme.

Ernster has reported the purification (11, p. 88-92) of a soluble enzyme from liver homogenates which he calls "DT Diaphorase". In 1960, he and Conover (7, p. 26-30) showed that the enzyme, stimulated by Vitamin K_3 and serum albumin, could catalyze the oxidation and phosphorylation of extra-mitochondrial NADPH_2 by mitochondria. The enzyme is nonspecific for NADH_2 or NADPH_2 with DCPIP as an acceptor. More recently, however, he has shown the enzyme to be identical with Vitamin K reductase. (9, p. 155-158). These studies indicate that the enzyme is not involved in the coupling of oxidative phosphorylation and is not an obligatory link in electron transport. Thus, the status of the enzyme's participation in the phosphorylation of extramitochondrial nicotinamide adenine dinucleotides has also been subjected to some doubt.

At least one other pathway exists for the oxidation of extra-mitochondrial NADH_2 , namely the reduction of pyruvate to lactate. This reaction simultaneously results in regeneration of the oxidized dinucleotide. The generation of lactic acid is, however, one of the few known metabolic "dead-ends", and it is difficult to reason that

this pathway provides sufficient oxidized dinucleotide for other metabolic processes.

The involvement of a soluble factor in oxidative phosphorylation is not a new idea. Such factors have most often been postulated to play a role in the coupling of phosphorylation to oxidation of various substrates and less often to take part in the fate of reduced nicotinamide adenine dinucleotides. Soluble compounds which have been suggested as important in oxidative phosphorylation include RNA, quinones, ATPase, the ADP--ATP exchange enzyme, and several other less well-defined factors. (27, p. 323-400).

The more undefined soluble factors have usually been isolated from cytoplasmic material. They may, in fact, be categorized as having originated in either the mitochondrial or the non-mitochondrial cytoplasm.

Extramitochondrial soluble factors have been reported of both a protein and non-protein nature. Pinchot, in particular, has suggested that from extracts of Alcaligenes faecalis, three components of the phosphorylating NADH_2 oxidase system may be obtained. (29, p. 1-9). Associated with the particulate NADH_2 oxidase is found a heat-stable factor which stimulates oxidative phosphorylation of NADH_2 by the oxidase in the presence of a heat-labile factor. The heat-stable

factor has been shown to be an adenine, guanine, and uracil-containing polynucleotide. (37, p. 257-264). He suggests that this polynucleotide may function as a coenzyme of oxidative phosphorylation.

Similar soluble factors have been observed by other workers with bacterial systems. The problem, in general, is that most bacteria do not possess so well-defined mitochondria as do higher animals and plants. Thus, it is difficult to say which factors are "mitochondrial" and which are "soluble" and/or extramitochondrial.

The work of Brodie and Gray is an example of the type of bacterial system obtained. (3, p. 534-537). After brief sonic digestion, they obtained particles similar to mitochondria that possessed a high degree of structural organization and lost their activity rather quickly in vitro. Soluble factors had to be added to the particles before oxidative phosphorylation could be observed.

Non-bacterial systems have also yielded extramitochondrial soluble factors concerned with oxidative phosphorylation. In investigating the respiratory activity of housefly sarcosomes, Slater and Van den Bergh found that a supernatant fraction from the thoracic muscle stimulated respiratory rates 10 to 20-fold when added to the reaction medium. (40, p. 362-371). The supernatant factor also established respiratory control with ADP and enabled the

attainment of high P:O ratios in the absence of serum albumin. They were able to show that the supernatant effect was due to the formation of rapidly oxidizable substrates, particularly pyruvate and α -glycerophosphate, from carbohydrate sources in the supernatant. They also found that the supernatant fraction seemed to alter the permeability barriers for these substrates in the sarcosomes.

Perhaps even more work has been done on the second type of soluble factor, namely that obtained from the mitochondrion itself. It was shown very early (17, p. 11) that the mitochondria release about 60% of their total nitrogen, mostly as soluble proteins, into solution upon disruption. Since that time, fractionation studies of isolated mitochondria have led to the discovery of several soluble factors, a few of which are capable of re-coupling oxidative phosphorylation when added back to the particulate enzyme fragments obtained simultaneously. Fragmentation is usually achieved via sonic digestion, vigorous shaking, or treatment with agents such as digitonin.

For example, Racker (31, p. 323-400) has prepared two particulate and two soluble fractions from mechanically disrupted mitochondria. His Coupling Factor I has ATPase activity and couples phosphorylation to the oxidation of various substrates by

Residue I. The Coupling Factor II has no ATPase activity but will, when combined with Coupling Factor I, couple phosphorylation by Residue II.

In 1960, Linnane and Titchener (24, p. 469-476) reported the separation of the electron transport particle of Green (ETP) into two components. In the presence of magnesium ion, these two factors would re-constitute oxidative phosphorylation. The soluble factor proved to be identical to Racker's Coupling Factor II.

Lehninger has used digitonin to produce sub-mitochondrial particles capable of oxidative phosphorylation. In addition, he has prepared at least three soluble factors involved either in oxidative phosphorylation or in the shrinking and swelling of mitochondria. Soluble factor R (22, p. 2459-2464) is a protein factor which releases mitochondria from their dependence upon a phosphate acceptor, but does not uncouple oxidative phosphorylation. Soluble factor U is heat stable, uncouples oxidative phosphorylation, and causes mitochondrial swelling. Finally, soluble factor C (23, p. PC 8-9), which leaks from swollen mitochondria, is capable of causing the contraction of mitochondria in the process of swelling.

In this laboratory, it was learned that a soluble fraction prepared from beef heart muscle homogenates was required for the coupled oxidative phosphorylation of nicotinamide adenine

dinucleotides by isolated beef heart mitochondria. (6, p. 1766-1767). Antimycin A and dinitrophenol proved to be effective inhibitors of the reaction (19, p. 771-779), indicating that the oxidation and phosphorylation indeed proceed via the electron transport system of the mitochondria.

In early experiments, some reaction was obtained with the soluble fraction, hexokinase, glucose, ADP, nicotinamide adenine dinucleotide, in the absence of the mitochondria. The oxidation was shown by the use of glycolytic inhibitors (19) to be due to glycolysis. Thus, in later experiments 2-deoxy-D-glucose was used in place of glucose in the phosphate trapping system. This effectively prevented observable glycolytic activity.

Flavin adenine dinucleotide, cytochrome c, albumin, vitamin K, and vitamin B₁₂ were also tested for activity with the nicotinamide adenine dinucleotide substrates. Results indicated that these compounds are not the active components of the soluble fraction. The absence of pyridine nucleotide transhydrogenase from the soluble fraction had also been reported. (19).

The manner in which these several substrates could be made available to the electron transport system was, however, not clear. It seemed of particular importance to elucidate the role of the soluble

fraction itself. Therefore, experiments were begun to try to find the answers to some of the basic questions proposed. For example, what is the chemical nature of the essential component(s) of the soluble fraction? What is the locus of action of the soluble fraction? That is, does the fraction react first with the substrate or with some part of the mitochondrial system? And finally, what characteristic property may account for the apparent non-specificity of the reaction, that is, in transforming both the oxidized and reduced forms of the nicotinamide adenine dinucleotide into available substrates?

METHODS AND MATERIALS

Methods

Beef heart mitochondria were prepared by the method of Hogeboom and Schneider (16, p. 513-519) as modified by Crane et al. (8, p. 475-487) with 0.25 M sucrose plus 1.85 g. KH_2PO_4 per liter. The pH of the heart homogenate was adjusted to pH 7.4 with 20% KOH. The mitochondria were washed twice with 0.25 M sucrose and suspended in a solution of 0.25 M sucrose. The preparations were frozen immediately after preparation and thawed just before use. Phosphorylating activity decreased with time, and the mitochondria were discarded after ten days to two weeks of storage.

Beef liver mitochondria were prepared from fresh beef liver, using essentially the same method as for the beef heart mitochondria. However, the liver tissue was chopped into approximately two cm. squares. Homogenization in the Waring Blendor was for 30 seconds at 3/4 the speed used for beef heart mitochondria. These mitochondria were also suspended in sucrose and frozen after washing, however, most of the phosphorylating activity disappeared within three days. These mitochondria proved to be quite "intact", that is, they did not oxidize or phosphorylate exogenous NADH_2 to any measurable extent.

Rat liver mitochondria were prepared by the method of Meyers and Slater (27, p. 558). Adult female white rats were killed by decapitation, and the livers were removed for immediate preparation of mitochondria. The mitochondria were suspended in 0.25 M sucrose, and the activity was assayed within an hour after preparation.

The soluble fraction was prepared by homogenizing 100 g. of fresh beef heart in 200 ml. of 0.9% KCl plus 0.001 molar EDTA in a Waring Blendor. The pH was adjusted to 7.4 with 20% KOH. The homogenate was centrifuged at 1000 x g for ten minutes; the supernatant layer was removed and centrifuged three hours at 25,000 x g. The supernatant liquid was dialyzed 24 hours against distilled water and then 24 hours against 0.02 M Tris buffer, pH 7.4, prior to use. The material precipitated during dialysis against Tris buffer was then removed by centrifugation for 15 minutes at 15,000 x g. This precipitated protein material showed similar activity to the soluble fraction if assayed immediately, but the activity was destroyed within 24 hours and may have been due to adsorbed soluble fraction. Dialysis up to 96 hours did not destroy the activity of the soluble fraction. The dialyzed supernatant fraction was referred to as "dialyzed soluble" (DS) and was stored frozen. The activity of the dialyzed soluble fraction was retained unimpaired for two to three months. Protein content was usually approximately 20 mg/ml, as assayed by the biuret method.

Oxidation was measured by conventional manometry. The ATP trapping system contained excess ADP, hexokinase, and 2-deoxy-D-glucose in 0.1 M potassium phosphate buffer, pH 7.4. The reaction was stopped and deproteinized by the addition of 0.5 ml. of 30% TCA, and an aliquot was removed for the phosphorus determination.

Phosphorylation was measured colorimetrically by the method of Fiske and SubbaRow.

The oxidation and reduction of the nicotinamide adenine dinucleotides was sometimes measured spectrophotometrically at 340 m μ . Measurements were made on the Beckman DU spectrophotometer equipped with a Gilford optical density converter and automatic cuvette positioner. This enabled the simultaneous measurement and recording of four reactions, for more accurate comparisons.

As shown by Tedeschi and Harris (39, p. 52-67), isolated mitochondria obey the osmotic law, and mitochondrial volume can be correlated to changes in optical density at 520 m μ . The measurements of mitochondrial swelling were also made simultaneously on four samples by using the Beckman DU spectrophotometer and the Gilford automatic cuvette positioner. The standard incubation medium used for oxidative phosphorylation assay (0.25 M sucrose, 2.3×10^{-3} M ADP, hexokinase, .012 M 2-deoxy-D-glucose, and 0.1 M phosphate buffer, pH 7.4) was used in the swelling experiments.

The oxidized and reduced nicotinamide adenine dinucleotides were also measured fluorometrically according to the procedure of Lowry et al. (25, p. 1047-1063). The reactions were stopped by centrifugation at 14,000 x g for five minutes to precipitate the mitochondria. Supernatant layers were decanted, and aliquots were used for assay of NAD and NADH₂. For extraction of the intramitochondrial dinucleotides, the mitochondria were mashed in a glass homogenizer for three minutes in 0.025 M Tris buffer, pH 8.0, at 85° C. The homogenates were centrifuged at 12,000 x g for one hour. Aliquots of the supernatant were used for nicotinamide adenine dinucleotide assay. Measurements were made on a Farrand Model "A" Fluorometer with primary Corning filter 5860 and secondary filters 5562 and 4308. Working standards of 0.05 to 0.1 µg. quinine in 0.1 N H₂SO₄ were used.

Materials

The NAD, NADP, NADH₂, NADPH₂, yeast hexokinase, 2-deoxy-D-glucose, and ADP were obtained from Sigma Chemical Company. Solutions of NADH₂ were prepared immediately before use to reduce as much as possible the type of deterioration reported on storage by Fawcett et al. (12, p. 210-212).

RESULTS

Several years ago in our laboratory we found that the addition of the soluble fraction prepared from beef heart muscle homogenates will permit oxidative phosphorylation when exogenous NADH_2 is a substrate. Mitochondria which are not "intact", i. e. will oxidize exogenous NADH_2 to some extent, show a stimulated rate of oxidation and phosphorylation in the presence of the soluble fraction.

In the absence of the mitochondria, however, the soluble fraction has no effect upon the nicotinamide adenine dinucleotide. Incubation of the soluble fraction with both NAD and NADH_2 in the complete phosphorylation medium results in no change in the optical densities at 340 m μ .

The rate of phosphorylation obtained is proportional to the amount of soluble fraction present, as is shown in Figure 1.

The soluble fraction prepared from beef heart muscle homogenate exhibits the same effect on intact mitochondria isolated from beef heart, rat liver, or beef liver.

It is not strictly valid to quantitatively compare the rate of disappearance of a given substrate from one mitochondrial preparation to another, as the oxidative activity of mitochondrial preparations may vary widely from one preparation to another and

with the same preparation after storage for only a few hours at 0° C. Qualitative differences remain constant, however, and may thus be compared. For example, the soluble fraction always stimulates the rate of NADH_2 oxidation, but this rate is more dependent upon the oxidative activity of the mitochondrial preparation than on any other factor.

It should be pointed out that the preparation of "intact" mitochondria is difficult, and very often a slow rate of NADH_2 oxidation is observed even in the absence of the soluble fraction. The rate of NADH_2 oxidation is always considerably below that of succinate oxidation in such preparations, though they may be considered as slightly damaged. Mitochondrial preparations damaged to the extent that they no longer couple phosphorylation to the oxidation of substrates as pyruvate or succinate are not used for assay.

To account for such variability in preparations, a control sample containing no soluble fraction has routinely been tested simultaneously with each experiment. The reaction rate in the absence of soluble fraction was then subtracted from the rate in its presence.

Figure 2 also illustrates the dependence of the rate of oxygen uptake upon the amount of NADH_2 present. The amount of substrate required varied with the activity of the mitochondrial preparation. With a very active preparation, the reaction rates in the presence of

8, 10, and 12 μ moles nicotinamide adenine dinucleotide were found to be identical. Ten μ moles was usually considered to be a sufficiently excess substrate concentration.

The rate of oxidation and/or phosphorylation can be measured during the course of the incubation period. Curves such as those shown in Figures 3, 4 and 5 are obtained.

If the incubation is continued for more than about fifteen minutes, the rate drops off sharply, particularly with the more fragile liver mitochondria as is seen in Figure 3. This is presumably due to lysis and damage of the intact mitochondria. Such lysis of rat liver mitochondria has often been observed (32, p. 376-378) and can be prevented by resuspending the mitochondria in fresh substrates.

As the permeability of mitochondria has been shown to be related to osmotic pressure, (39, p. 52-67) the effect of the sucrose concentration of the incubation medium was studied. Results are shown in Figure 6. A maximum amount of phosphorylative activity was observed with 0.25 M sucrose. This was true for a substrate such as pyruvate as well as with NADH_2 plus the soluble fraction.

To investigate the effect of the soluble fraction on the structural integrity of the mitochondrion, the swelling or shrinking was measured optically at 520 m μ . Results are shown in Figure 7.

The soluble fraction did not itself cause the mitochondria to swell (or shrink). Some swelling was observed with NADH_2 alone; more swelling was observed with NADH_2 plus the soluble fraction. Some shrinkage of the mitochondria was seen with NAD, both in the presence and absence of the soluble fraction. Inorganic phosphate and malate were used as positive controls to demonstrate mitochondrial swelling. In summary, some swelling was observed upon incubation of the complete system, but the effect of the soluble fraction itself was not to make the mitochondria swell.

Preparations of the soluble fraction contain from 20-25 mg protein/ml. The heat stability of the soluble fraction was investigated as is shown in Figure 8.

Removal of the protein by heat denaturation destroys the activity of the soluble fraction. If the heat-treated protein is separated from its supernatant fraction by centrifugation, the supernatant portion remains capable of stimulating the action of the original soluble fraction. The supernatant itself, however, is not able to bring about oxidative phosphorylation of NADH_2 by intact mitochondria.

The effect of the soluble fraction on the activity with other substrates was also investigated. As exogenous isocitrate, citrate, NAD, and NADH_2 , have all been shown unavailable to isolated mitochondria (14, p. 275-286), these four were studied.

Isocitrate and citrate utilization seemed not to be consistently affected by the addition of the soluble fraction.

On the other hand, NAD in the presence of soluble fraction seemed to be a very good substrate for the reaction. In order to elucidate possible differences in the reactions with NAD and NADH_2 , simultaneous measurements were made of the activity with the two substrates. Both oxidative and phosphorylative reactions were measured during the course of the incubation, yielding the results shown in Figures 9 and 10.

It is seen that with the more active mitochondrial preparation in Figure 9, the oxygen consumption with NAD as substrate began to reach a plateau earlier than it did with NADH_2 . The manometric method of measurement used did not permit measurement of differences during the first five minutes. In Figure 10, the phosphorylation measurements show the rate with NAD to lag behind that with NADH_2 and then both rates reach a plateau at approximately the same time. The mitochondrial preparation was five days old in the experiment for Figure 10, thus the longer-than-usual lag period was to be expected. The earlier plateau in phosphorylative rates can probably be attributed to lysis, as the aged preparation would be expected to be more fragile.

Simultaneous measurements of the rates of oxidation of NAD and NADH_2 in the presence and absence of the soluble fraction were made in the Beckman DU spectrophotometer equipped with Gilford automatic cuvette positioner. Measurements were complicated somewhat by the high turbidity of the mitochondrial assay system, but the method did permit better evaluation of the system during initial reaction times. Results are shown in Figures 11 and 12.

Oxidation of nicotinamide adenine dinucleotide was observed with both NAD and NADH_2 as substrate. The rate of oxidation was more rapid with NADH_2 . Reduction of the oxidized substrate was not observed.

With both the oxidized and reduced compounds as substrates, the reaction seen is that of oxidation.

Because of the important role of succinate as a substrate for the mitochondrial respiratory chain, the effect of the soluble fraction on succinate oxidation was determined. The effect was tested with both a coupled mitochondrial preparation and a Keilin-Hartree respiratory particle preparation.* Results are shown in Figures 13 and 14.

The Keilin-Hartree particle is a non-phosphorylating respiratory particle; thus no effect on phosphorylative ability could be measured.

*Kindly supplied by Dr. T. E. King.

(The soluble fraction did not restore phosphorylative ability to the particles.)

As seen in the figures, the soluble fraction is slightly stimulatory to succinate oxidation with the Keilin-Hartree particle, but exhibits a 15-20% inhibition of mitochondrial succinate oxidation.

A fluorescent method was also used to determine the incorporation of the reduced nicotinamide adenine dinucleotide into the mitochondria. Results are summarized in Table I. At the same time, duplicate samples were assayed for phosphorylative activity. Results are given in Table II.

Table I also shows the results obtained in a similar experiment in which the incorporation of the oxidized dinucleotide was measured. A simultaneous measurement of phosphorylative activity is shown in Table II. It is to be noted that the mitochondrial preparation was less active in this experiment than in the one with NADH_2 .

With the oxidized dinucleotide added as substrate, NADH_2 is found in the supernatant fraction after completion of the reaction. This occurs both in the presence and absence of the soluble fraction, but lends support to the idea that NADH_2 is the actual substrate for the oxidative and phosphorylative reactions.

There is a definite increase in the NAD content of the soluble fraction-treated mitochondria after incubation with either of the two dinucleotides. The effect is particularly pronounced when NAD is used as the substrate.

DISCUSSION

The data presented do not suggest a mechanism for the action of the soluble fraction.

Although the observed time lag at first made the idea of some type of "shuttle" very inviting, the total lack of substrate oxidation in the absence of the mitochondria, as well as the lack of activity of the DS as a substrate in the absence of NAD or NADP, seem to preclude this possibility. It should be noted, of course, that any slow or rate-limiting reaction could give rise to the observed lag period--not only a reaction of the soluble fraction itself. The heat lability of the soluble fraction, indicating an active protein component, makes an enzymatic process seem quite likely.

The soluble fraction does not provide the same sort of additional substrate as does the supernatant fraction of Slater (cf. p. 10 this thesis). Slater's fraction, in contrast to ours, is capable of acting as a substrate to the mitochondrion without any further additions. Our fraction has been so extensively dialyzed that any small molecular weight substrates would seemingly have been removed.

The apparent anomaly that both oxidized and reduced compounds were serving as substrates for electron transport has been resolved

by the fluorometric data which show that the NAD is reduced extra-mitochondrially if both DS and mitochondria are present.

Alteration of a permeability barrier remains an enticing possibility. This seems to be supported by the fluorometric data indicating an increased amount of oxidized dinucleotide in the mitochondria if the soluble fraction is present. The lack of swelling observed with the soluble fraction does not contradict this idea. It has been generally recognized for some time that the phosphorylative ability of mitochondria is closely related to their structural integrity. In recent studies on the uncoupling of oxidative phosphorylation, however, Sanadi (13, p. 276) has been able to show that structural modification--at least that measurable at 520 m μ --is independent of and follows uncoupling. Thus, if one assumes the reverse to be true, that coupling does not necessarily provoke an observable structural change, the lack of DS-induced swelling is not at all surprising.

In retrospect, why do isolated mitochondria exclude exogenous NADH₂ and NADPH₂ as substrates? The question is a fascinating one, and the answers which have been suggested are numerous and varied. Racker (31, p. 323-400) has suggested that this lack of efficient utilization "can in fact be looked upon as a precautionary measure by nature to preserve the important and multifunctional reduced pyridine

nucleotides for synthetic processes." Green, (15, p. 73-130) in commenting upon the observed phenomena that whereas undamaged mitochondria oxidize NADH_2 slowly or not at all, mitochondria damaged by any one of several pretreatments are quite capable of its oxidation, suggests that sites are occupied in the undamaged mitochondria. He considers the sites at which the exogenous dinucleotide could be reduced to be occupied by internal nucleotide which must be reversibly ejected in order to make the sites available. Lehninger (21, p. 176) further suggests that because the mitochondria oxidize intramitochondrial NADH_2 very rapidly, they become impermeable to NADH_2 .

The data given in this thesis do not permit an answer to this question. In addition, one must be especially careful in giving in vivo significance to data obtained with isolated portions of an organism, such as mitochondria. As Schneider points out (36, p. 1-72), it is always possible that the phenomena observed with isolated mitochondria are the result of a synergistic action between the mitochondria and contaminating cytoplasm. It could, of course, also be true that the cytoplasm is not at all "contaminating", but represents the natural reaction. However, even with these reservations well in mind, these data fit others (cf. Slater) to suggest that perhaps natural extramitochondrial enzymes or cofactors are capable of altering the apparent

permeability barrier of mitochondria. In this way, then, extramitochondrial nicotinamide adenine dinucleotide may be made available to the electron transport system.

SUMMARY

The beef heart muscle dialyzed soluble fraction is necessary for the oxidative phosphorylation of exogenous nicotinamide adenine dinucleotide by mitochondria from beef heart, beef liver, and rat liver.

The soluble fraction is heat labile, but contains a stimulatory heat-stable component.

Mitochondrial swelling is not induced by the soluble fraction.

Both NAD and NADH_2 can serve as substrates for the reaction, but the NAD is reduced extramitochondrially in the presence of the DS and the mitochondria. The soluble fraction itself cannot reduce the oxidized NAD.

More oxidized NAD is found within the mitochondria in the presence of the dialyzed soluble fraction with both NAD and NADH_2 as a substrate.

BIBLIOGRAPHY

1. Birt, L. M. and W. Bartley. The distribution and metabolism of intra- and extra-mitochondrial pyridine nucleotides in suspensions of liver mitochondria. *Biochemical Journal* 75:303-315. 1960.
2. Boxer, George E. and Thomas M. Devlin. Pathways of intracellular hydrogen transport. *Science* 134:1495-1501. 1961.
3. Brodie, Arnold F. and Clarke T. Gray. Bacterial particles in oxidative phosphorylation. *Science* 124:534-537. 1957.
4. Bücher, T. and M. Klingenberg. Wege des Wasserstoffs in lebendigen Organisation. *Angewandte Chemie* 70:552-570. 1958.
5. Chance, Britton and Bo Thorell. Localization and kinetics of reduced pyridine nucleotide in living cells by microfluorometry. *Journal of Biological Chemistry* 234:3040-3050. 1959.
6. Cheldelin, Vernon H., Carl Widmer, Sudha Joshi, and R. W. Newburgh. Phosphorylation coupled with pyridine nucleotide oxidation. *Journal of the American Chemical Society* 79:1766-1767. 1957.
7. Conover, Thomas E. and Lars Ernster. Mitochondrial oxidation of extramitochondrial TPNH mediated by DT diaphorase. *Biochemical and Biophysical Research Communications* 2:26-30. 1960.
8. Crane, F. L., J. L. Glenn and D. E. Green. Studies on the electron transfer particle. IV. The electron transfer particle. *Biochimica et Biophysica Acta* 22:475-487. 1956.
9. Danielson, Lennart and Lars Ernster. Lack of relationship between mitochondrial oxidative phosphorylation and the dicumural-sensitive flavoenzyme "DT Diaphorase" or vitamin K reductase. *Nature* 194:155-158. 1962.

10. Devlin, Thomas M. and Bernice H. Bedell. Stimulation by acetoacetate of DPNH oxidation by liver mitochondria. *Biochimica et Biophysica Acta* 36:564-566. 1959.
11. Ernster, Lars and Margareta Ljunggren. Purification and some properties of a highly dicumarol-sensitive liver diaphorase. *Biochemical and Biophysical Research Communications* 2:88-92. 1960.
12. Fawcett, C. P., M. M. Ciotti and N. O. Kaplan. Inhibition of dehydrogenase reactions by a substance formed from DPN. *Biochimica et Biophysica Acta* 54:210-212. 1961.
13. Fluharty, Arvan L. and D. R. Sanadi. On the mechanism of oxidative phosphorylation. IV. Mitochondrial swelling caused by arsenite in combination with 2, 3-dimercaptopropanol and by cadmium ion. *Biochemistry* 1:276-281. 1962.
14. Green, David E. and F. L. Crane. Structure of the mitochondrial electron transport system. In: *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo, Tokyo, Pan Pacific Press, 1958.* p. 275-286. (I.U.B. Symposium Series, vol. 2.)
15. Green, David E. Electron transport and oxidative phosphorylation. *Advances in Enzymology* 21:73-130. 1959.
16. Hogeboom, George H. and Walter C. Schneider. Cytochemical studies. IV. Physical state of certain respiratory enzymes of mitochondria. *Journal of Biological Chemistry* 194:513-519. 1952.
17. Holter, H. Localization of enzymes in cytoplasm. *Advances in Enzymology* 13:1-18. 1952.
18. International Union of Biochemistry. Commission on Enzymes. Report. New York, Pergamon Press, 1961. (I.U.B. Symposium Series, vol. 20).
19. Joshi, Sudha, R. W. Newburgh, and Vernon H. Cheldelin. Oxidation of pyridine nucleotides coupled to phosphorylation. *Journal of Biological Chemistry* 229:771-779. 1959.

20. Kaplan, Nathan O., Sidney P. Colowick and Elizabeth F. Neufeld. Pyridine nucleotide transhydrogenase. III. Animal tissue dehydrogenases. *Journal of Biological Chemistry* 205: 1-16. 1953.
21. Lehninger, A. L. Oxidative phosphorylation. In: *The Harvey Lectures*, vol. 49, New York, Academic Press Inc., 1955. p. 176.
22. Lehninger, A. L. and LeMar F. Remmert. An endogenous uncoupling and swelling agent in liver mitochondria and its enzymic formation. *Journal of Biological Chemistry* 234: 2459-2464. 1959.
23. Lehninger, Albert L. and Gerald S. Golterer. A soluble protein required in mitochondrial contraction. Leakage of active factors from mitochondria. *Journal of Biological Chemistry* 235: PC8-9. 1960.
24. Linnane, Anthony B. and Edward B. Titchener. Studies on the mechanism of oxidative phosphorylation. VI. A factor for coupled oxidation in the electron transport system. *Biochimica et Biophysica Acta* 39:469-476. 1960.
25. Lowry, Oliver H., Nira R. Roberts, and Joyce I. Kapphahn. The fluorometric measurement of pyridine nucleotides. *Journal of Biological Chemistry* 224:1047-1063. 1957.
26. Maley, Gladys Feldott. Phosphorylation associated with the oxidation of reduced DPN by rat liver mitochondria. *Journal of Biological Chemistry* 224:1029-1038. 1957.
27. Meyers, D. K. and E. C. Slater. The enzymic hydrolysis of adenosine triphosphate by liver mitochondria. I. Activities at different pH values. *Biochemical Journal* 67:558-571. 1957.
28. Newburgh, R. W. and Vernon H. Cheldelin. Intracellular distribution of pentose cycle activity in rabbit kidney and liver. *Journal of Biological Chemistry* 218:89-96. 1956.

29. Pinchot, Gifford B. A polynucleotide coenzyme of oxidative phosphorylation. *Archives of Biochemistry and Biophysics* 94:257-264. 1961.
30. Purvis, J. L. Pyridine nucleotides in rat-liver mitochondria. I. The demonstration of a new form of diphosphopyridine nucleotide and triphosphopyridine nucleotide. *Biochimica et Biophysica Acta* 38:435-446. 1960.
31. Racker, Efraim. Mechanism of synthesis of adenosine triphosphate. *Advances in Enzymology* 23:323-400. 1961.
32. Roodyn, D. B. and J. W. Suttie. Prolonged maintenance of oxidative phosphorylation in rat liver mitochondria. *Biochimica et Biophysica Acta* 52:376-378. 1961.
33. Sacktor, B. A biochemical basis of flight muscle activity. In: IV. International Congress of Biochemistry, vol. 12, Vienna, 1958. p. 138-152.
34. Sacktor, B. Pathways of hydrogen transport in flight muscle. In: XI. International Kongress für Entomologie, Wien, 1960. p. 180-183. (Symposium 3: Insect Chemistry).
35. Schäfer, Günter and Walther Lamprecht. 2-Hydroxysäure-dehydrogenase aus Lebermitochondrien. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* 326:259-287. 1961.
36. Schneider, Walter G. Mitochondrial metabolism. *Advances in Enzymology* 21:1-72. 1959.
37. Shibko, S. and Gifford B. Pinchot. Fractionation of the polynucleotide coenzyme of oxidative phosphorylation. *Archives of Biochemistry and Biophysics* 94:257-264. 1961.
38. Stein, Abraham M., Nathan O. Kaplan, and Margaret M. Ciotti. Pyridine nucleotide transhydrogenase. VII. Determination of the reactions with coenzyme analogues in mammalian tissues. *Journal of Biological Chemistry* 234:979-986. 1959.

39. Tedeschi, Henry and Daniel L. Harris. The osmotic behavior and permeability to non-electrolytes of mitochondria. *Archives of Biochemistry and Biophysics* 58:52-67. 1955.
40. Van den Bergh, S. G. and E. C. Slater. The respiratory activity and permeability of housefly sarcosomes. *Biochemical Journal* 82:362-371. 1962.

APPENDIX

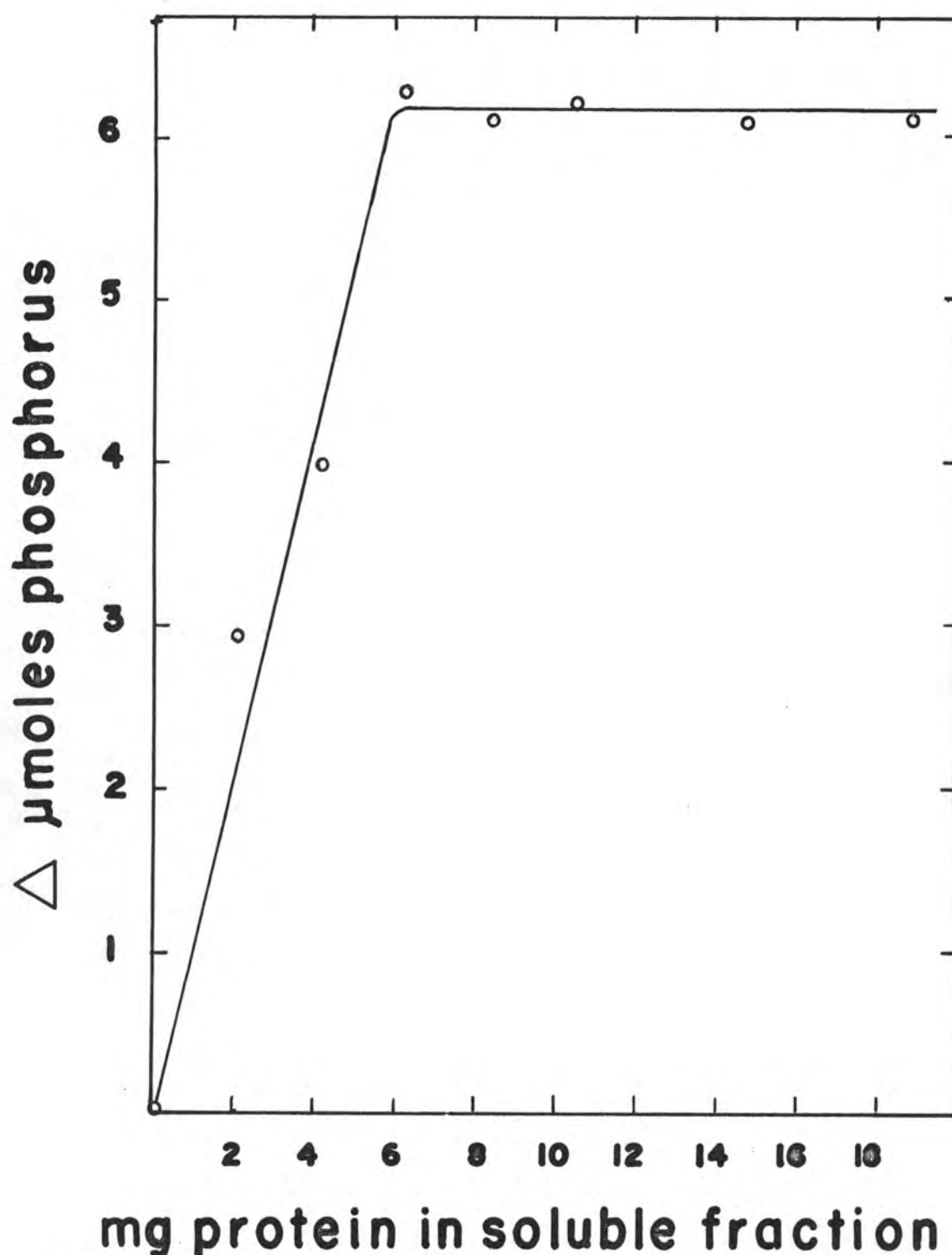


Figure 1. Each vessel contained 15 mg BHM protein, 20 μmoles MgCl_2 , 2 μmoles NADH_2 , 3mg ADP, 6 mg 2-deoxy-D-glucose, 4 mg hexokinase, 0.01 M phosphate buffer, pH 7.4, soluble fraction as indicated, and 0.25 M sucrose to 3.0 ml.

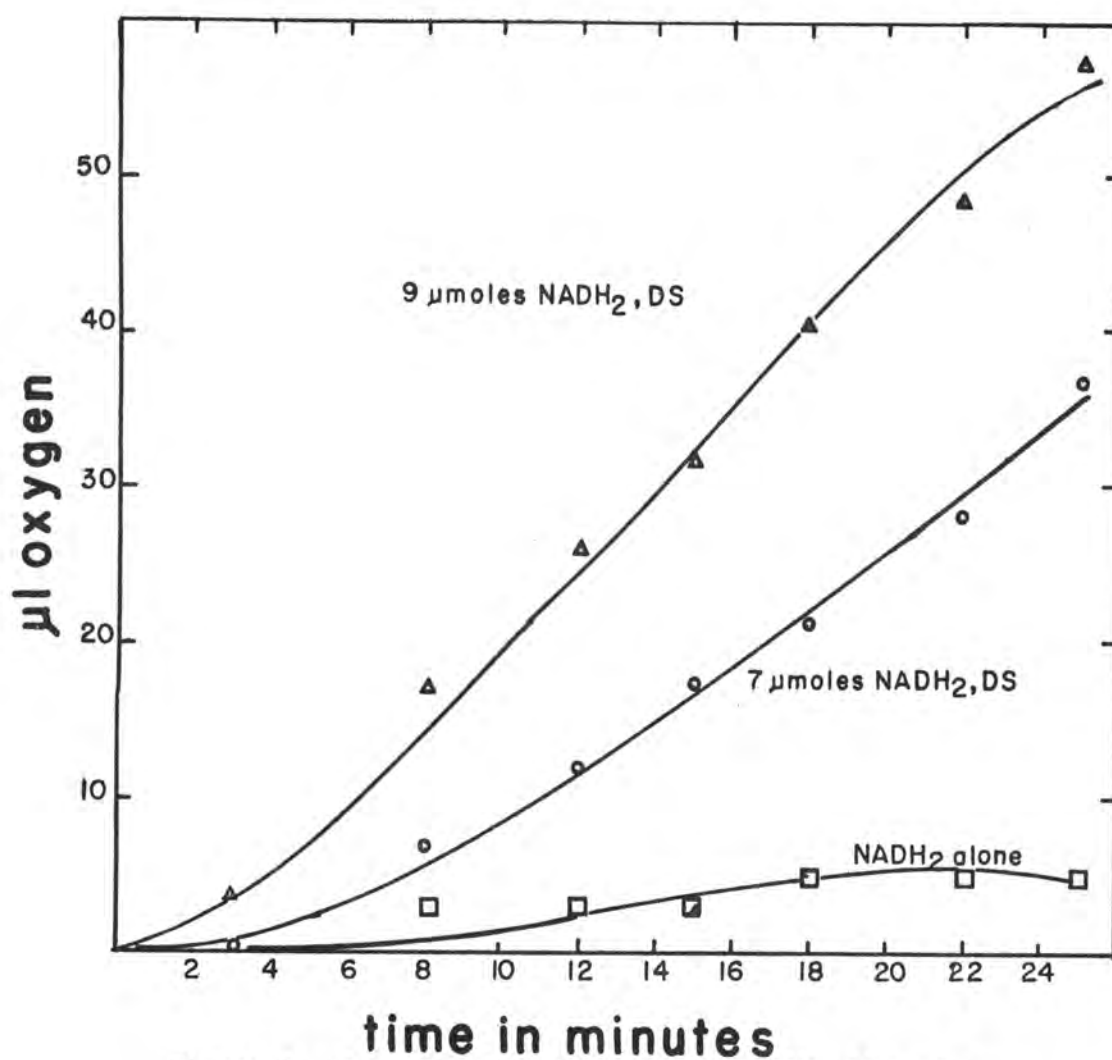
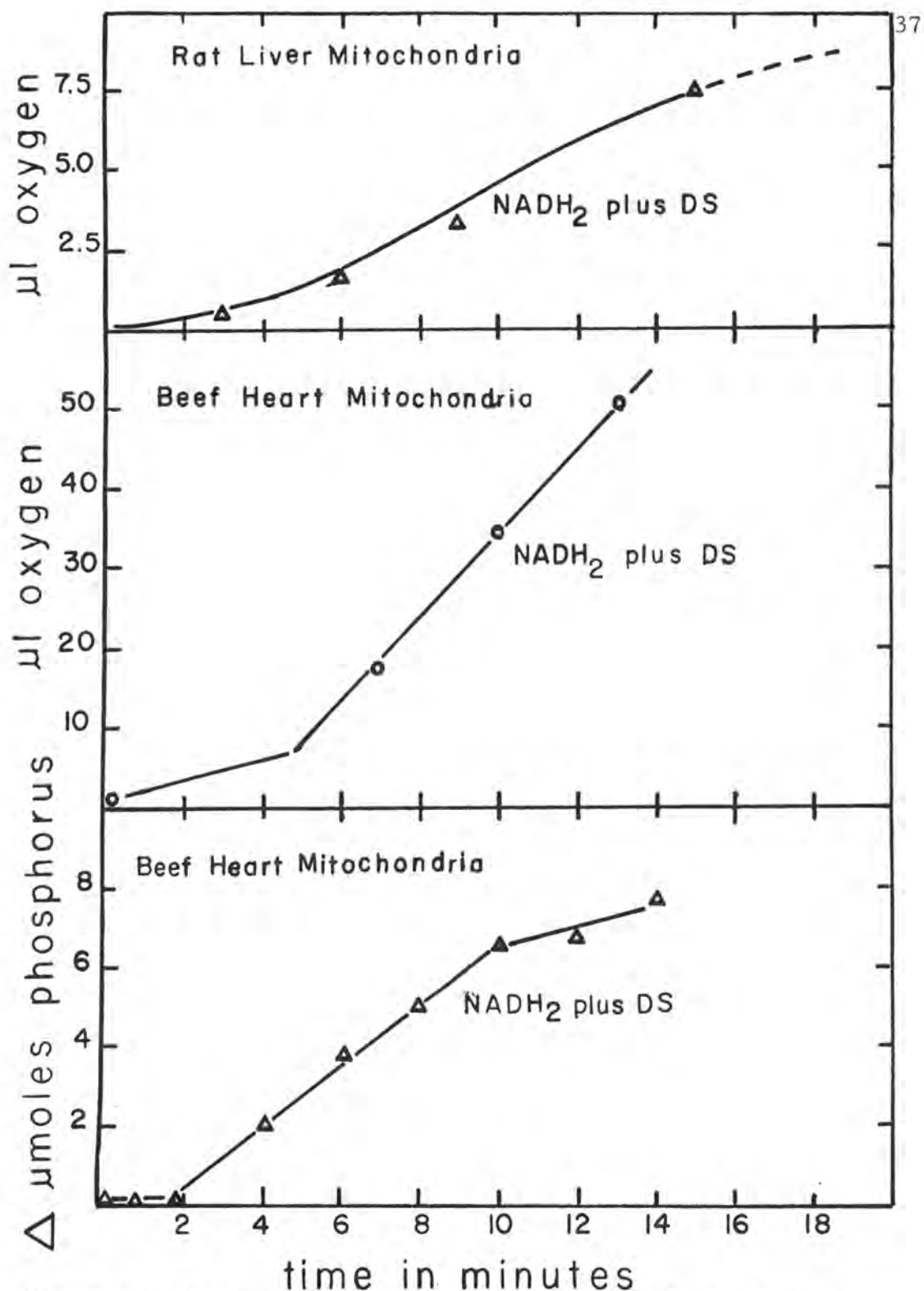


Figure 2. Each flask contained 1.0 ml RLM, 20 $\mu\text{moles MgCl}_2$, 3 mg ADP, 4 mg hexokinase, 6 mg 2-deoxy-D-glucose, 0.01 M phosphate buffer, pH 7.4, 0.1 ml 20% KOH in center well, 0.25 M sucrose to 3.0 ml, plus where indicated 4 mg DS protein and NADH₂.



Figures 3, 4, and 5. Flasks contained 1.0 ml RLM or 15 mg BHM protein, 20 $\mu\text{moles MgCl}_2$, 3 mg ADP, 4 mg hexokinase, 6 mg 2-deoxy-D-glucose, 0.01 M phosphate buffer, pH 7.4, 10 $\mu\text{moles NADH}_2$, 4 mg DS protein, 0.1 ml 20% KOH in center well, plus 0.25 M sucrose to 3.0 ml.

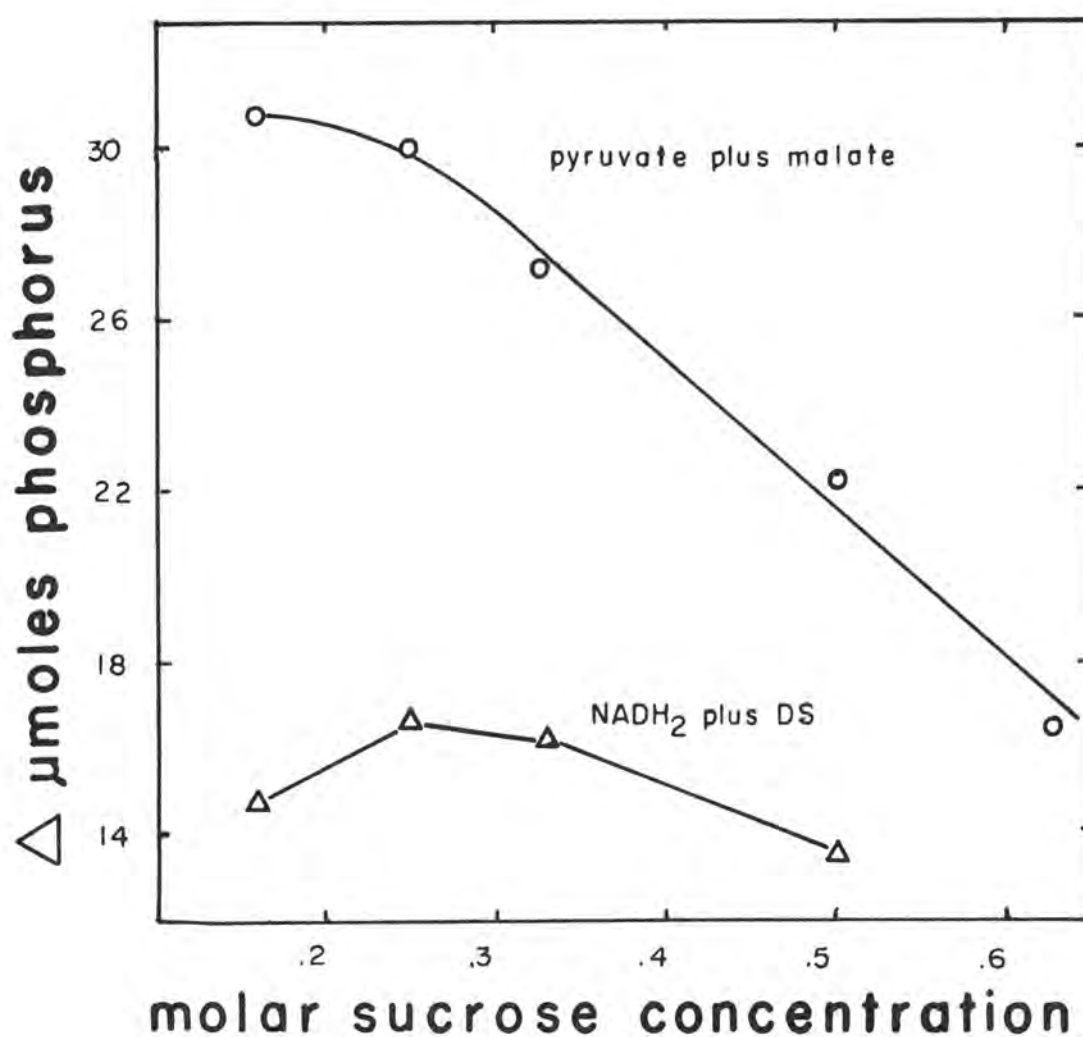


Figure 6. Each flask contained 8 mg BHM protein, 20 μmoles MgCl_2 , 3 mg ADP, 6 mg 2-deoxy-D-glucose, 4 mg hexokinase, 0.01 M phosphate buffer, pH 7.4, plus where indicated: 20 μmoles pyruvate, 5 μmoles malate, 2 μmoles NADH_2 , and 4 mg DS protein. Final sucrose concentration is shown.

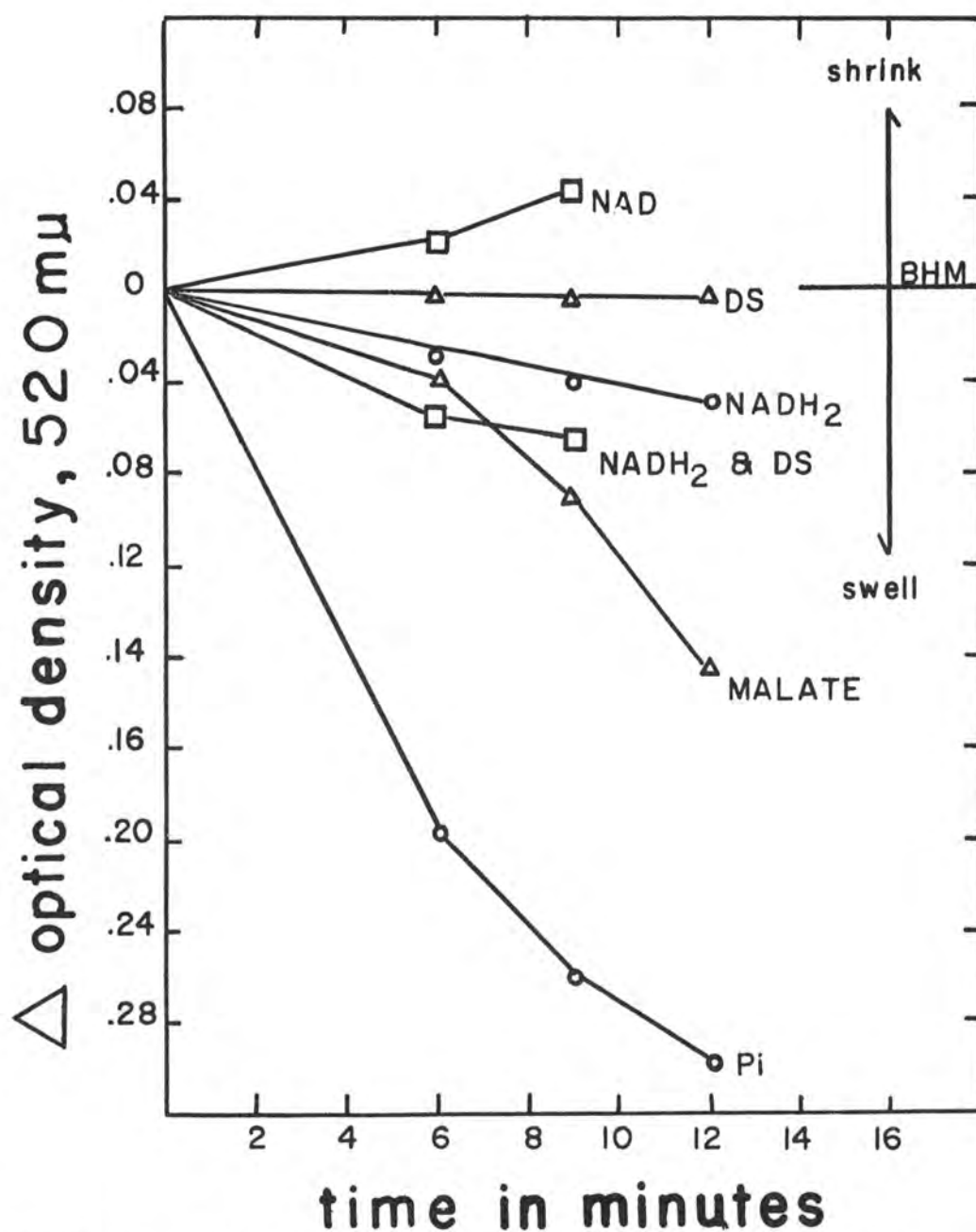


Figure 7. Each flask contained 1.5 mg BHM protein, 2 umoles $MgCl_2$, 0.3 mg ADP, 0.4 mg hexokinase, 0.6 mg 2-deoxy-D-glucose, phosphate buffer (0.001 M), pH 7.4, 0.25 M sucrose to 3.0 ml, plus where indicated: 0.2 umole $NADH_2$, 0.2 umole NAD, 0.02 M KH_2PO_4 , 0.007 M malate. Points on curves are the average of three or more determinations.

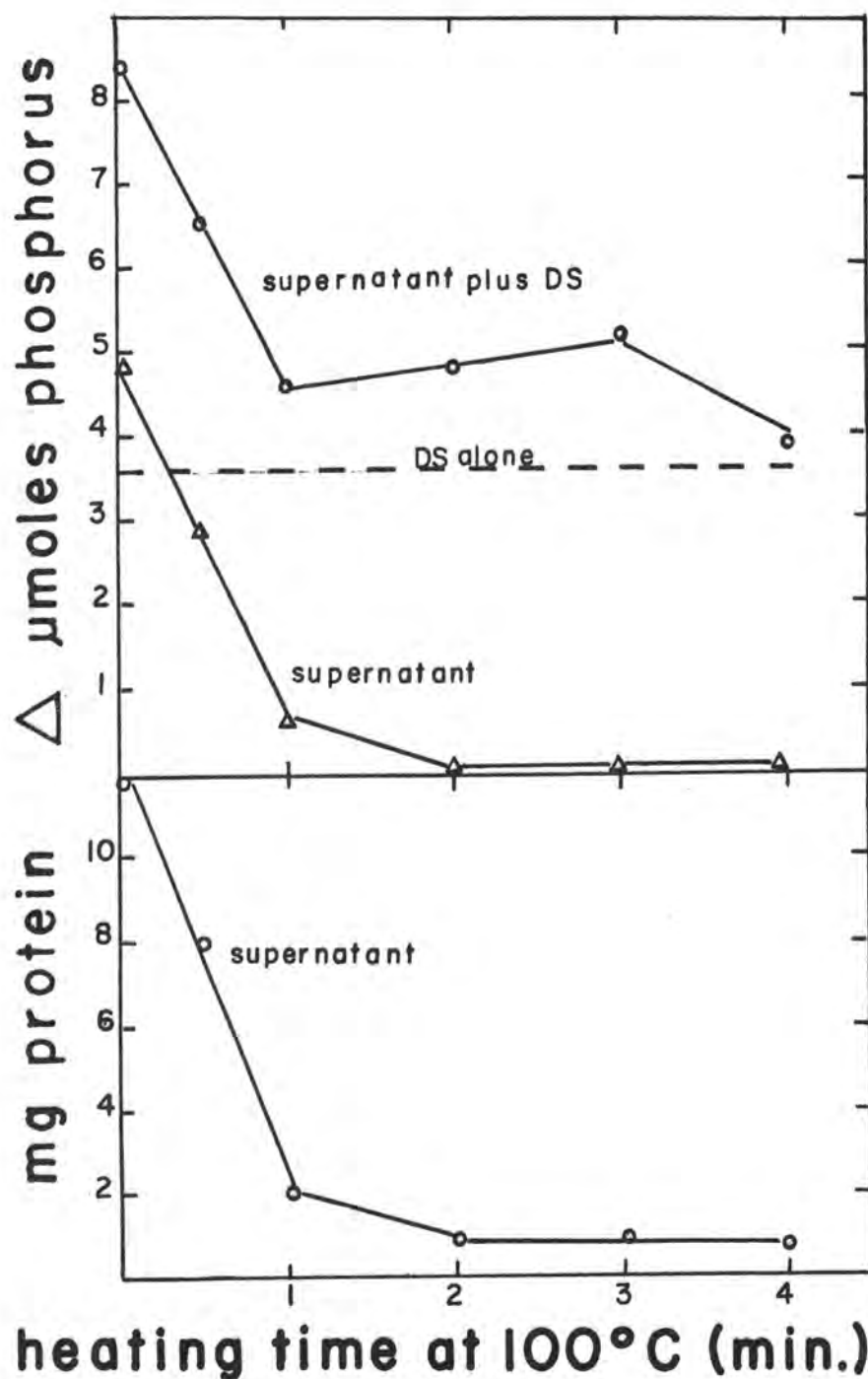


Figure 8. The phosphorus assay medium consisted of 15 mg BHM protein; 20 μ moles $MgCl_2$, 3 mg ADP, 6 mg 2-deoxy-D-glucose, 4 mg hexokinase, 2 μ moles $NADH_2$, 0.01 M phosphate buffer, pH 7.4, and 0.25 M sucrose to 3.0 ml in each vessel. The "supernatant" was obtained by centrifugation of the heat-treated DS.

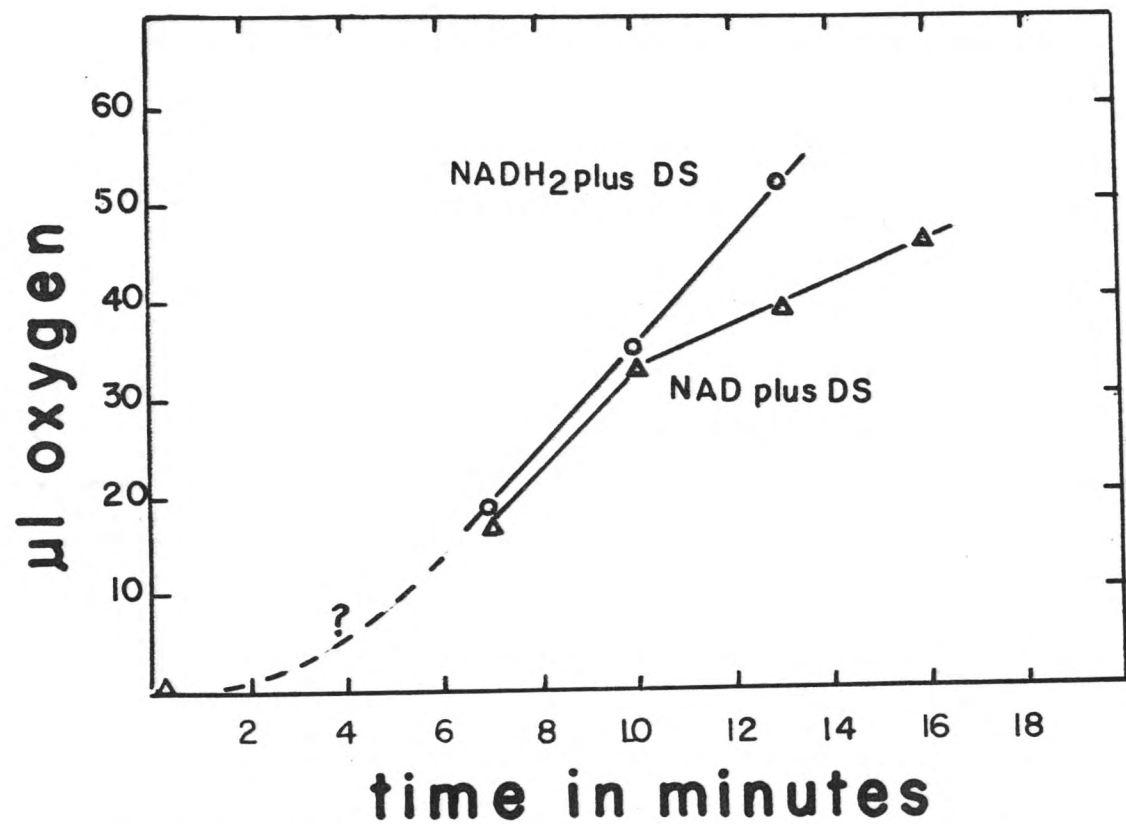


Figure 9. Each flask contained 16 mg BHM protein, 4 mg DS protein, 0.1 ml 20% KOH in center well, 20 umoles $MgCl_2$, 3 mg ADP, 4 mg hexokinase, 6 mg deoxyglucose, 0.01 M phosphate buffer, pH 7.4, 0.25 M sucrose to 3.0 ml, plus where indicated 10 umoles NAD or NADH₂.

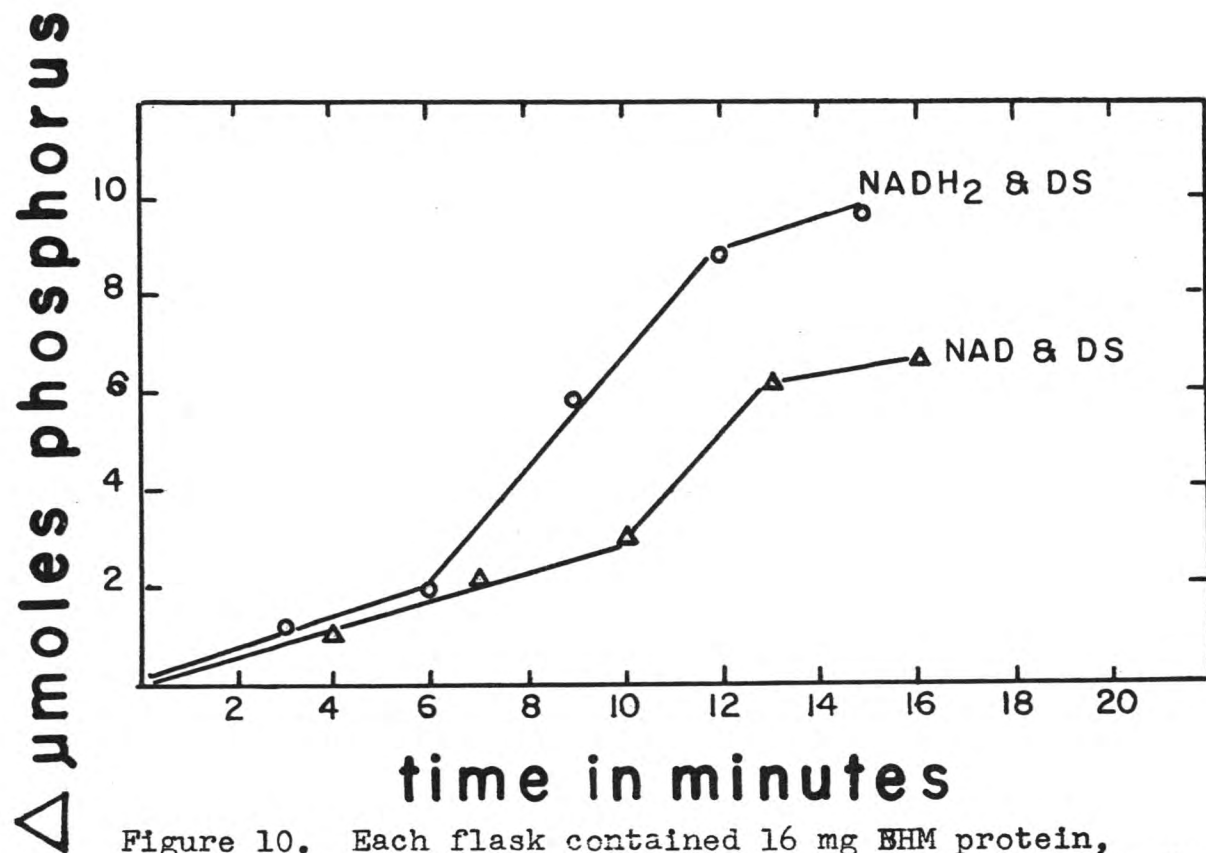


Figure 10. Each flask contained 16 mg BHM protein, 4 mg DS protein, 0.1 ml 20% KOH in center well, 20 μmoles MgCl_2 , 3 mg ADP, 4 mg hexokinase, 6 mg 2-deoxy-D-glucose, 0.01 M phosphate buffer, pH 7.4, 0.25 M sucrose to 3.0 ml, plus where indicated 10 μmoles NAD or NADH_2 .

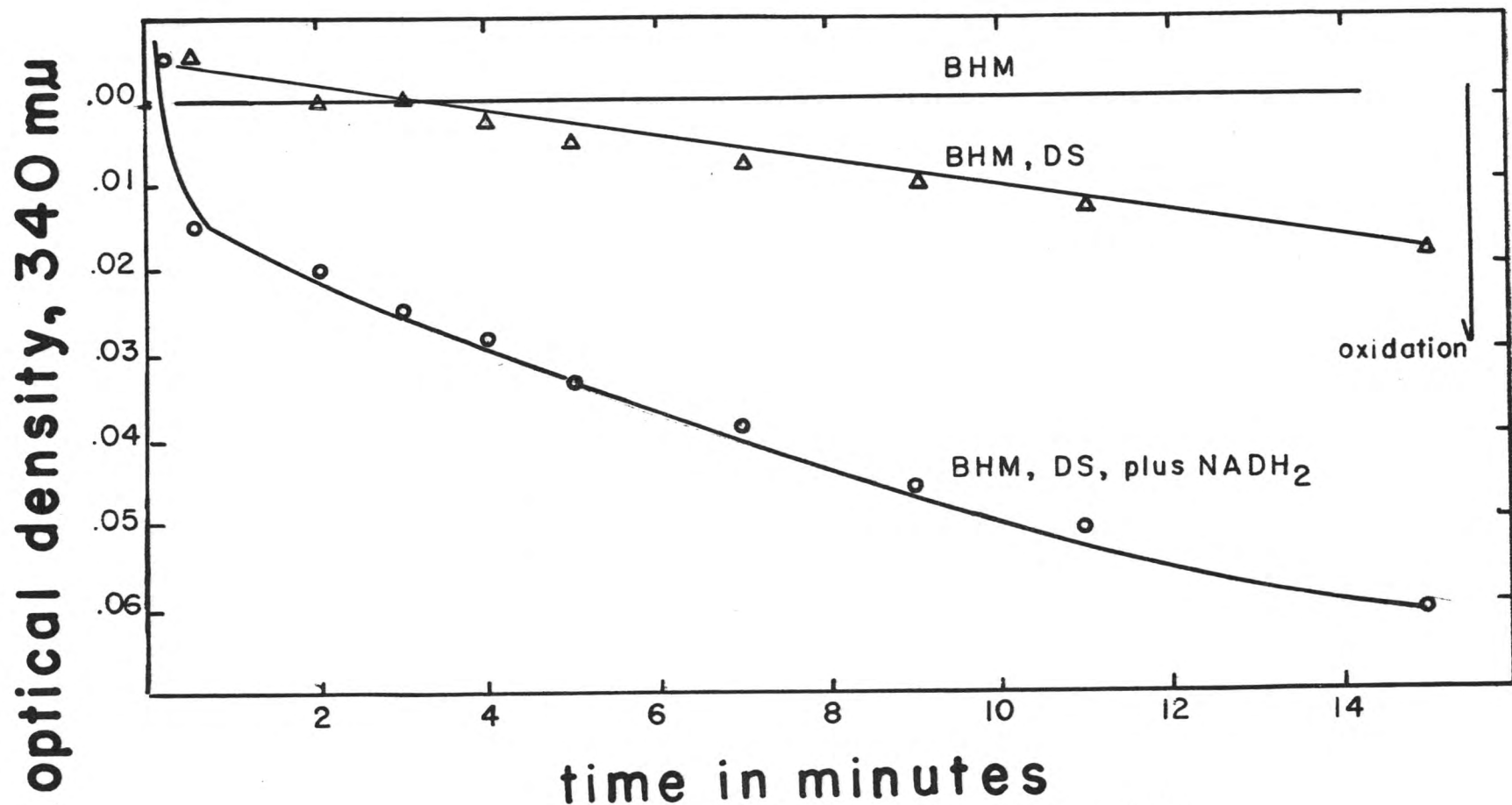


Figure 11. Each flask contained 1.5 mg BHM protein, 0.3 mg ADP, 0.6 mg 2-deoxy-D-glucose, 0.4 mg hexokinase, 2 umoles $MgCl_2$, 0.001 M phosphate buffer, pH 7.4, 0.25 M sucrose to 3.0 ml, plus 0.2 umole $NADH_2$ and 0.3 mg DS protein where indicated.

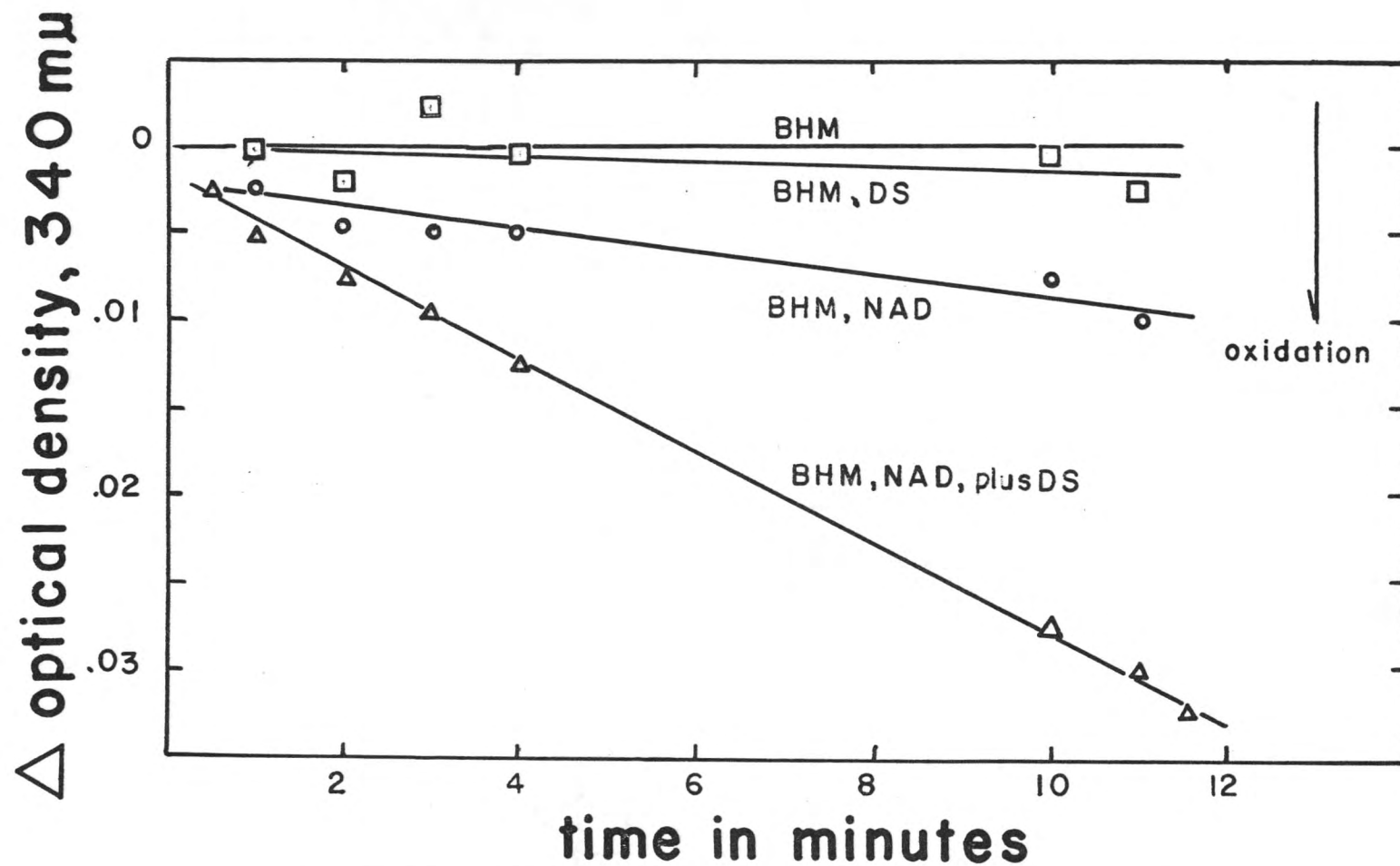


Figure 12. Each flask contained 1.5 mg BHM protein, 0.3 mg ADP, 0.6 mg 2-deoxy-D-glucose, 0.4 mg hexokinase, 2 umoles $MgCl_2$, 0.001 M phosphate buffer, pH 7.4, 0.25 M sucrose to 3.0 ml, plus 0.2 umole NAD and 0.3 mg DS protein where indicated.

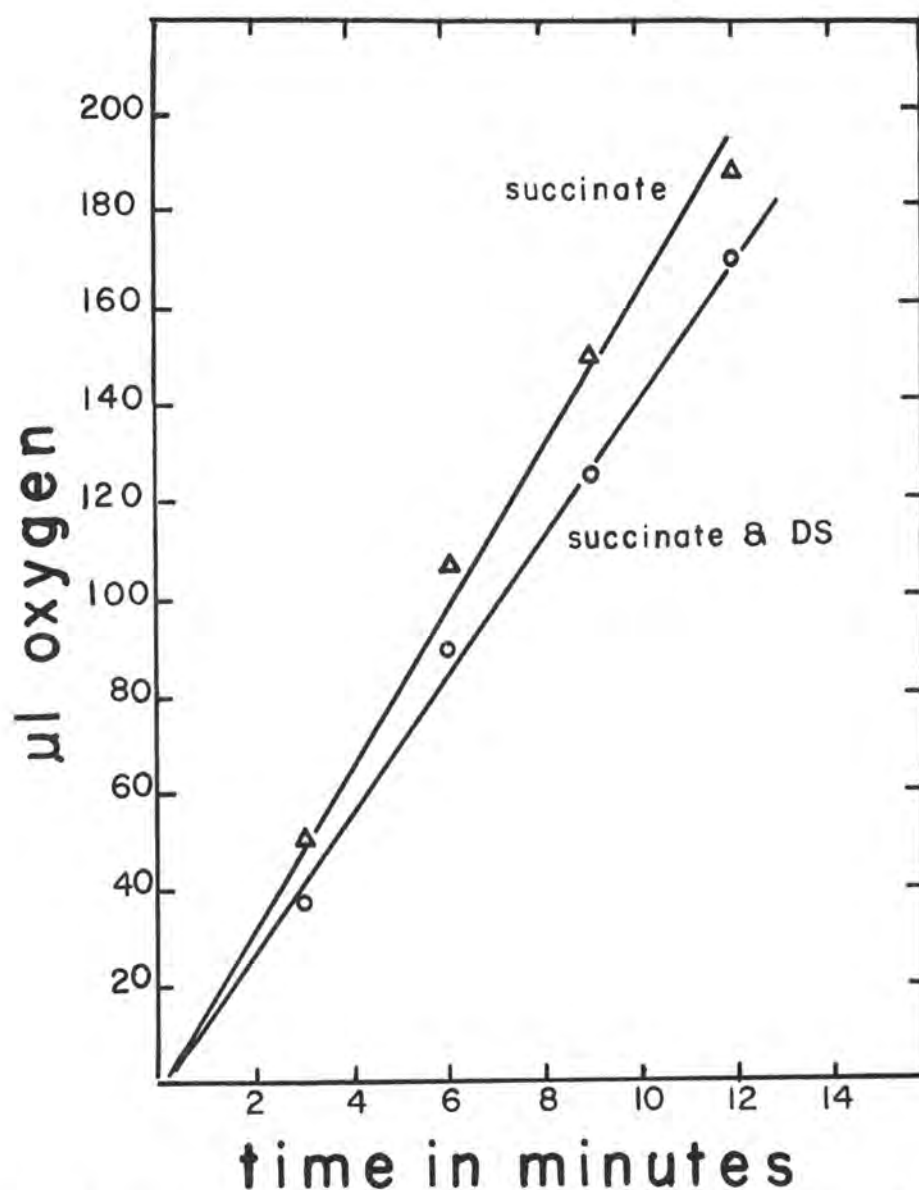


Figure 13. Each flask contained 1.0 ml RLM, 3 mg ADP, 6 mg hexokinase, 6 mg 2-deoxy-D-glucose, 20 μmoles MgCl_2 , 0.1 ml 20% KOH in center well, 0.25 M sucrose to 3.0 ml, 0.01 M phosphate buffer, pH 7.4, plus 4 mg DS protein and 20 μmoles succinate where indicated.

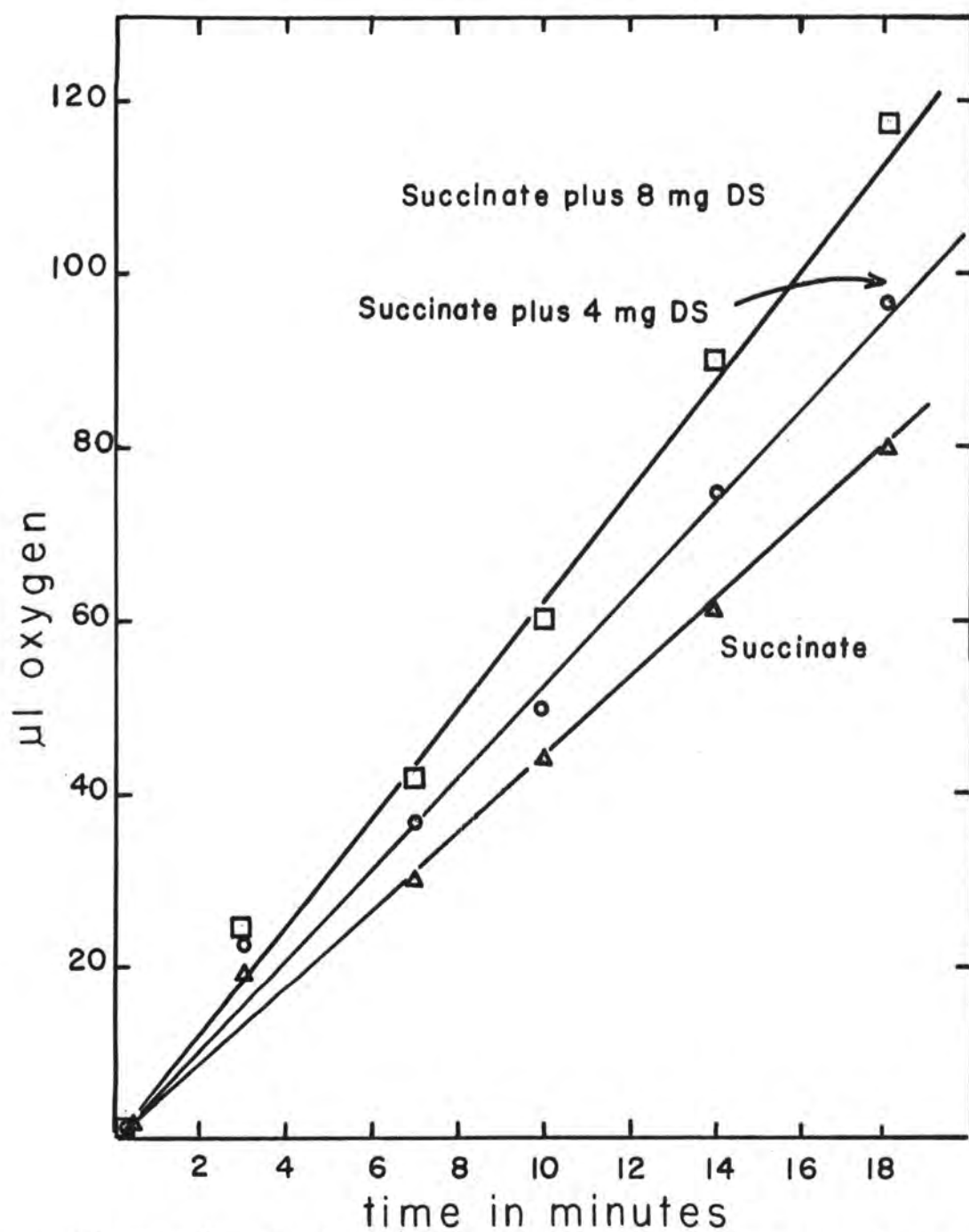


Figure 14. Each flask contained 2 mg HMP protein, 20 umoles $MgCl_2$, 3 mg ADP, 6 mg 2-deoxy-D-glucose, 4 mg hexokinase, 0.01 M phosphate buffer, pH 7.4, 0.1 ml 20% KOH in center well, 0.04 M succinate, plus 0.25 M sucrose to 3.0 ml.

Table I. Each flask contained BHM = mg protein, 3.1 mg ADP, 6 mg 2-deoxy-D glucose, 4 mg hexokinase, 20 μ moles MgCl_2 , 0.01 M phosphate buffer pH 7.4, 0.25 M sucrose to 3.0 ml, plus (where indicated) 5 μ moles NAD or NADH_2 , 4 mg DS protein. Reaction stopped at 9 min by centrifugation at 12,000 \times g.

#	ADDITION	SUPERNATANT		MITOCHONDRIA		
		μ moles NADH_2	μ moles NAD	μ moles NADH_2	μ moles NAD	μ moles Δ NAD
5/31	None	0.03	0.04	0.05	0.12	---
	NADH_2	0.11	1.75	0.05	0.29	0.17
	NADH_2 +DS	0.12	1.61	0.05	0.29	0.23
	DS	0.04	0.05	0.05	0.06	---
6/1	None	0.03	0.03	0.04	0.13	---
	NAD	0.08	2.48	0.04	0.37	0.24
	NAD+DS	0.09	3.22	0.04	0.49	0.40
	DS	0.04	0.11	0.04	0.09	---

Table II.

#	ADDITION	$\mu\text{mP}/$ 9 min
5/31	None	---
	NADH_2	2.1
	NADH_2 +DS	7.0
	DS	0.0
6/1	None	---
	NAD	0.6
	NAD+DS	1.3
	DS	0.0

Each flask contains the same components as listed in Table I. Reactions were stopped at 9 min. with 0.5 ml 30% TCA. Phosphorus determinations were made on the deproteinized material.