DETECTION AND CHARACTERIZATION OF A COMPONENT(S) IN COMMERCIAL LOTS OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE OXIDIZABLE BY INTACT YEAST CELLS

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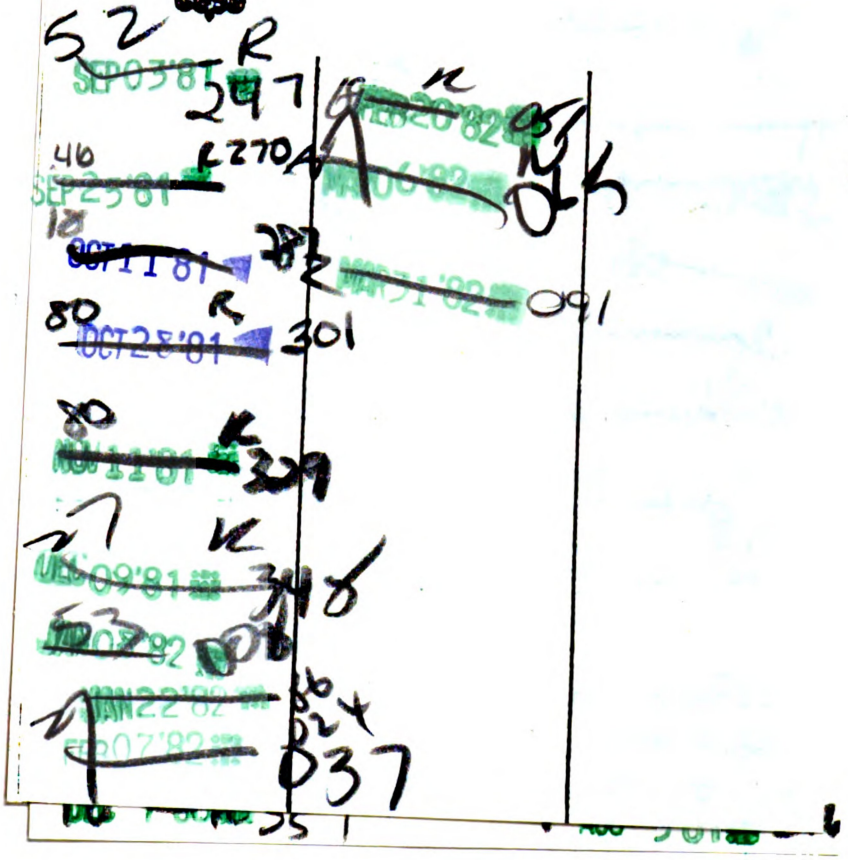
1969



ABSTRACT

DETECTION AND CHARACTERIZATION OF A COMPONENT(S) IN COMMERCIAL LOTS OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE OXIDIZABLE BY INTACT YEAST CELLS

By

Ralph Leonard Somack

Intact yeast cells were found to oxidize some component(s) of reduced nicotinamide adenine dinucleotide (NADH) solutions. This oxidation was also observed with solutions of oxidized nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as substrates; but only one commercial lot of each of these compounds was tested. The extent of the oxygen uptake observed varied from lot to lot (0.25 to 1.13 umoles of O_2 uptake/mg NADH). The respiratory quotient of the oxidation with NADH was observed to be in the order of 0.67, and no free amino nitrogen was released during the oxidation. The extent of this oxidation was as high at pH 4.0 as at pH 7.0, even though the NADH in the solution was all in the form of the acid modification product at pH 4.0. The oxidizable component(s) was separated from the NADH by the use of anion exchange resin; and, thus, proven to be a contaminant(s) of the preparation. This compound(s) was found to be neutral at both acid and basic pHs; as indicated by its failure

to adsorb to either anion or cation resins at these pHs. The eluted component(s) expressed no characteristic absorption spectrum between 220-440 nm. O_2 uptake was not observed when the eluted component(s) was dried at either acid or basic pHs under a vacuum at 40 C; thus indicating that the component(s) was either volatile or unstable under these conditions. Inhibition of both O_2 uptake and CO_2 evolution was observed when the component(s) was oxidized by yeast in the presence of azide, malonate or iodoacetate. This indicated that metabolism of the component(s) was dependent on the electron transport system, the tricarboxylic acid cycle and sulfhydryl containing enzymes. The low levels of the oxidizable component(s) present, the high cost of NADH preparations, and the difficulty in concentrating the component(s) precluded further efforts toward identification at this time.

DETECTION AND CHARACTERIZATION OF A COMPONENT(S) IN COMMERCIAL LOTS OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE OXIDIZABLE BY INTACT YEAST CELLS

By

Ralph Leonard Somack

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DEDICATION

This thesis is dedicated to my wife, Diana, for her love, her understanding, and her patience.

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INTRODUCTION

Commercially prepared oxidized and reduced nicotinamide adenine dinucleotide (NAD and NADH respectively) have been used in a great number of scientific investigations. These and other pyridine nucleotides are involved in a large number of oxidation-reduction reactions catalyzed by dehydrogenases.

Anderson (1), in his studies on the primary site of inhibition of yeast respiration by sorbic acid, found that sorbate inhibited aerobic oxygen uptake by yeast cells at pH 4.0 when solutions of commercial NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were used as substrates. These oxidations were markedly inhibited by both KCN and azide indicating that the oxidation proceeded via the cytochrome system. Since these coenzymes are known to be converted to products inactive in dehydrogenase reactions under acid conditions (11, 20), it was unlikely that these coenzymes were metabolized in the manner outlined by Anderson.

The present study was initiated in an attempt to identify the nature of these oxidations.

REVIEW OF LITERATURE

Oxidation of NADH and NADPH

Anderson (1), showed that solutions of NADH and NADPH supported oxygen uptake at acid pH by yeast cells. The oxidation, which was observed in the presence of 33 umoles of NADH, was shown to be inhibited 100% by KCN (6.7×10^{-1}M) and 68% by sorbic acid (5.3×10^{-3}M). The oxidation observed with NADPH solutions was inhibited 39% by atabrin (5.3×10^{-2}M), 100% by azide (7.7×10^{-3}M), and 88% by sorbic acid (5.3×10^{-3}M). Further evidence that the oxidation proceeded via electron transport (ETS) came from the observation that methylene blue was reduced when cytochrome oxidase was inhibited by KCN with an NADH solution as substrate. The experiment was performed with yeast cells at pH 5.1 - 5.3. Studies with 2-4 dinitrophenol (DNP) indicated that the oxygen uptake by whole cells observed with NADH solutions required active transport since DNP lowered the resulting Qo_2, while it failed to affect the rates of endogenous respiration or glucose oxidation. As Anderson pointed out, this would be expected since substrate phosphorylation occurs via glycolysis during glucose metabolism.

Studies with crude extracts of yeast cells showed that these preparations also oxidized NADH and that the oxidation was sensative to azide, antimycin A and KCN.

Although the oxygen uptake with intact cells was inhibited by CO, there was no significant inhibition of oxygen uptake with the crude yeast extracts (Anderson, 1963, unpublished data). These results were explained on the basis of the peroxidase activity observed in the isolated crude yeast mitochondria preparations but not in the intact cells.

Formation and Properties of The Acid Product of NADH and NADPH

NADH, NADPH and other n^1-substituted dihydropyridines have been shown to be unstable in acid (11, 20). The characteristic 340 nm absorbtion peaks of the reduced coenzymes are lost with the concurrent formation of a new peak at 290 nm. This latter peak disappears rapidly at pH 1 but can be stabilized by the addition of bisulfite or by neutralization immediately after the formation of the primary acid product. The primary acid product decomposes relatively slowly between pH 3 and 5. Burton and Kaplan (4) have suggested that the primary acid product is formed by the opening of the heterocyclic ring yielding an amino aldehyde. Bisulfite is thought to stabilize the acid product by addition through the oxygenated grouping of the aldehyde at the 6 position of the ring.

The results presented by Anderson (1) are complicated by the fact that two different systems seem to have been

operative. Since NADH and NADPH are rapidly converted to enzymically inactive coenzymes under acid conditions, these would have been inactive in the ETS oxidations carried out by Anderson with whole yeast cells. However, his experiments with crude yeast extracts were carried out at neutral pH, and these would have involved true coenzyme oxidations in the systems studied.

EXPERIMENTAL METHODS

Culture and Cultural Techniques

The yeast used in these experiments was from two sources. In one experiment, a stock strain of baker's yeast from our laboratories was used to produce fresh cells in the following manner: 250 ml volumes of dextrose broth (Difco) were adjusted to pH 4.0 with tartaric acid, innoculated with stock yeast and allowed to grow in shake flasks at 30 C for 24 hours. The cells were harvested by centrifugation, washed two times with 0.1 M KH_2PO_4 and stored at 0 C until use. In all other experiments, baker's yeast purchased locally was air dried and maintained in the freezer. Just prior to use, the dried yeast was washed three times with distilled water, re-suspended in distilled water and incubated on a rotary shaker for three hours to lower the endogenous metabolic rate. Cell weights are reported as dry weights and were determined by placing 1 ml of cell suspension at 110 C for 24 hours.

Oxidation Techniques

Standard Warburg techniques as described by Umbreit *et al*. (17) were employed for measurements of O_2 uptake and CO_2 evolution. All components of the reaction mixture were added to the main compartment of

the Warburg flask except substrates which were tipped in from a side arm after thermal equilibrium was attained. Air was used as the gas phase in all experiments. Carbon dioxide was determined by the direct method; and 0.2 ml of 20% KOH along with a strip of filter paper was placed in the center well of the cups used to measure oxygen uptake. Reaction mixtures were adjusted to the pH of the experiment with potassium phthalate, glycine-hydrochloride buffer or potassium phosphate buffer. The pH was determined at the termination of the experiment (final pH). In each case where used, the respiratory quotient (RQ) was defined as umoles of CO_2 evolved/ umole O_2 consumed.

Assay and Cleavage of NADH

The NADH and related compounds were obtained from various commercial sources as specified in the Results, and were stored at 0 C until use. A modification of the lactic dehydrogenase assay of Kornberg (13) was employed to measure the coenzymatic purity of a number of commercial preparations of NADH. Snake venom phosphodiesterase was purchased from Sigma Chemical Company and was used to split NADH by the method of Razzel and Khorana (15).

Purification of the Oxidizable Contaminant

Purification of an oxidizable fraction(s) present in NADH was achieved with 200-400 mesh Dowex 1-X8, OH^-

form. The resin was washed overnight in 4 N NaOH followed by five volumes of distilled water. Further purification was achieved with 200-400 mesh Dowex 50W-X4, H^+ form. The resin was activated for use in the same way as the Dowex 1-X8 except that 5 N HCl was substituted for the NaOH. Columns of about 0.5 x 3 cm were used with each resin. The columns were washed with at least ten volumes of distilled water prior to use.

RESULTS

Studies on The Oxidation of NADH by Yeast Cells

Nature of the oxidation

Varying amounts of O_2 uptake and CO_2 evolution by yeast cells were noted when similar concentrations of various commercial brands of NADH were added as substrate. In earlier work, the pH of the reaction mixtures was below 5 suggesting that the acid modification product of NADH was the species being oxidized. Figure 1 shows the oxidation observed with two commercial brands of NADH. The respiratory quotient (RQ), adjusted for endogenous activity, for the oxidation of the lot obtained from Sigma was 0.62 while the RQ for that from Boehringer and Soehne was 0.72. Yeast cells for these oxidations were from two different sources, as indicated. A direct comparison of the extent of O_2 uptake by commercial dried yeast was then made using a number of different lots of NADH from different sources. As is evident in Fig. 2, the yeast oxidized some component(s) of all lots, but there was considerable variation in the extent of oxidation among lots. The total O_2 uptake with the lot obtained from Sigma was very much higher than with any of the others.

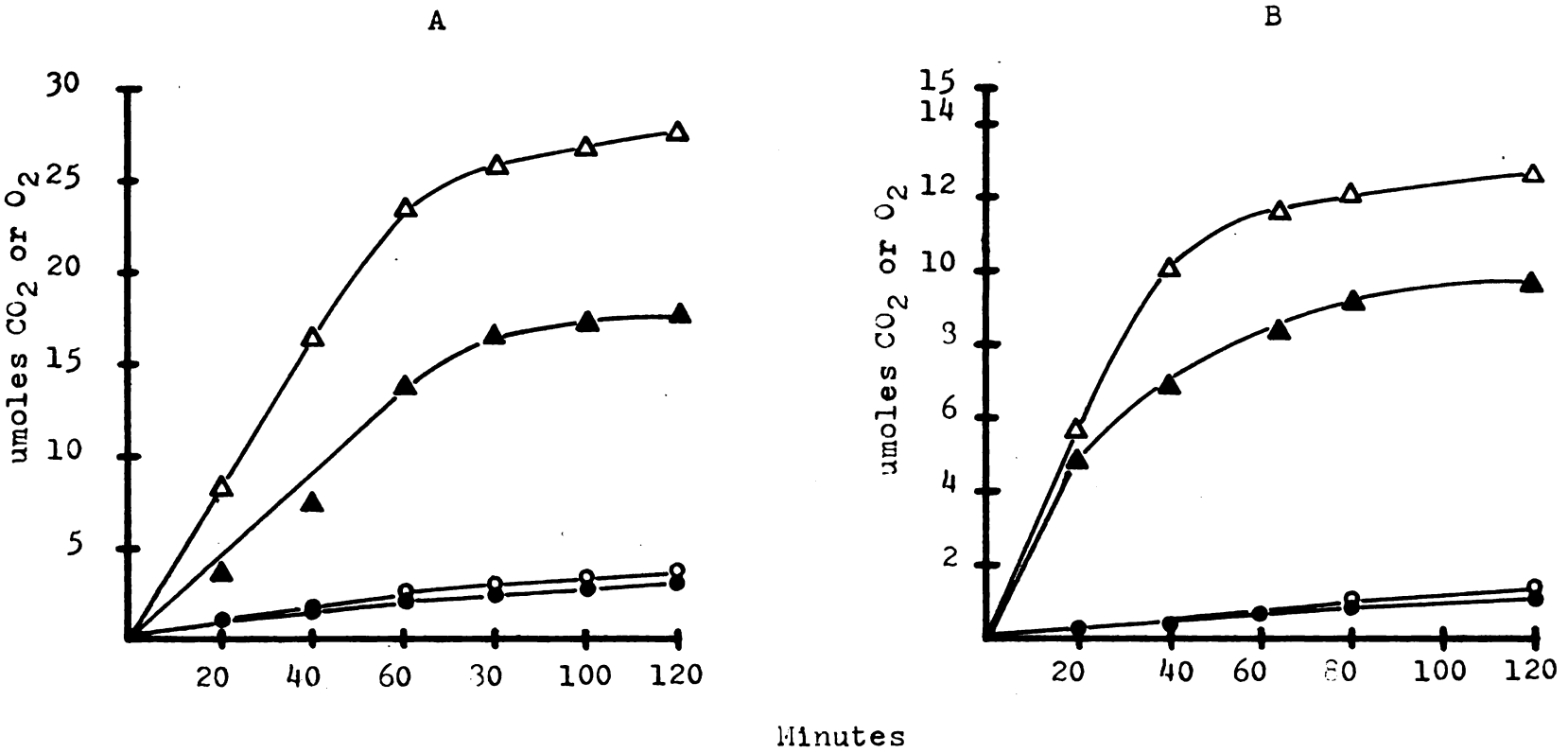


Figure 1.--Effect of NADH on O_2 uptake and CO_2 evolution by yeast cells. Values were determined manometrically at 30 C. (A) The Warburg vessels contained: 250 umoles potassium phthalate, 23 mg (dry weight) commercial dried yeast and, where indicated, 30 umoles Sigma NADH (lot #106B-6550) assayed at 340 nm to be 70.4% pure. 0.2 ml of 20% KOH was added to the center wells to measure O_2 uptake. The gas phase was air. The total volume of each cup was 3.0 ml and the final pH 4.0 - 4.1. (B) The cups were identical to A except that 300 umoles potassium phthalate, 11.5 mg cloned yeast cells and, where indicated, 9.4 umoles Boehringer and Soehne NADH (lot #6304264) were used. The NADH assayed at 340 nm to be 70.3% pure. NADH, CO_2 evolution (▲); NADH, O_2 uptake (Δ); endogenous, CO_2 evolution (●); endogenous, O_2 uptake (o).

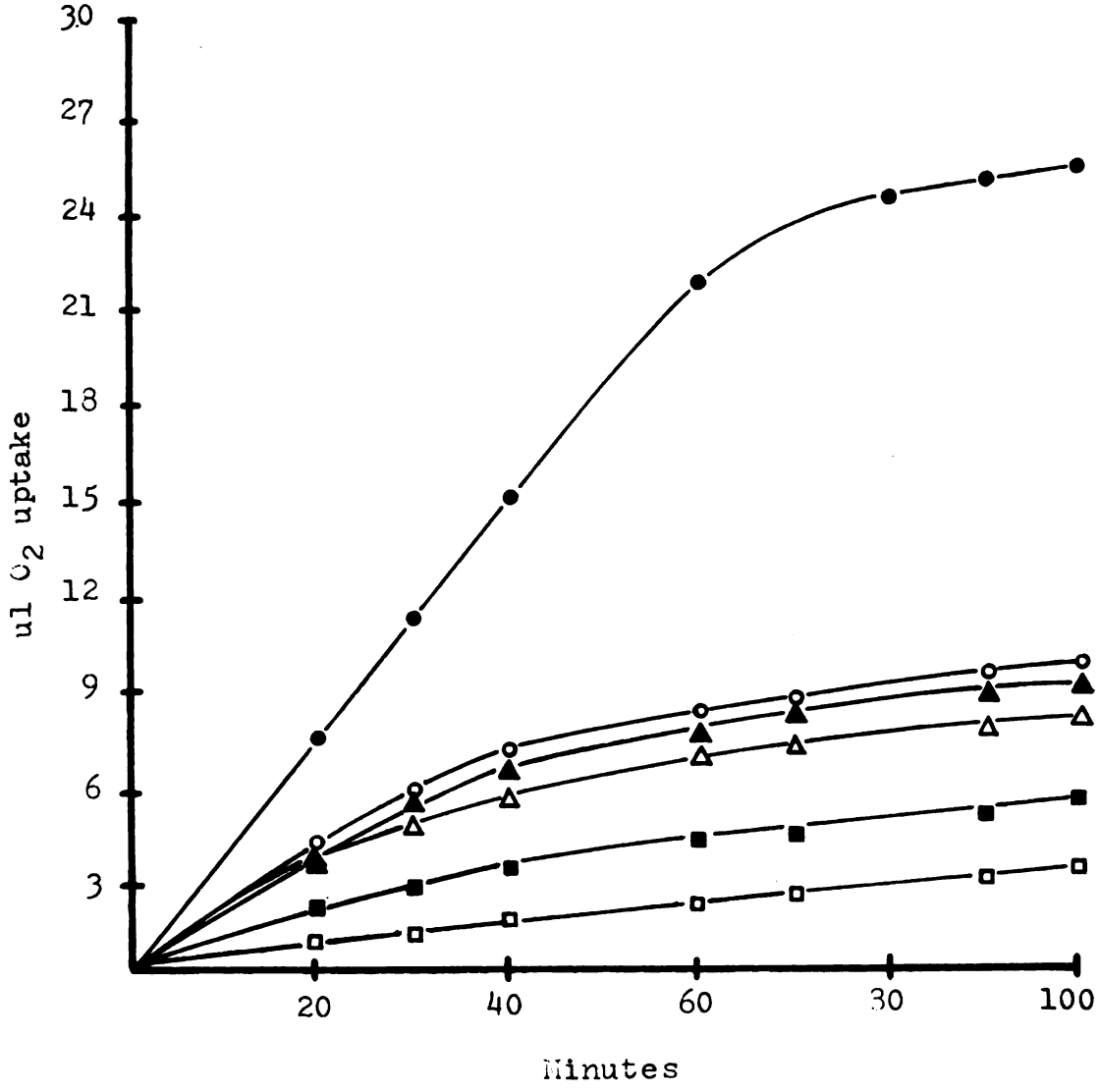


Figure 2.--Oxidation of various brands of NADH. The experimental procedure was as outlined in Figure 1(A) except that 25.9 mg (dry weight) of yeast was used. Amounts of 10.66 µmoles of the lyophilized and 30 µmoles of the other lots of NADH were prepared in H_2O and tipped in from the side arms at zero time. The final pH was 4.7 - 4.8. Sigma,disodium Lot #106B-6550 (●); General Biochemicals, disodium Lot #83789 (o); Calbiochem, disodium Lot #801865 (▲); General biochemicals, dilithium Lot #85232 (Δ); Sigma, disodium Lot #6-84-7 (■); endogenous (□).

Yeast cells also oxidized some component(s) of the solutions of a few other related compounds tested. A brand of NADPH supported O_2 uptake while NADP from the same company did not. One brand of NAD showed some O_2 uptake over endogenous levels. Fig. 3 shows that the initial rates of O_2 uptake observed with the NADH solutions were the same as the rate observed with glucose as substrate.

Purity analyses of NADH preparations

Equal samples of the NADH lots tested with yeast (Fig. 2) were assayed for purity based on the amount of 340 nm absorbing material present. Activity was then determined by the lactic acid dehydrogenase reaction. The results indicate that there was no correlation between purity, rate and extent of activity, and amount of O_2 uptake by yeast cells (Table 1). The large differences in the rate of the lactic dehydrogenase reaction with the different NADH preparations may have resulted from the presence of inhibitors in some of them as previously reported (9). This experiment suggested that a contaminant present in some commercial lots of NADH, and not the acid modification product of the coenzyme, might be responsible for this oxidation.

Effects of cleavage of NADH on oxidation by yeast cells

To determine what the effect of altering the intact nature of the NADH molecule would have on the

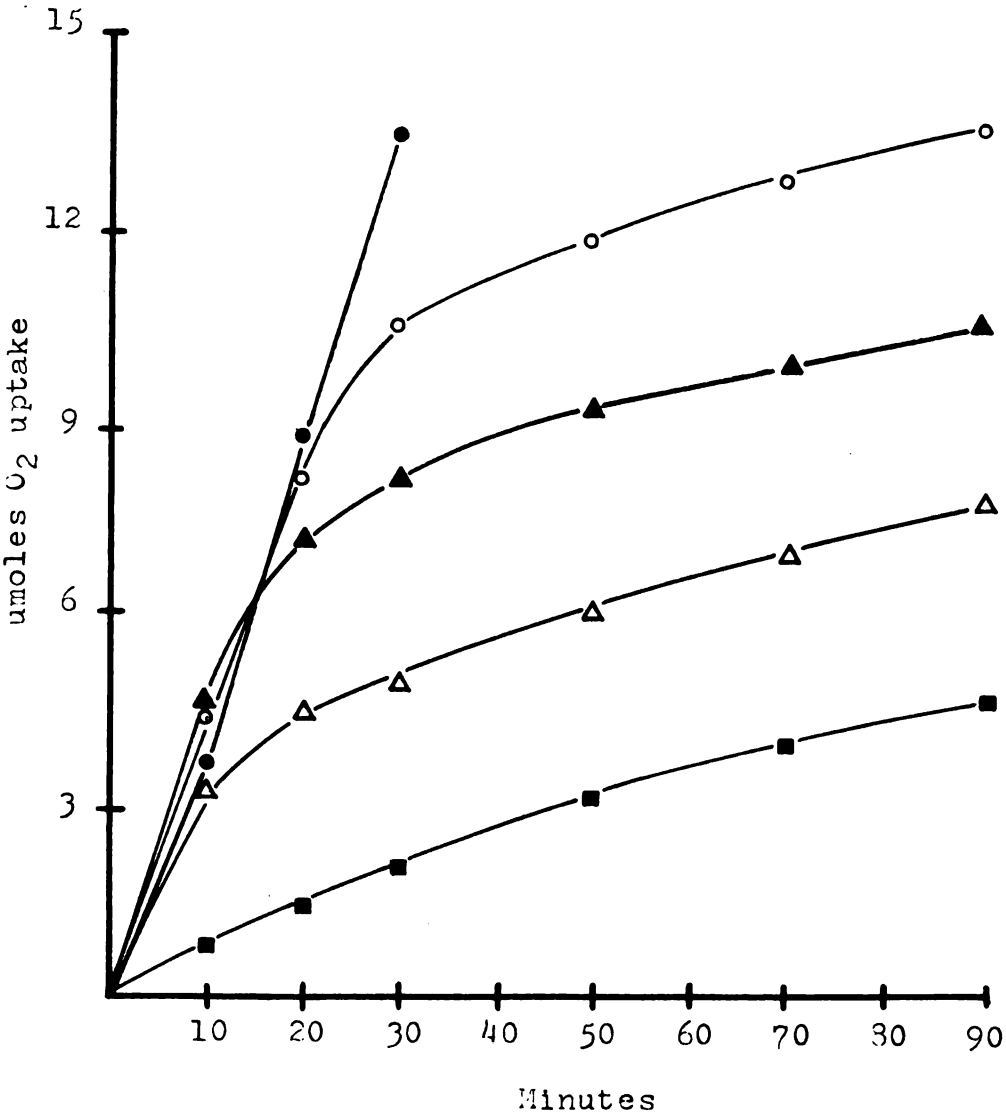


Figure 3.--Oxidation of NADPH, NADP and NAD. See Figure 1(A) for procedure. The Warburg vessels contained: 300 µmoles potassium phthalate, 9.1 mg yeast cells (cloned); and, where indicated, 10 µmoles of glucose or dinucleotide. The final pH was 3.9 - 4.2. Glucose (●); NADPH, Boehringer and Soehne Lot #6331344 (o); NADH, Boehringer and Soehne Lot #6304264 (▲); NAD, Calbiochem Lot #63991 (Δ); Endogenous and NADP Boehringer and Soehne Lot #6043507 (■).

TABLE 1.--Oxidation by yeast of various brands of NADH tested for spectrophotometric and functional purity.

Sample NADH	umoles per vessel*	umoles O_2 uptake*	% purity**	Δ OD per minute***
Gen BCH 83789	30	7.6	65.75	0.186
Gen BCH 85232	30	5.1	74.27	0.236
Sigma 106B-6550	30	24.0	70.41	0.216
Calbiochem 801865	30	6.5	72.18	0.322
Sigma 6-84-7 (lyophylized)	10.66	2.6	62.70	0.192

* Values are from Fig. 2 and were adjusted for endogenous activity.

** Cuvettes contained 0.1 umole NADH (based on 100% purity) in 1.0 ml 0.05 M KPO_4 buffer (pH 7.5). The blank contained just buffer. The optical density was read at 340 nm and the extinction coefficient (6.22 M^{-1} cm^{-1}) used to calculate % purity.

*** Each cuvette contained 0.5 umoles NADH (based on 100% purity), 0.025 ug rabbit muscle lactic dehydrogenase, 50 umoles KPO_4 buffer (pH 7.5) and 10 umoles sodium pyruvate, in 1.0 ml. The blank consisted of all elements except NADH, which was added, and the decrease in optical density at 340 nm recorded with time. The rates were calculated from linear portion of the curves. All reactions went essentially to completion.

oxidation, snake venom phosphodiesterase was employed. This enzyme is known to split the molecule at the pyrophosphate linkage yielding reduced nicotinamide mononucleotide (NMNH) and adenylic acid (15). Cleavage of 22 umoles of NADH was verified by assaying with the lactic dehydrogenase system until no loss in optical density at 340 nm was observed (Fig. 4). The cleavage products (7.88 umoles) were tested for oxidation by yeast compared to an untreated NADH control. The results are shown in Fig. 5 and indicate that cleavage of the molecule in this manner does not affect the oxidation phenomena.

Spectrum before and after oxidation

A spectrum of NADH both before and after acidification and after oxidation was performed to determine if products which absorb between 240 nm and 460 nm were produced as a result of the oxidation. Fig 6 shows that following oxidation no peaks were either produced or lost within this range. The primary NADH acid modification spectrum is characterized by loss of the band at 340 nm, greatly increased absorption in the region of 280 to 290 nm and a heightened maxima which is shifted from 260 to 265 nm. This agrees with the reports of Haas (11) and Rafter et al. (14). If the reaction proceeds by a mechanism which involves the NADH acid product, it does nothing to alter the spectral

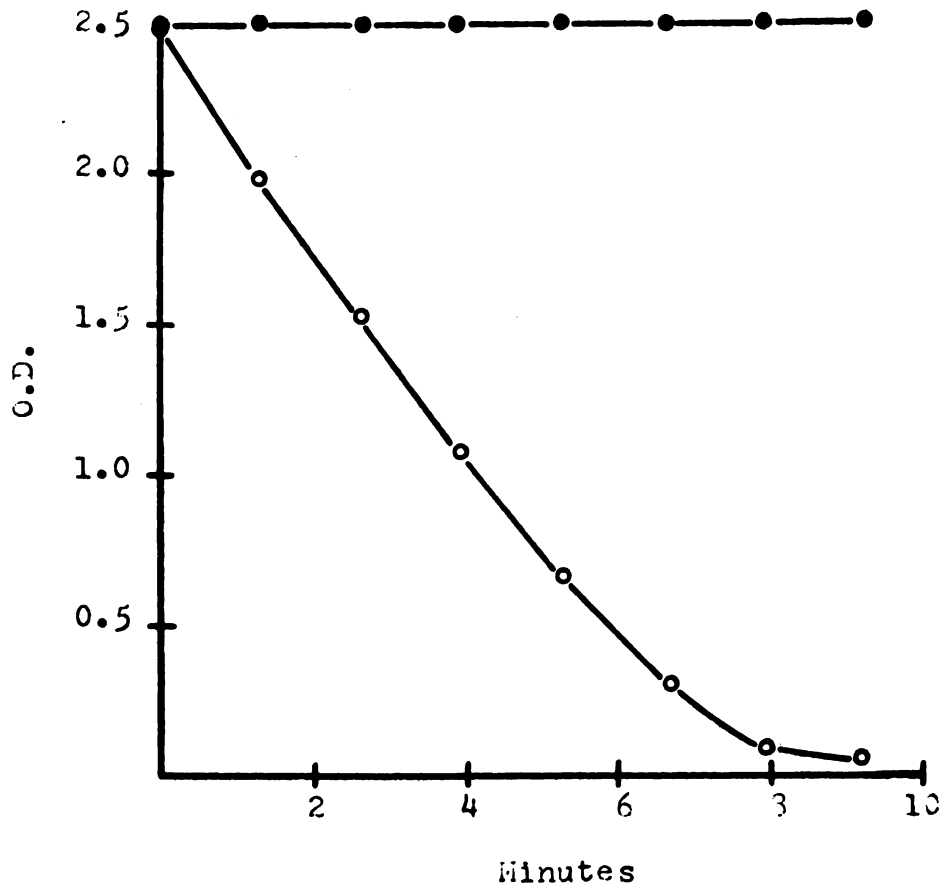


Figure 4.--Phosphodiesterase cleavage of NADH. A reaction mixture containing 22 µmoles NADH (Boehringer and Soehne, Lot #6304264), 200 µmoles Tris-HCl (pH 8.9) and 0.1 mg snake venom phosphodiesterase was incubated at 30 C for 2 hours. A control of 22 µmoles of NADH without phosphodiesterase was treated similarly along with 2 blank solutions; one without NADH and one without NADH or diesterase. All volumes equalled 2 ml. The diesterase treated mixture and the NADH control were assayed for intact nucleotide by the lactic dehydrogenase assay using the blank solutions as reference cuvettes. Cuvettes contained .055 ml of the diesterase treated mixture, NADH control or appropriate blank solution, 1 µmole sodium pyruvate, 50 µmoles potassium phosphate (pH 7.5) and .025 ug rabbit muscle lactic dehydrogenase in a total volume of 1 ml. Diesterase treated NADH (●); NADH control (o).

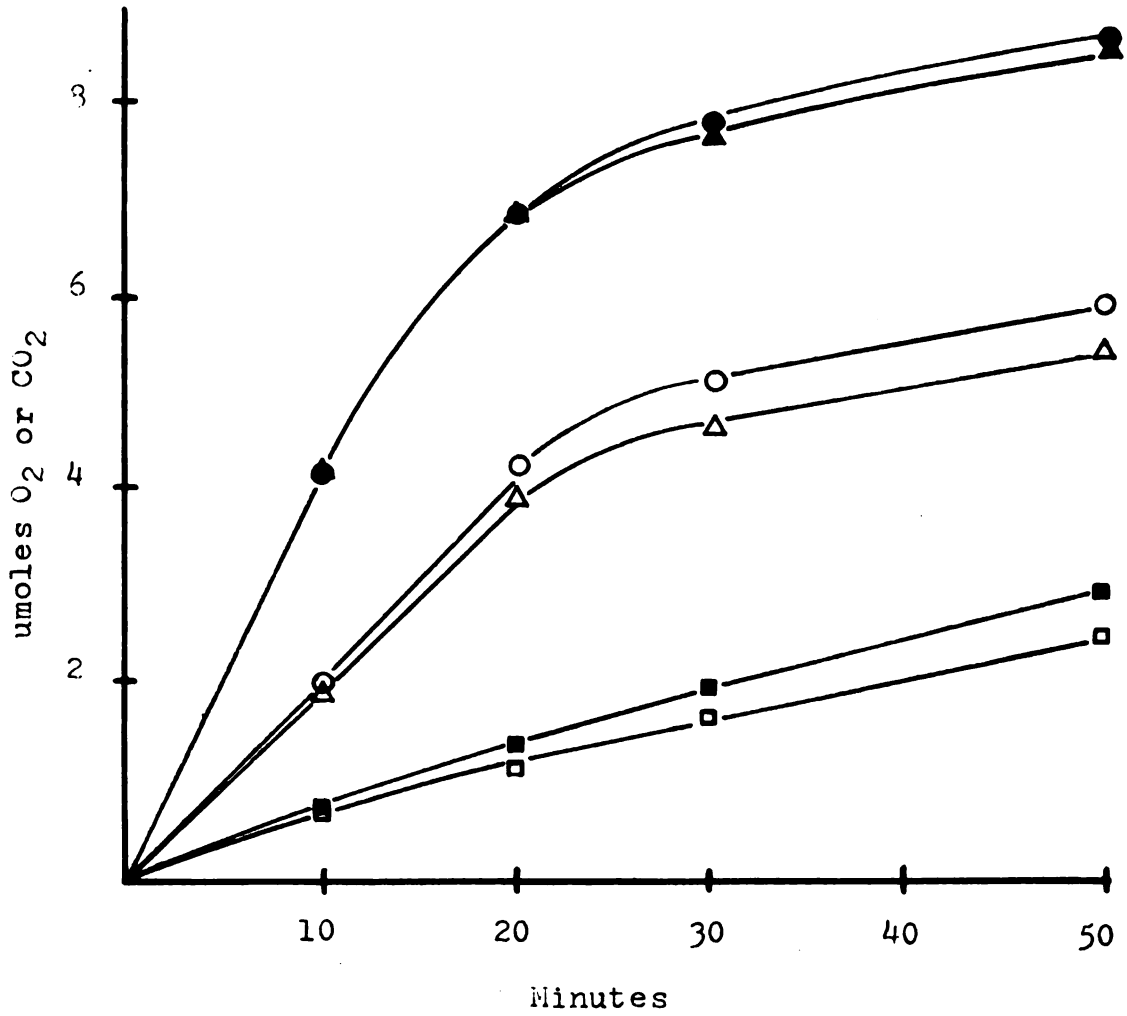
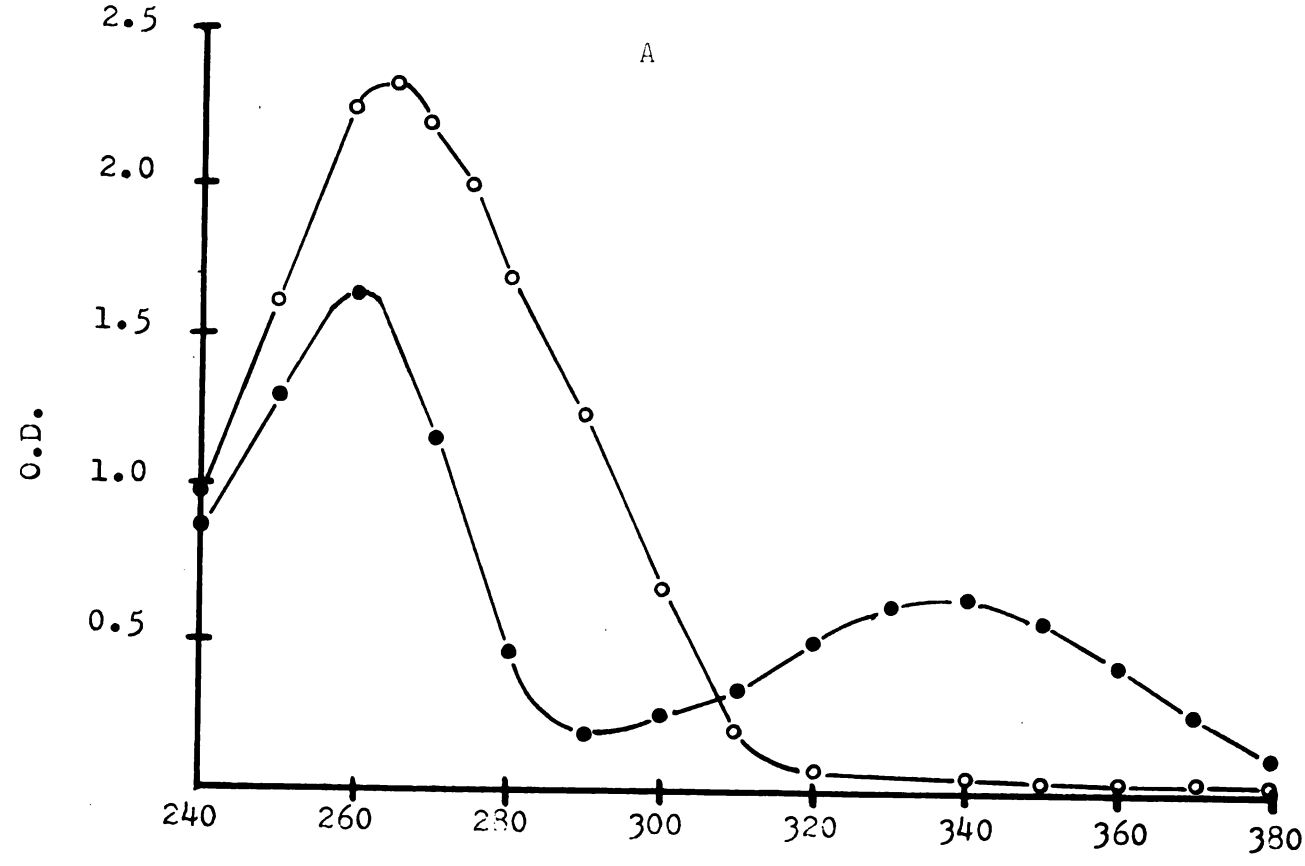


Figure 5.--Oxidation of NADH and of phosphodiesterase treated NADH by yeast. Aliquots of 1 ml from the control and phosphodiesterase treated solutions of NADH (Figure 4) were tested manometrically for O_2 uptake and CO_2 evolution by yeast cells. See Figure 1(A) for procedure. The Warburg vessels contained 1 ml glycine-HCl buffer (0.1M), pH 4.3, 20 mg cells, and substrates as indicated. The final pH was 4.5. The vessels containing the blank solutions (Figure 4) showed no O_2 uptake. Diesterase treated O_2 and CO_2, respectively, (●,o); NADH control O_2 and CO_2, respectively, (▲,Δ), endogeneous O_2 and CO_2, respectively (■,□).

Figure 6.--(A) Spectrum of NADH and the NADH acid product. A solution containing 0.104 µmoles of NADH per ml was compared with a solution of the same lot of NADH at the same concentration after acidification to pH 3.0 with HCl and incubation for 2 hr. NADH (●); acid treated NADH (○). (B) Spectrum of the NADH acid product after oxidation by yeast cells. Oxidation by yeast of 7.65 µmoles of NADH from the same stock solution used in A above was conducted as described in Figure 1(A). The cells were discarded, the supernatants diluted 1 to 20 and the spectrum determined.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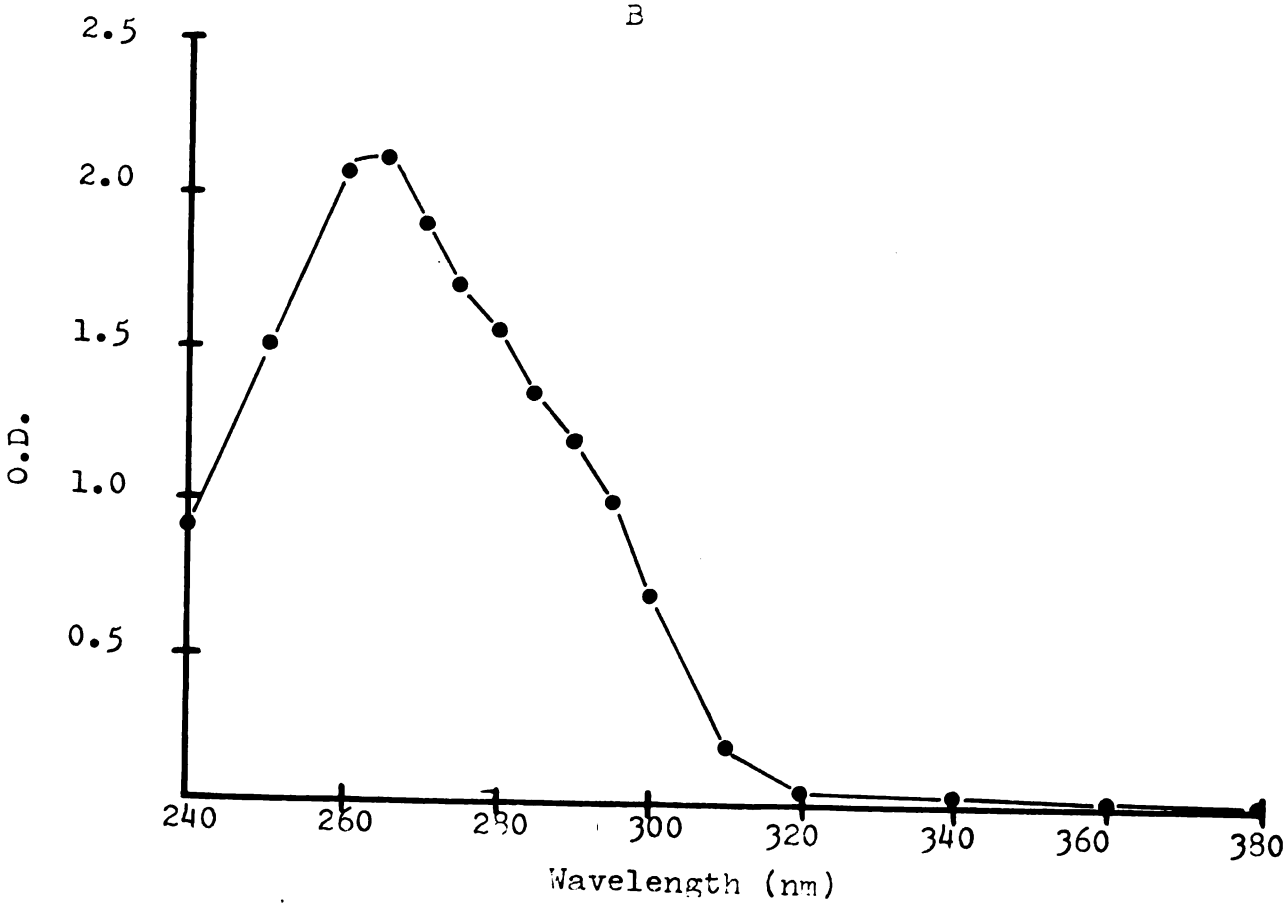
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Wavelength (nm)

characteristics of that compound. Also, yeast cells failed to show any oxygen uptake when solutions of commercial adenylic acid or of nicotinamide monomucleotide (NMN) were used as substrates in experiments such as outlined in Fig. 1 (A). These experiments provide increased evidence for the existence of some oxidizable component other than the NADH acid product in the solutions tested.

Studies With The NADH Contaminant

Separation on anion exchange resin

Direct evidence for the hypothesized contaminant was attained when 100 umoles of NADH (Sigma, lot #106B6550) was passed through a Dowex 1-X8, OH^- form column. The NADH was adsorbed and the eluate tested for oxidation with yeast. The NADH was eluted with HCl-NaCl buffer and similarly tested. The details and results of this experiment are summarized in Table 2. Although fractions I and II contained less than 0.2 umoles of NADH acid product, they accounted for literally all the O_2 uptake of all fractions when tested with yeast. Fraction IV contained 48 umoles of NADH acid product, 76% of the total recovered from the column, but supported no O_2 uptake with yeast. Fractions I and II potentially represent enough material for 78 umoles of O_2 uptake based on the actual oxidation of 0.8 ml of each fraction. The

TABLE 2.--Separation of an oxidizable contaminant from NADH by anion exchange chromatography[a].

Fraction #	pH of fraction	umoles NADH (acid product)/vessel	umoles O_2 uptake
I	12.0	.051	13.05
II	12.0	.046	2.59
III	10.5	.058	----
IV	9.5	9.9	.00
V	5.5	2.24	----
VI	5.0	0.8	----
13 umoles NADH	---	---	11.22

[a]One hundred umoles of NADH (Sigma, lot # 106B-6550) in 1.0 ml of H_2O was added to a Dowex 1-X8 column (see the methods section for details of preparation). Fraction I (4.0 ml) was collected by washing with H_2O. The column was then eluted with 3.9 ml of 5 x 10^{-4} M HCl in 0.1 N NaCl to collect fraction II and then with 3.9 ml volumes of 5 x 10^{-4} N HCl in 1.0 N NaCl for fractions III through VI. The pH of all fractions was adjusted to 4.0 - 4.6 with 2 N HCl and assayed at 260 nm for NADH acid product using an extinction coefficient of 2.5 x 10^{-4} M^{-1} cm^{-1} (7). O_2 uptake was measured manometrically and corrected for endogenous activity. See Fig. 1 (A) for procedure. The Warburg vessels contained 250 umoles potassium phthalate, 20 mg of cells and, where indicated, 0.8 ml of fractions I through VI, or NADH. The final pH was 4.2 - 4.4.

ratio of umoles of O_2 uptake to NADH in the control vessel suggests that this is 90% of the value expected if 100% of the contaminant had been recovered from the column. The contaminant must be either positively charged or neutral at high pH since it was not adsorbed by the column.

Free amino nitrogen determination

The Nessler's reaction was applied to determine if there was any free amino nitrogen in fraction I or in the oxidation product of fraction I. No significant amounts of free amino nitrogen were noted in either case (Table 3).

Effects of substrate drying on the oxidation

An attempt was made to concentrate the contaminant(s) separated in fraction I by drying under a vacuum at 40 C. However, when the sample was re-hydrated and tested with yeast for O_2 uptake, none was observed. This result suggested that the contaminant(s) was either volatile or inactivated by such treatment.

Table 4 summarizes the effects of various treatments of both an NADH solution and fraction I. The data indicate that drying at 40 C at an acid pH of either solution totally destroyed the oxidizable component(s). Drying the NADH solution and fraction I at a basic pH resulted in decreases in the O_2 uptake with yeast of

TABLE 3.--Determination of free amino nitrogen before and after oxidation of fraction I by yeast.[a]

Sample	umoles free amino nitrogen	umoles O_2 uptake
endogenous vessel (2.8 ml)	1.94	3.7
fraction I vessel (2.8 ml)	0.97	11.8
fraction I - untreated (0.5 ml)	0.17	---

[a]An amount of 0.5 ml of fraction I (prepared as outlined in Table 2 and stored at 5 C until use) was oxidized as outlined in Fig. 1 (A). The cells were spun down and discarded. The supernatants were stored frozen until the Nessler's determination for free nitrogen. Amounts of 0.7 ml of the endogenous and fraction I supernatants and 0.2 ml of unoxidized fraction I were brought to 2.0 ml volumes with H_2O. Free amino nitrogen was determined by direct nesslerization (17). The results, where indicated, are reported as umoles of free amino nitrogen per vessel or 0.5 ml of fraction I - untreated.

87.5% and 96.5% respectively. When an acid solution of fraction I was allowed to evaporate without heat, brought to volume and tested, no oxidation was detected. These results indicated that the contaminant was either volatile or unstable under acid or basic conditions.

The stochiometry of the oxidation of fraction I is similar to that of untreated NADH. The RQ, adjusted for endogenous activity, of the reaction was 0.67 (Fig. 7). Fig. 8 demonstrates the effect of carrying out the reaction at pH 7.0. The rate was lowered but the total

TABLE 4.--Effect of various treatments of NADH and fraction I on O_2 uptake by yeast.[a]

Substrate	Treatment	umoles NADH per vessel	umoles O_2 uptake[b]
NADH	none, pH 8.9	20.0	15.2
NADH	dried (40 C), pH 8.9	20.0	1.9
NADH	dried (40 C), pH 3.0	20.0	0.4
0.8 ml fraction I	none, pH 12.0	.04	12.5
"	dried (40 C), pH 12.0	.04	0.4
"	dried (40 C), pH 3.0	.04	0.0
"	dried (no heat), pH 3.0	.04	0.0

[a]180 umoles (based on 100% purity) of Sigma NADH (Lot #128B-6210) was made up in 1.8 ml H_2O. 0.2 ml aliquots (20 umoles) were treated as indicated and oxidized. The remaining 100 umoles in 1.0 ml was put through a Dowex 1 column. Fraction I was collected in 4 ml of H_2O and 0.8 ml aliquots were treated as indicated. Fractions were assayed as basic solutions at 340 mu for NADH. Acidification was with 0.1 N HCl, where indicated. Drying was accomplished with a vacuum rotary evapomix at 40 C. O_2 uptake was determined as outlined in Fig. 1(A). [b]The Warburg vessels contained 250 umoles potassium phthalate, 10 mg cells, and substrate, as indicated. The final pH was 4.1 - 4.6

[b]Adjusted for endogenous activity.

O_2 uptake was not significantly altered. This result might have been due to a decrease in substrate permeability at the higher pH. The endogenous O_2 uptake level was unchanged.

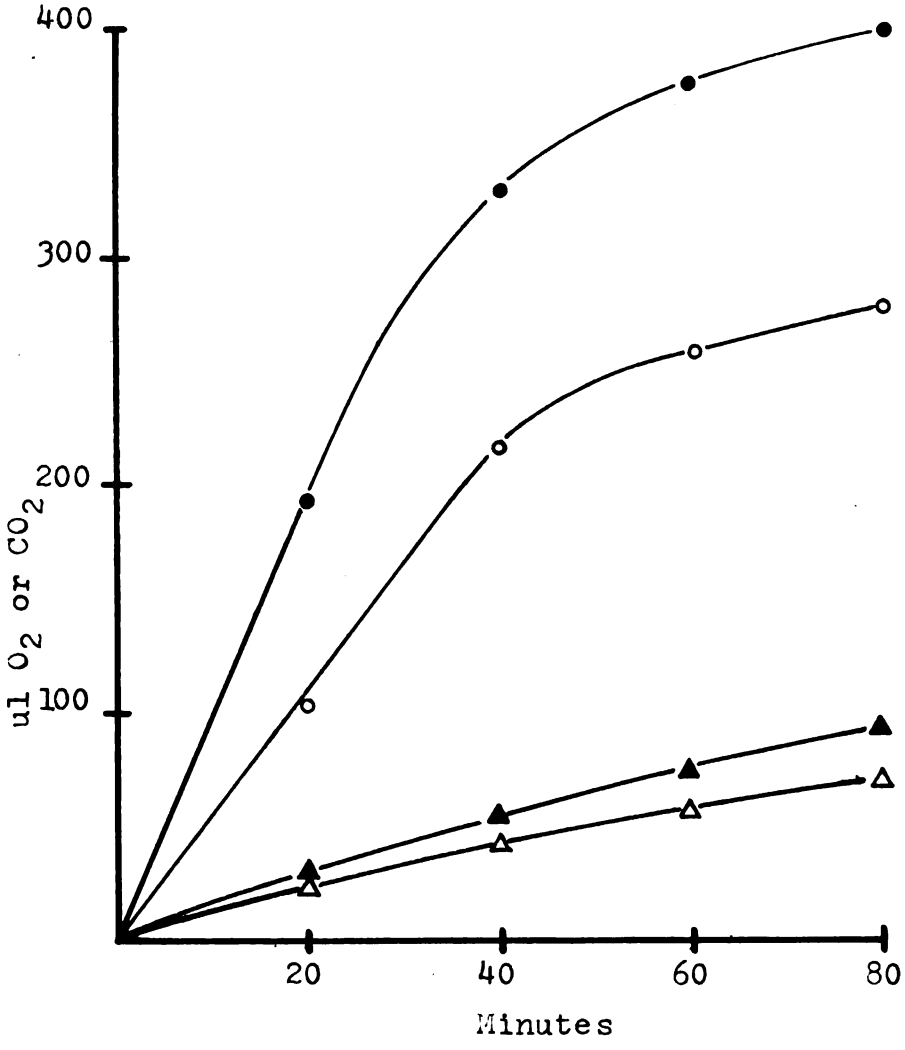

Figure 7.--Oxidation of fraction I by yeast cells at pH 4.0. See Figure 1(A) for procedure. The Warburg vessels contained 250 μmoles potassium phthalate, pH 4.0, 12.9 mg cells and, where indicated, 0.8 ml fraction I adjusted to pH 4.0 with 0.1N HCl. The final pH was 4.0. Fraction I, O_2 and CO_2, respectively (●,o); endogenous, O_2 and CO_2, respectively (▲,Δ).

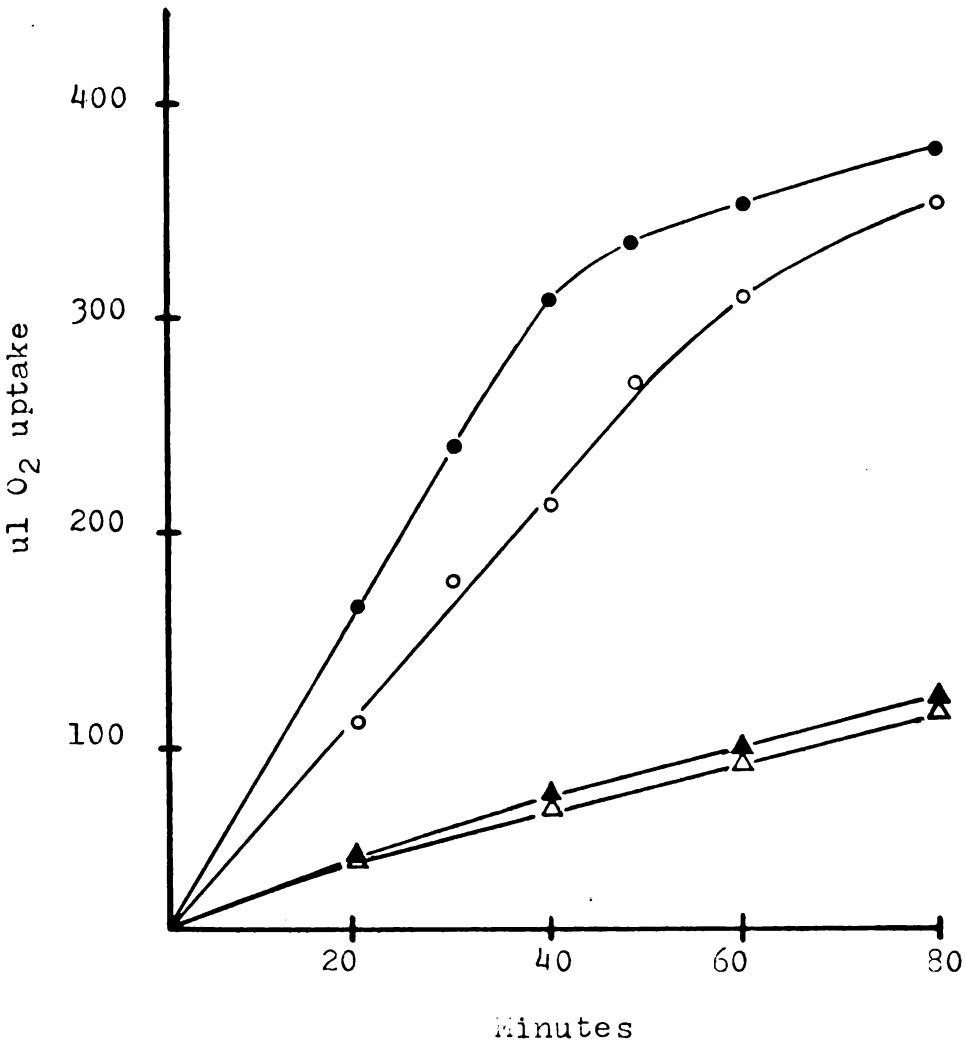

Figure 8---Oxidation of fraction I by yeast cells at pH 7.0. See Figure 1(A) for procedure. The Warburg vessels contained 12.9 mg cells and, where indicated, 250 µmoles potassium phthalate, pH 4.0, or 2000 µmoles potassium phosphate buffer, pH 7.2, and 0.8 ml fraction I, pH 12. Fraction I, pH 4.4 and 7.1, respectively (●,o); endogenous, pH 4.0 and 7.0, respectively (Δ,▲).

Further purification with cation exchange resin

To determine whether the contaminant was positively charged or neutral, fraction I was adjusted to pH 4.0 and added to a Dowex 50 W, H^+ form, cation exchange column and washed with water. The water eluate was tested for oxidation with yeast as shown in Fig. 9. Based on the fraction I control from the anion exchange resin, 91% of the activity was recovered after passing fraction I through the cation exchange resin. The results suggested that the contaminant was uncharged at pH 4.0.

Absorption spectrum of the contaminant

An amount of 0.5 ml of the eluate from the acidic resin was added to 0.5 ml H_2O, and the absorption spectrum recorded from 200 to 950 nm using H_2O as a blank. This amount of eluate in 1.0 ml represents a potential of approximately 4 umoles of O_2 uptake with yeast. No significant absorption peaks were observed.

Studies Using Inhibitors of Cellular Metabolism

Azide is known to inhibit respiration in yeast by blocking electron transport. When the oxidation of fraction I by yeast was allowed to proceed in the presence of azide (3.3×10^{-3} M), both O_2 uptake and CO_2 evolution were completely inhibited (Table 5).

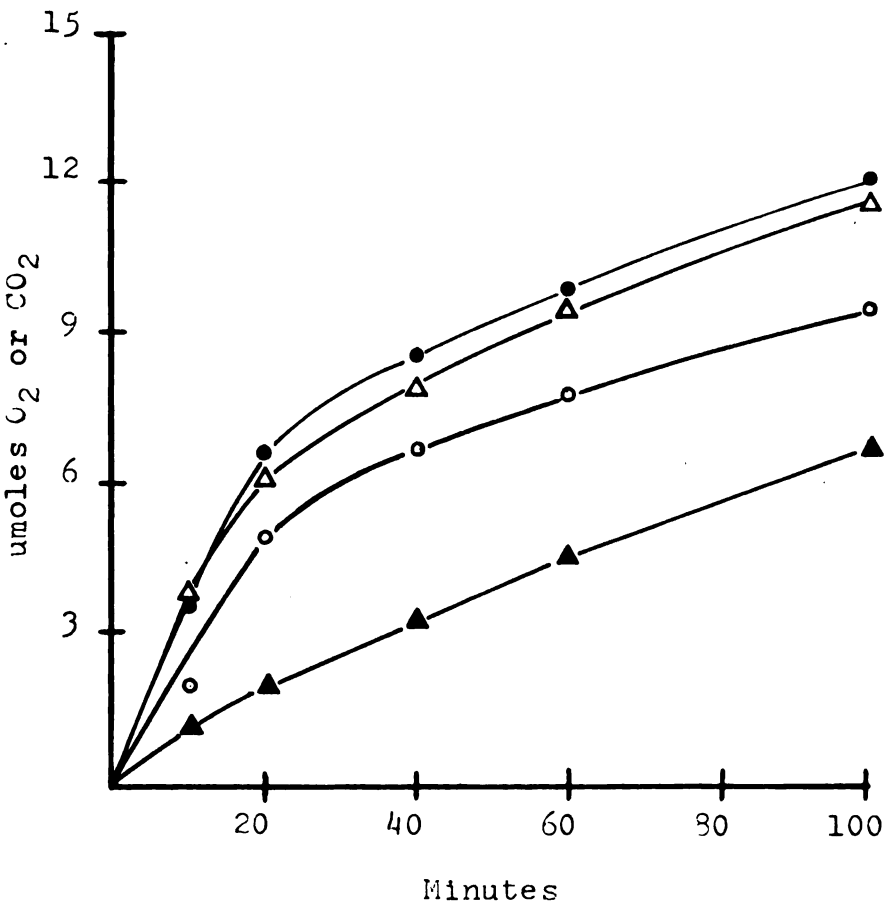

Figure 9.--Oxidation of fraction I by yeast cells after passage through a cation exchange column. An amount of 100 µmoles of NADH (Sigma Lot #128B-6210) in 1 ml H_2O was added to a Dowex I-X8, OH^- form, column. Fraction I (3.8 ml) was collected by washing with H_2O. An amount of 0.9 ml of fraction I was acidified to pH 4.0 with HCl and added to a Dowex 50 W, H^+ form, column, and fraction II (3.0 ml) collected by washing with H_2O. O_2 uptake and CO_2 evolution were determined as outlined in Figure 1(A). The Warburg vessels contained 250 µmoles potassium phthalate, pH 4.0, 26 mg yeast cells and, where indicated, 0.2 ml of fraction I or 0.8 ml of fraction II. The final pH was 4.0 - 4.1. Eluate from Dowex I-X8 (fraction I), O_2 uptake (Δ); eluate from Dowex 50 W (fraction II), O_2 uptake and CO_2 evolution, respectively (●,o); endogenous, O_2 uptake (▲).

TABLE 5.--Effect of azide, malonate and iodoacetate on the oxidation of fraction I by yeast cells.[a]

Experiment	Substrate	Inhibitor	umoles CO_2	umoles O_2	% inhibition CO_2	% inhibition O_2
A	endogenous		3.34	3.17		
	endogenous	azide	2.2	0.21	34	93.4
	pyruvate		13.9	13.5		
	pyruvate	azide	0.0	0.0	100	100
	fraction I		3.52	5.25		
	fraction I	azide	0.1	0.0	97.2	100
B	endogenous		3.4	4.0		
	endogenous	malonate	1.64	1.23	47.7	69.2
	pyruvate		1.96	2.12		
	pyruvate	malonate	0.46	0.88	76.5	58.5
	fraction I		1.99	4.10		
	fraction I	malonate	0.0	1.81	100	55.9
C	endogenous			4.22		
	endogensou	iodoacetate		.07		98
	glucose			12.4		
	glucose	iodoacetate		0.0		100
	pyruvate			13.5		
	pyruvate	iodoacetate		.21		98
	fraction I			6.1		
	fraction I	iodoacetate		.08		99

[a]Table 5.--Effect of azide, malonate, and iodoacetate on the oxidation of fraction I by yeast cells. See Fig. 1 for procedure. The % inhibition was adjusted for endogenous activity with or without the appropriate inhibitor.

Experiment A. Vessels contained 23 mg cells, 250 umoles potassium phthalate, and 10 umoles sodium azide, 0.2 ml fraction I acidified to pH 2.0 with 2 N HCl, or 14.8 umoles potassium pyruvate, where indicated. The final pH was 4.0.

Experiment B. Vessels contained 20 mg cells, 2000 umoles potassium phosphate (pH 6.2) and 2500 umoles sodium malonate, 0.2 ml acidified fraction I, or 7.4 umoles potassium pyruvate, where indicated. The fianl pH was 6.2 to 6.3.

Experiment C. Vessels contained 23 mg cells, 250 umoles potassium phthalate and 13.3 umoles iodoacetic acid, 10 umoles glucose, 0.3 ml of fraction I or 7.4 umoles potassium pyruvate, where indicated. The final pH was 4.0 - 4.1.

Malonate (8.3×10^{-1} M) was employed to study the effects of inhibition of the TCA cycle on the metabolism of fraction I. Malonate is a competitive inhibitor which, at sufficient concentration, ties up succinate dehydrogenase preventing the formation of fumerate with the resultant accumulation of succinate. The reaction was run at pH 6.2 in the presence of high concentrations of potassium phosphate buffer since it was impossible to lower the pH further with the quantity of malonate needed for inhibition without adding excessive amounts of ions. To overcome the error due to the solubility of CO_2 in the buffered vessels at this pH, the solubility coefficient α' at pH 6.3 was used to compute the KCO_2 values used in measuring CO_2 evolution (17).

The results indicated that CO_2 evolution was inhibited 100% while O_2 uptake was inhibited 56%. However, this may be in error due to the method used in estimating CO_2.

Iodoacetate (4.4×10^{-3} M) inhibited oxidation of both fraction I and pyruvate by 99%. This data indicates that the oxidation is dependent on a sulfhydryl containing enzyme(s).

DISCUSSION

Data has been presented showing that some commercial preparations of NADH and related nucleotides contain some component(s), other than the nucleotides themselves or their acid modification products, which supports O_2 uptake and CO_2 evolution by yeast cells. The oxidizable componant(s) was not adsorbed on either anionic (pH 12) or cationic (pH 4.0) exchange resins; and, thus, is believed to be uncharged.

Drying at 40 C under vacuum of an NADH solution or separated contaminant at either acid or basic pHs resulted in the loss of the component(s) oxidizable by yeast cells. Evaporation at an acid pH without heat gave the same result. Either the component(s) is volatile under these conditions or it is inactivated by drying with or without heat, or both. Drying an NADH solution with heat at basic pHs lead to a recovery of 12.5% of the original O_2 uptake. Lyophylization at various pHs was not attempted.

The RQ from the oxidation of the separated component(s) was 0.67 suggesting that it might be a lipid or fatty acids. However, this seems unlikely since fats would not be expected to be volatile or destroyed by the treatments employed. Since fatty acids are negatively charged, they would not likely pass

through the strong anion exchange columns employed to separate the contaminant from NADH.

The compound(s) appears to be colorless and showed no characteristic absorption peaks between 220-440 nm. If one assumes that only 1 umole of material is required to yield even 4 umoles of O_2 uptake than at least 1 umole of material/ml was tested for light absorption. Saturated hydrocarbons, alcohols and ethers are transparent in this region. Since monofunctional olefins, acetylenes, carboxylic acids, esters, amides and oximes have absorption maxima at 200 nm, non-specific absorption in this area might have blanked out absorption from any one of these compounds under the conditions employed (16). Aldehydes, ketones, aliphatic nitro compounds and nitrate esters have absorption maxima near the ultraviolet region but the intensities are very low. Acetone, for example, has a molecular extinction coefficient of 16 at 270 nm and such a compound would certainly have been missed if only a few umoles of material was present as was probably the case with the oxidizable contaminant.

The inhibition of the oxidation noted with azide indicates that the oxidation proceeds via ETS. Anderson's (1) observation that the oxidation of "NADH" proceeded in the presense of KCN with methylene blue as an electron accepter, indicated that flavo proteins were involved in the oxidation.

Malonate at high concentrations inhibited CO_2 evolution 100% while inhibiting O_2 uptake 56%. As pointed out previously, the extent of inhibition of CO_2 evolution by malonate might have been in error due to the method used in estimating CO_2 at the higher pH of the reaction. Since the oxidation (Fig. 8), at a neutral pH, proceeded without a loss in total O_2 uptake compared to the reaction at an acid pH, the results with malonate are meaningful, however limited. The metabolism of the contaminant seems to involve, at least in part, the TCA cycle.

Iodoacetate inhibited the oxidation of the contaminant by 99%. However, no conclusions can be drawn concerning the role of the glycolytic pathway since O_2 uptake with pyruvate was inhibited to the same extent. Since iodoacetate is known to combine with sulfhydryl group containing enzymes thus rendering them inactive, the oxidation seems to be dependent on a sulfhydryl containing enzyme(s). Anderson's (1) observation that the oxidation of "NADH by whole cells was prevented by DNP indicated that active transport was required. Glucose was unaffected, presumably because its oxidation proceeded via the glycolytic pathway thus supplying its own ATP for active transport through substrate level phosphorylation. This indicates that the metabolism of the contaminant possibly does not proceed via glycolysis or DNP would have had no effect, as with glucose.

Addition products between pyridine nucleotides and a number of nucleophilic substances have been characterized and the possible relation of these compounds to the contaminant should be discussed. Carbonyl addition reactions are thought to take place at the 4 position of oxidized NAD through the formation, in the presence of alkali, of carbonyl anions (2, 5, 6, 8). Such active carbonyl compounds include dihydroxyacetone, glyceraldehyde, acetone and its α-substituted halide derivatives and acetophenone. The rate of addition of these to NAD is related to the nucleophilic strength of the α group. The addition compounds have spectra almost identical with that of NADH and form almost identical acid modification products. They differ, however, from NADH in that they are inactive as coenzymes in dehydrogenase systems. Other addition compounds of NAD with hydroxylamine (3), mercaptans (18), organic sulfinates (21), imidazoles (19), and para aminobenzoic acid and related compounds (10) have been described.

The reactivity of the pyridinium ring to the above mentioned compounds presents the possibility that the contaminant found in some commercial preparations of NADH might be unobstrusively formed during coenzyme purification from yeast as an addition product with the coenzyme. In this form, the contaminant might escape notice when assayed spectrophotometrically. However, this hypothesis might reasonably be excluded since the

addition compounds are inactive as coenzymes. The nucleotides present in the commercial preparations used in these experiments seemed equivalent both coenzymatically and spectrophotometrically on a dry weight basis. Organic sulfinates, on the other hand, form addition products which can be converted to coenzymatically active reduced NADH simply by neutralization. However, these compounds and in fact all the addition compounds mentioned, seem unlikely as candidates for the oxidizable contaminant since most of them form stable addition products with NAD and many of them are either positively or negatively charged compounds. None of these compounds would have been expected to pass through the strongly acidic or basic columns used to separate the contaminant from NADH.

The potent inhibitors described by Fawcett et al. (9) would also seem to be unlikely as oxidizable contaminants since they appear to be nucleotides formed only in frozen solutions of NADH, and attempts to separate them from NADH have failed.

Based on 340 nm absorbancies, the commercial lots of NADH were only about 65-75% pure. Thus, there could be considerable amounts of a variety of contaminants present based on dry weight. For example, a weight containing 10 umoles of NADH would contain 1.77 to 2.48 mg of extraneous matter including oxidized NAD. If a contaminant had a molecular weight of 100, then this would represent 17.7 to 24.8 umoles of material.

This would be more than required to yield the results obtained with the Sigma NADH even if only 1 umole of O_2 was consumed per umole contaminant. The possibility that the contaminant is a smaller compound and/or the oxidation of a umole of material contributes more than a umole of O_2 uptake emphasizes the fact that much less than 50% of the extraneous matter present in a sample of NADH need actually be contaminant to yield typical results.

The identification of the contaminant cannot be made on the basis of the results presented in this thesis alone. NADH is very expensive and one would have to find a method for concentration and purification of this material in order to proceed with an accurate identification.

SUMMARY

During the ivestigation of the oxidation of the primary acid modification product of NADH by yeast cells, it was observed that the extent of oxygen uptake by the cells was much higher when some brands of NADH were used than when equivalent amounts of other brands were tested. This lead to the hypothesis that a contaminant(s) present in some preparations and not the acid product of the coenzyme was actually responsible for the oxidation.

That the contaminant(s) actually existed was demonstrated by the spearation of an oxidizable fraction from NADH through ion exchange chromatography. The contaminant(s) was characterized as a neutral compound with no characteristic absorption spectrum between 220-440 nm, which was either volatile or unstable when dried at acid or basic conditions under a vacuum at 40 C. The RQ of the oxidation was in the order of 0.67, and no free amino nitrogen was released during the oxidation. Use of metabolic inhibitors indicated that the oxidation by yeast was dependent on the electron transport system, the tricarboxylic acid cycle and sulfhydryl containing enzymes.

Unfortunately, the identity of the contaminating compound(s) remains obscure. The characteristics observed appear to eliminate a number of substrates readily

oxidized by yeast cells; e.g., stable sugars, acids, and stable glycolytic intermediates. The low levels of the oxidizable component(s) present, the high cost of NADH preparations, and the difficulty in concentrating the contaminant(s) has precluded further efforts toward identification at this time.

BIBLIOGRAPHY

1. Anderson, T. E. 1962. The primary site of inhibition of yeast respiration by sorbic acid. Ph.D. Thesis, Michigan State University.

2. Burton, R. M., and N. O. Kaplan. 1954. The reaction of pyridine nucleotides with carbonyl compounds. J. Biol. Chem. 206: 283-297.

3. Burton, R. M., and N. O. Kaplan. 1954. A chemical reaction of hydrozylamine with diphosphopyridine nucleotide. J. Biol. Chem. 211: 447-463.

4. Burton, R. M., and N. O. Kaplan. 1963. The reaction of reduced pyridine nucleotide with acid. Arch. Biochem. Biophys. 101: 150-159.

5. Burton, R. M., A. San Pietro, and N. O. Kaplan. 1957. Preparation of pyridine nucleotide analogs by the carbonyl addition reaction. Arch. Biochem. Biophys. 70: 87-106

6. Burton, R. M., and N. O. Kaplan. 1957. Enzymatic properties of chemically prepared diphosphopyridine nucleotide analogs. Arch. Biochem. Biophys. 70: 107-120.

7. Chaykin, S., J. O. Meinhart, and E. G. Krebs. 1956. Isolation and properties of a reduced diphosphopyridine nucleotide derivative. J. Biol. Chem. 220: 811-820.

8. Colowick, S. P., N. O. Kaplan, and M. M. Ciotti. 1951. The reaction of pyridine nucleotide with cyanide and its analytical use. J. Biol. Chem. 191: 447-459.

9. Fawcett, C. P., M. M. Ciotti, and N. O. Kaplan. 1961. Inhibition of dehydrogenase reactions by a substance formed from reduced diphosphopyridine nucleotide. Biochem. Biophys. Acta. 54: 210-212.

10. Guardiola, A. L., D. Paretsky, and W. McEwen. 1958. The interaction of DNP with p-amino benzoic acid and analogous compounds. J. Am. Chem. Soc. 80: 418-421.

11. Haas, E. 1936. Notiz über das Verhalten der Absorption-spektren der Dihydro-pyridinverbindungen. Biochem. Z. 282: 157-205.

12. Johnson, M. J. 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137: 575-586.

13. Kornberg, A. 1955. Lactic dehydrogenase of muscle. p. 441-443. In S. P. Colowick, and N. O. Kaplan (ed.), Methods in enzymology, Vol. 1, Academic Press, Inc., New York.

14. Rafter, G. W., S. Chaykin, and E. G. Krebs. 1954. The action of glyceraldehyde-3-phosphate dehydrogenase on reduced diphosphopyridine nucleotide. J. Biol. Chem. 208: 799-811.

15. Razzell, W. E., and H. G. Khorana. 1959. Studies on polynucleotides. III. Enzymatic degradation. Substrate specificity and properites of snake venom phosphodiesterase. J. Biol. Chem. 234: 2105-2117.

16. Shriner, R. L., R. C. Fuson, and D. Y. Curtin. 1965. The systematic indentification of organic compounds. p. 206-219. Academic Press, Inc., New York.

17. Umbriet, W. W., R. H. Burris, and S. F. Stauffer. 1964. Manometric techniques. 4th ed. Burgess Publishing Co., Minneapolis 15, Minn.

18. van Eys, J., and N. O. Kaplan. 1957. The addition of sulfhydryl compounds to diphosphopyridine nucleotide and its analogues. J. Biol. Chem. 228: 305-314.

19. van Eys, J. 1958. The reaction of quaternary pyridine derivatives with imidazoles. J. Biol. Chem. 233: 1203-1209.

20. Warburg, O., W. Christian, and A. Griese. 1935. Wasserstoffübertragendes co-frement, seine zusammensetzung und wirkungsweise. Biochem. Z. 282: 157-205.

21. Yarmolinsky, M. B., and S. P. Colowick. 1956. On the mechanism of pyridine nucleotide reduction by dithionite. Biochem. et Biophys. Acta. 20: 177-189.