REGULATION OF PYRIDINE NUCLEOTIDE METABOLISM IN SACCHAROMYCES CEREVISIAE

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

BY

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The levels of total nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), and their redox states were determined as the function of growth in S. cerevisiae. Cells growing in a medium containing 0.8% glucose exhibit two phases of exponential growth, utilizing glucose and ethanol, respectively. The NAD pool is 50% reduced during both stages of growth while the NADP pool is 67% reduced in glucose growth and 48% reduced in ethanol growth. The NAD/NADP ratio is constant during growth on glucose and a two-fold increase in the NAD/NADP ratio occurs upon exhaustion of glucose. The increased ratio is maintained during growth on ethanol.

This alteration in the regulation of the relative levels of NAD and NADP may be due to a change in the regulation of NAD kinase and/or NADP phosphatase activities. These changes may be related to the redox state of the NADP pool.

INTRODUCTION

The pyridine nucleotides[1], nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are present in all living cells and act as coenzymes for a large and diverse number of cellular oxidation-reduction enzymes. NAD^+ is normally used in catabolism and NADPH is a primary source of reducing equivalents for anaerobic pathways such as the biosynthesis of fatty acids and of deoxyribonucleotides. Recently other roles for pyridine nucleotides in metabolism have become apparent. NAD^+ serves as the substrate for the enzyme DNA ligase in Escherichia coli (Laipis et al., 1969), and also for poly (ADP-ribose) polymerase in rat liver (Sugimura, 1973; Honjo and Hayaiski, 1973).

The biosynthetic pathways which produce the pyridine nucleotides have not been completely characterized, although they have been studied in bacteria, fungi, and mammals. Quinolinic acid is a common intermediate in at least three different pathways of de novo biosynthesis of NAD in various organisms. These include the tryptophan pahtway present in Neurospora (Partridge, 1952) and mammals (Henderson and Hankes, 1956);

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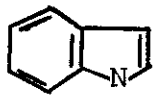
[1]The terms NAD and NADP have been used in context to represent the total pyridine nucleotide pool. NAD^+, NADH and $NADP^+$, NADPH have been used to denote the specific oxidezed and reduced forms.

the formylaspartate pathway of Clostridia (Scott et al., 1969); and the aspartate-three-carbon fragment pathway of higher plants and bacteria (Gholson, 1966). Yeast is capable of converting tryptophan to quinolinic acid under aerobic conditions (Scheme I) (Ahmad and Moat, 1966; Schott and Staudinger, 1971). In this tryptophan-quinolinic acid pathway, two of the enzymatic steps, tryptophan pyrrolase and 3-hydroxyanthranilic acid oxidase require molecular oxygen for activity. Thus, under anaerobic conditions, yeast utilize an alternative pathway for the biosynthesis of quinolinic acid from aspartate and glutamate (Ahmad and Moat, 1966; Heilmann and Lingens, 1967). No evidence has yet been obtained for the formation of a stable free intermediate between aspartate and quinolinic acid.

Two salvage routes for the biosynthesis of pyridine nucleotides from nicotinic acid or nicotinamide have been described (Scheme II) (Preiss and Handler, 1958; Nishizuka and Hayaishi, 1963). Nicotinic acid can be converted to NAD by a salvage pathway that depends upon phosphoribosyl pyrophosphate (PRPP) and ATP, and is accomplished by three consecutive reactions in which nicotinic acid mononucleotide (NicMN) and nicotinic acid adenine dinucleotide (dNAD) are formed as intermediates. This pathway is often referred to as the "Preiss-Handler Pathway". The enzymes, nicotinate phosphoribosyl transferase, dNAD pyrophosphorylase and NAD synthetase, catalyze these reactions and these enzymes have

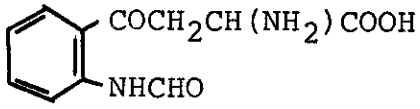
SCHEME I. The de novo pathways of NAD biosynthesis in yeast

AEROBIC



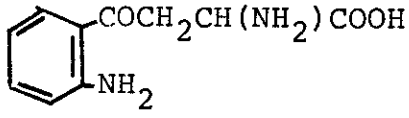
Tryptophan

O_2 (Tryptophan pyrrolase)



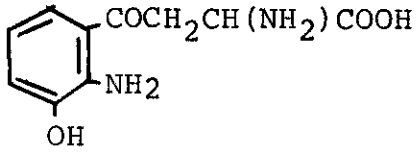
N-Formyl kynurenine

(N-Formyl kynurenine formamidase)

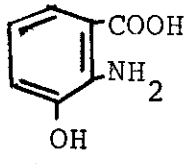


Kynurenine

O_2 (Kynurenine3-hydroxylase)



3-Hydroxykynurenine



3-Hydroxyanthranilic acid

O_2 (3-hydroxyanthranilic acid oxidase)

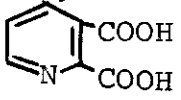
ANAEROBIC

Aspartate +

C_3-Fragment

O

O

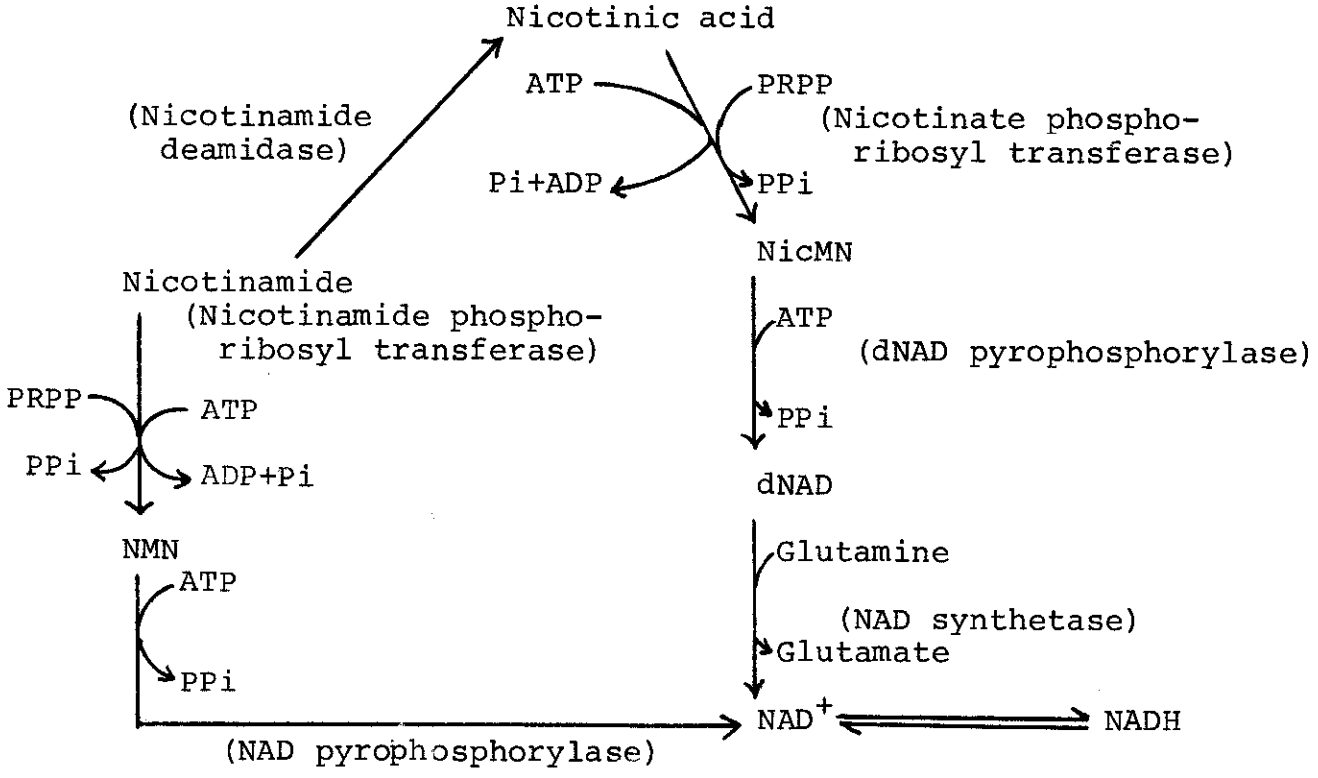


Quinolinic acid

(Quinolinate phosphoribosyl transferase)

Pyridine nucleotide cycles

SCHEME II. Alternate salvage pathways of NAD biosynthesis in yeast.


Nicotinic acid
ATP
PRPP
(Nicotinamide deamidase)
(Nicotinate phospho-ribosyl transferase)
Pi+ADP
PPi
NicMN
Nicotinamide
(Nicotinamide phospho-ribosyl transferase)
ATP
(dNAD pyrophosphorylase)
PRPP
ATP
PPi
PPi
ADP+Pi
dNAD
NMN
Glutamine
ATP
(NAD synthetase)
Glutamate
PPi
NAD+
NADH
(NAD pyrophosphorylase)

been partially purified and studied (Preiss and Handler, 1958). Nicotinamide can be converted to NAD via the Preiss-Handler pathway after conversion to nicotinic acid by nicotinamide deamidase in most bacteria, such as Mycobacterium phlei and M. tuberculosis (Halpern and Grossowioz, 1957) and Aspargillus niger (Sarma et al., 1964), and in rabbit liver (Su et al., 1969). Nicotinamide can also form NAD with nicotinamide mononucleotide (NMN) as an intermediate in animal tissue (Dietrich et al., 1968) and in Lactobacillus fructosus (Ohtsu et al., 1967). This latter salvage route has been termed the "Dietrich Pathway".

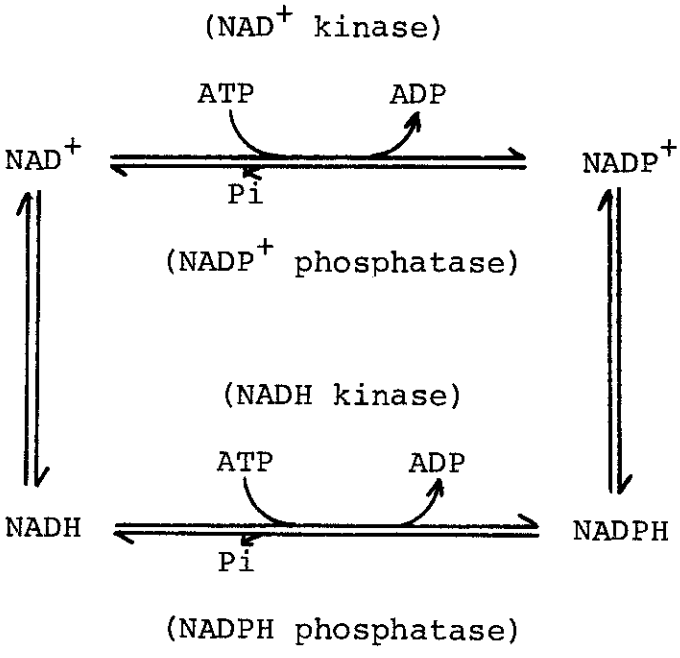
Chaykin and co-workers have studied nicotinamide metabolism in a nicotinamide-requiring yeast (Chaykin et al., 1967). Nicotinamide was rapidly taken up by the cells and converted to NAD. Intermediates were not detected, and the appearance of NADP in the cells lagged behind that of NAD. Although the Preiss-Handler pathway has been shown to exist in yeast (Preiss and Handler, 1958), the pathway of utilization of nicotinamide is unclear since NAD at physiological concentrations is a competitive inhibitor of nicotinamide deamidase in yeast (Calbreath and Joshi, 1971).

The interconversion of NAD nucleotides and NADP nucleotides is shown in Scheme III. NAD is converted to NADP by phosphorylation via NAD kinase (ATP:NAD 2'-phosphotransferase). At least two NAD kinases exist in yeast. A cytoplasmic enzyme is specific for NAD^+ and is inhibited by NADH (Apps, 1970);

and a mitochondrial enzyme is specific for NADH as substrate (Griffiths and Bernofsky, 1972). NAD kinase had also been partially purified and studied in other organisms including Azotobacter vinelandii (Chung, 1967; 1968), rat liver (Yero et al., 1968) and pigeon liver (Wang and Kaplan, 1954). NADP is converted to NAD by the action of NADP phosphatases. NADP can be converted to NAD in vitro by treatment with intestinal phosphatase. Specific NADP phosphatases have not been isolated or characterized as yet, although activity capable of regenerating NAD by hydrolytic cleavage of the 2'-phosphate of NADP has been reported in preparations of yeast mitochondria (Bernofsky and Utter, 1968). Several lines of evidence suggest that the rate of interconversion of NAD and NADP is rapid compared to the rate of pyridine turnover (Lundquist and Olivera, 1973; Stollar and Kaplan, 1961).

The mechanisms by which the cellular levels of nicotinamide nucleotides are maintained within cells are still poorly understood. However the importance of their levels in fungi have been shown by previous studies on the biochemical basis of morphogenesis in Neurospora crassa. A decrease of reduced nicotinamide adenine dinucleotide phosphate (NADPH) dramatically affects the morphology of the hyphae, leading to a decreased apical growth rate and branched, bulbous type of cells (Brody, 1970).

SCHEME III. The interconversion of NAD and NADP nucleotides.


(NAD+ kinase)
ATP
ADP
NAD+
NADP+
Pi
(NADP+ phosphatase)
(NADH kinase)
ATP
ADP
NADH
NADPH
Pi
(NADPH phosphatase)

Yeast is a favorable organism to use for the study of the metabolism of the pyridine nucleotides. It can be grown as a homogeneous cell type in a defined environment. The principle organelles of mammalian cells have their counterparts in yeast and the use of nicotinamide-requiring strains can insure good incorporation of labeled precursors into pyridine nucleotides. Furthermore, yeast grown in a complex medium containing limiting glucose under aerobic conditions, exhibit two phases of exponential growth. In the first exponential phase, glucose is consumed and glycolysis is the major metabolic pathway; in subsequent growth, a second exponential phase requiring functional mitochondria is maintained by a respiratory metabolism using the products of glucose fermentation.

In this study, the regulation of the levels of NAD and NADP nucleotides in yeast is examined. Efficient methods for extraction of the oxidized and reduced pyridine nucleotides from yeast were found. The cellular levels of total nicotinamide nucleotides, the relative levels of NAD and NADP, and the redox states of NAD and NADP pools were determined in yeast under different growth conditions. The changes of these nucleotide levels are discussed and compared with those in the literature.

MATERIALS AND METHODS

Materials

Bicine, cycloheximide, chloramphenicol, phenazine ethosulfate, MTT tetrazolium, L-leucine, D,L-isocitric acid, bovine serum albumin, NAD^+, NADH, $NADP^+$, NADPH, alcohol dehydrogenase (Cat. No. A1762) and isocitrate dehydrogenase (Cat. No. I 2002) were obtained from the Sigma Chemical Co. $CaCl_2$, NaOH, KOH, $MgSO_4$, KH_2PO_4, dextrose, $MgCl_2$, and phenol reagent solution were obtained from Fisher Scientific Co. $(NH_4)_2SO_4$ was from Baker Chemical Co. Agar, Bacto-yeast extract, Bacto-Peptone, and Bacto-yeast nitrogen base without amino acids were obtained from the Difco Laboratory. Membrane filters were purchased from Gelman, Omnifluor was from New England Nuclear and L-(4,5-^{3}H)-leucine (specific activity = 55 mCi/mmol) was from Amersham/Searle Co.

Methods

Organism and Culture Conditions

Saccharomyces cerevisiae (isolated from commercial Red Star yeast) was used in this study and was maintained on 1% yeast extract, 2% Bacto-Peptone, 2% glucose agar plates. Yeast cells were grown in a liquid complex medium which contained 0.8% glucose, 1.0% yeast extract, 0.5% Bacto-Peptone, and salts: 0.9% KH_2PO4, 0.5% $MgSO_4$, o.6% $(NH_4)_2SO_4$, and 0.03% $CaCl_2$. Cells were also grown in a semidefined medium contain-

ing 0.8% glucose, 0.1% yeast extract, and 0.67% Difco nitrogen base without amino acids. All cultures were grown at 30C on a rotary incubator. Inoculums were grown for 24 hours and were in stationary phase when experimental cultures were initiated by injecting fresh medium with a 1% volume of inoculum. Culture growth was monitored turbidimetrically by measuring the absorbance at 600_{nm} in a Beckman 25 spectrophotometer. When the absorbance readings exceeded 0.4, the samples were diluted until the readings fell below 0.4. Cell numbers were determined using a Petroff-Hauser counting chamber.

Medium Transfers, Glucose phase cultures. Duplicate 50 ml aliquots of a log phase culture, corresponding to 1 to 1.5 $A_{600\ nm}$ turbidimetric units per ml, growing on glucose in the semidefined medium were filtered through membrane filters (47 mm diam. 0.45μ pore size). Filtered cells were washed with 30 ml of fresh medium and resuspended in 50 ml of medium containing: (1) 0.37% (80 mM) ethanol, 0.1% yeast extract, and 0.67% Difco nitrogen base without amino acids; or (2) 0.53% (35 mM) glucose, 0.1% yeast extract and 0.67% Difco nitrogen base without amino acids. Less than 5 minutes was required for the transfers.

Ethanol phase cultures. Duplicate 10 ml aliquots of a log phase culture, corresponding to 7.6 - 8.6 $A_{600\ nm}$ turbidimetric units per ml, growing on semidefined medium in the ethanol phase were shifted to 100 ml of medium containing:

(1) 0.8% (40 mM) glucose, 0.1% yeast extract, and 0.67% Difco nitrogen base without amino acids; or (2) 0.32% (70 mM) ethanol, 0.1% yeast extract, and 0.67% Difco nitrogen base without amino acids.

Medium Containing Protein Synthesis Inhibitors. Transfers from glucose medium to ethanol medium and to glucose medium as control were done in the presence of either 4.0 mg/ml of chloramphenicol, or 2 µg/ml of cycloheximide (Clark-Walker and Linnane, 1966) with cultures growing in the semidefined medium.

Extraction of Pyridine Nucleotides

Acid Extraction. Duplicate aliquots of culture medium corresponding to 5 to 10 $A_{600\ nm}$ turbidimetric units were harvested by centrifugation at 1500 x g for 10 minutes, and the medium was removed by aspiration. The oxidized pyridine nucleotides were extracted by adding 2.0 ml of 0.5 M $HClO_4$ and incubating at 60C for 10 minutes with stirring. This treatment destroys reduced pyridine nucleotides. The extracts were chilled and centrifuged at 1500 x g for 10 minutes, and the supernatants were adjusted to pH 7.0-7.5 by adding 1.0 M KOH, 0.33 M K_2HPO_4-KH_2PO_4, pH 7.5, with rapid mixing. After standing on ice for at least 15 minutes, the insoluble $KClO_4$ was removed by centrifugation at 1500 x g for 10 minutes, and the final supernantant fractions were stored frozen until assayed for pyridine nucleotide.

Alkaline Extraction. Reduced pyridine nucleotides were extracted by adding 2.0 ml of 0.25 M NaOH and incubating at 60C for 10 minutes with stirring. This treatment destroys the oxidized pyridine nucleotides. The extracts were chilled and centrifuged at 1500 x g for 10 minutes, and the supernatants were neutralized to pH 7.2-7.5 by adding 0.37 M H_3PO_4 with rapid mixing. Phenazine ethosulfate (2mM) was added to a final concentration of 0.16 mM with vortex mixing, and the samples were placed in the dark for 10 minutes at room temperature. This treatment oxidizes the reduced pyridine nucleotides.

Estimation of Total Pyridine Nucleotide and Redox State. The total NAD or NADP nucleotides in the cells was taken as the sum of the amount of pyridine nucleotide measured in the acid and alkaline extracts. However, since the centrifugation of the cells might be expected to alter the redox state of the pyridine nucleotide pools (Lundquist and Olivera, 1971), the relative amounts of oxidized and reduced forms obtained by this method do not necessarily reflect the in vivo redox state of the pyridine nucleotides. In order to obtain estimates of the redox state of the pools in vivo, NaOH was added directly to cultures to a final concentration of 0.25 M with rapid mixing to inactivate all enzymatic activities and to extract the reduced pyridine nucleotides. The mixing was achieved by rapid vortex mixing of the culture immediately

(less than 1 second) after the addition of the NaOH. The total volumes were kept to a minimum (usually 1-2 ml) to facilitate complete mixing as rapidly as possible. The extracts were heated at 60C for 10 minutes to destroy oxidized pyridine nucleotides and were treated further as described above for the alkaline extraction of the cell pellets. The redox state was taken as the amount of pyridine nucleotide in the direct alkaline extract divided by the total pyridine nucleotide determined as described above.

Analysis of Pyridine Nucleotides

Analysis of NAD^+. Frozen supernatant fractions from pyridine nucleotide extractions were thawed and centrifuged at 1500 x g for 10 minutes to remove additional $KClO_4$ which precipitated when the samples were exposed to temperatures below 0C. NAD^+ was determined by a modification of an enzymic cycling assay of Bernofsky and Swan (Bernofsky and Swan, 1973). Each assay tube contained the following in a final volume of 1.2 ml: 0.1 M Na-Bicine, pH 8.0; 0.5 M ethanol; 0.42 mM MTT Tetrazolium; 1.66 mM phenazine ethosulfate; 4.16 mM EDTA; 0.8 mg/ml bovine serum albumin; 0.1 ml of alcohol dehydrogenase (0.5 mg/ml in 0.1 M Na-Bicine, pH 8.0); and 0.6 ml of acid or alkaline extraction medium containing either cellular extracts or NAD^+ standards. The acid and alkaline extraction media were prepared exactly as

described in extraction of pyridine nucleotides only in the absence of cell samples. The cycling assay was initiated by addition of alcohol dehydrogenase and was terminated after 30 minutes at 30C by addition of 0.5 ml of 12 mM iodoacetate, and absorbance was determined at 570 nm.

Analysis of $NADP^+$. A cycling assay similar to the NAD^+ assay has been developed for $NADP^+$. Each assay tube contained the following in a final volume of 1.2 ml: 100 mM Na-Bicine, pH 8.0; 10 mM $MgCl_2$; 10 mM D,L-isocitrate; 0.42 mM MTT Tetrazolium; 1.66 mM phenazine ethosulfate; 0.8 mg/ml bovine serum albumin, 0.25 mg isocitrate dehydrogenase; and 0.6 ml of acid or alkaline extraction medium containing either cellular extracts or $NADP^+$ standards. The cycling assay was initiated by the addition of isocitrate dehydrogenase, and the tubes were incubated at 30C for 30 minutes. The assays were terminated by the addition of 0.5 ml of 2.5 mM p-hydroxymercuriobenzoate in 0.05 M glycylglycine, and the abosrbance was measured at 570 nm.

Protein Determination

Duplicate aliquots of culture (5-10 $A_{600\ nm}$ units) were centrifuged at 1500 x g for 10 minutes, and the medium was aspirated. The cell pellets were suspended in 2 ml of ice cold 10% (w/v) trichloroacetic acid (TCA). After at least 15 minutes on ice, the samples were centrifuged at 1500 x g for 10 minutes. The pellets were suspended in 0.1 M NaOH

and were frozen until assayed for protein by the method of Lowry (Lowry et at., 1951), using crystalline bovine serum albumin as standard.

RNA Determination

RNA was analyzed by Fleck and Munro's (Fleck and Munro, 1962) modification of the Schmidt and Thannhauser procedure (Schmidt and Thannhouser, 1945). Briefly, the method is as follows: The cell pellets were suspended in 20% (w/v) ice cold TCA for 10 minutes to remove acid soluble materials. RNA was then extracted from the pellets with 0.3 M NaOH at 37C for 45 minutes. The mixture was acidified with perchloric acid to a final concentration of 0.5 M and centrifuged. The resulting supernatant was analyzed spectrophotometrically ($A_{260\ nm}$ = 1 = 34 µg of RNA).

Incorporation of L-(4,5-^{3}H)-Leucine

The incorporation of L-(4,5-^{3}H)-leucine by whole yeast cells was measured by adding to exponentially growing cultures L-(4,5-^{3}H)-leucine (1.0 µCi/ml, 9.1 mM), in the presence of either 2 µg/ml of cycloheximide or 4 mg/ml of chloramphenicol. At different time intervals, 0.1 ml aliquots of the culture were spotted on Whatman filter papers (3 mm diam.); these filters were added to 200 ml of 20% (w/v) ice cold TCA. After standing for at least 15 minutes, filters were washed with another 200 ml of 20% TCA,

and were then washed with 100 ml of 95% ethanol. Filters were dried at 60C for 10 minutes. 5 ml of scintillation solution (4 g of Omnifluor per liter of toluene) was added and radioactivity was measured in a Beckman LS-100 liquid-scintillation counter.

RESULTS

Growth of Yeast

Figure 1 shows a typical growth pattern of yeast in the complex medium containing 0.8% glucose. After an initial lag, the cells grow exponentially with a generation time of 68 - 72 minutes. During the first exponential phase, glucose is removed from the medium and ethanol accumulates to a maximum at about 400 minutes (Jacobson and Bernofsky, 1974). At this point, cells enter a non-growing stage for about 1 hour at 4.3×10^7 cells per ml of culture. In this stage, functional mitochondria are formed to maintain a second phase of exponential growth by oxidative metabolism (Wallace and Linnane, 1964; Polakis *et al.*, 1964), with a generation time of 370 - 390 minutes. During this exponential phase, ethanol is consumed. When ethanol is depleted, the cells enter a stationary phase, at cell numbers of 1.9×10^8 cells per ml of culture, and the culture turbidity is around 25. Figure 2 shows a similar biphasic growth pattern of yeast in semidefined medium containing 0.8% glucose as carbon source. The cells have slower rates than observed for the complex medium, with generation times of 80 - 87 minutes and 550 - 570 minutes

Figure 1. Typical growth curve of yeast in complex medium. The medium contains 0.8% glucose, 1% yeast extract, 0.5% Bacto-Peptone and salts. Culture turbidity and cell numbers were determined as described in "Materials and Methods". The two exponential growth phases have generation times of 68-72 minutes and 370-390 minutes.

Symbols: Turbidimetric readings at 600 nm (●); and cell numbers (0).

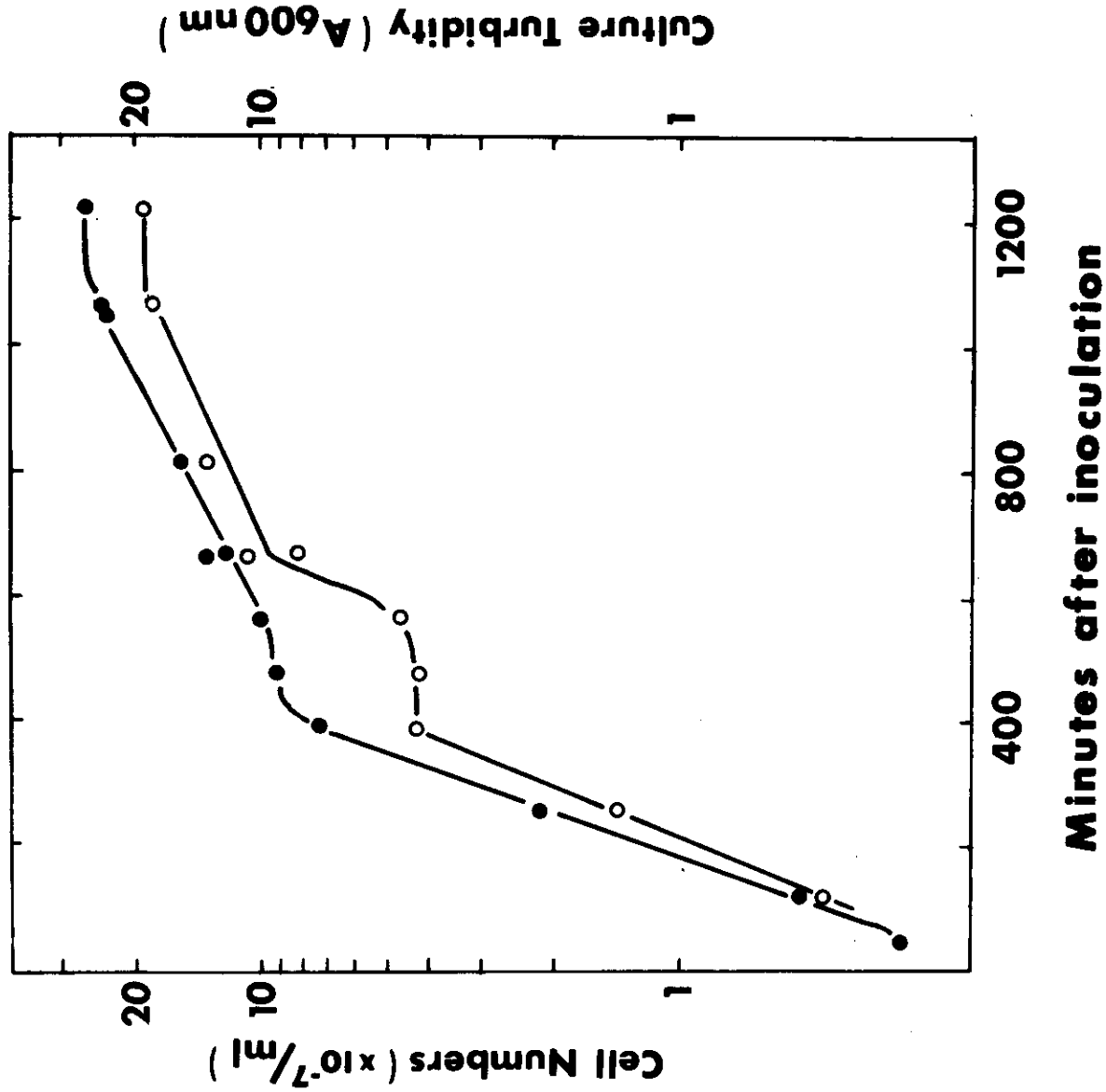
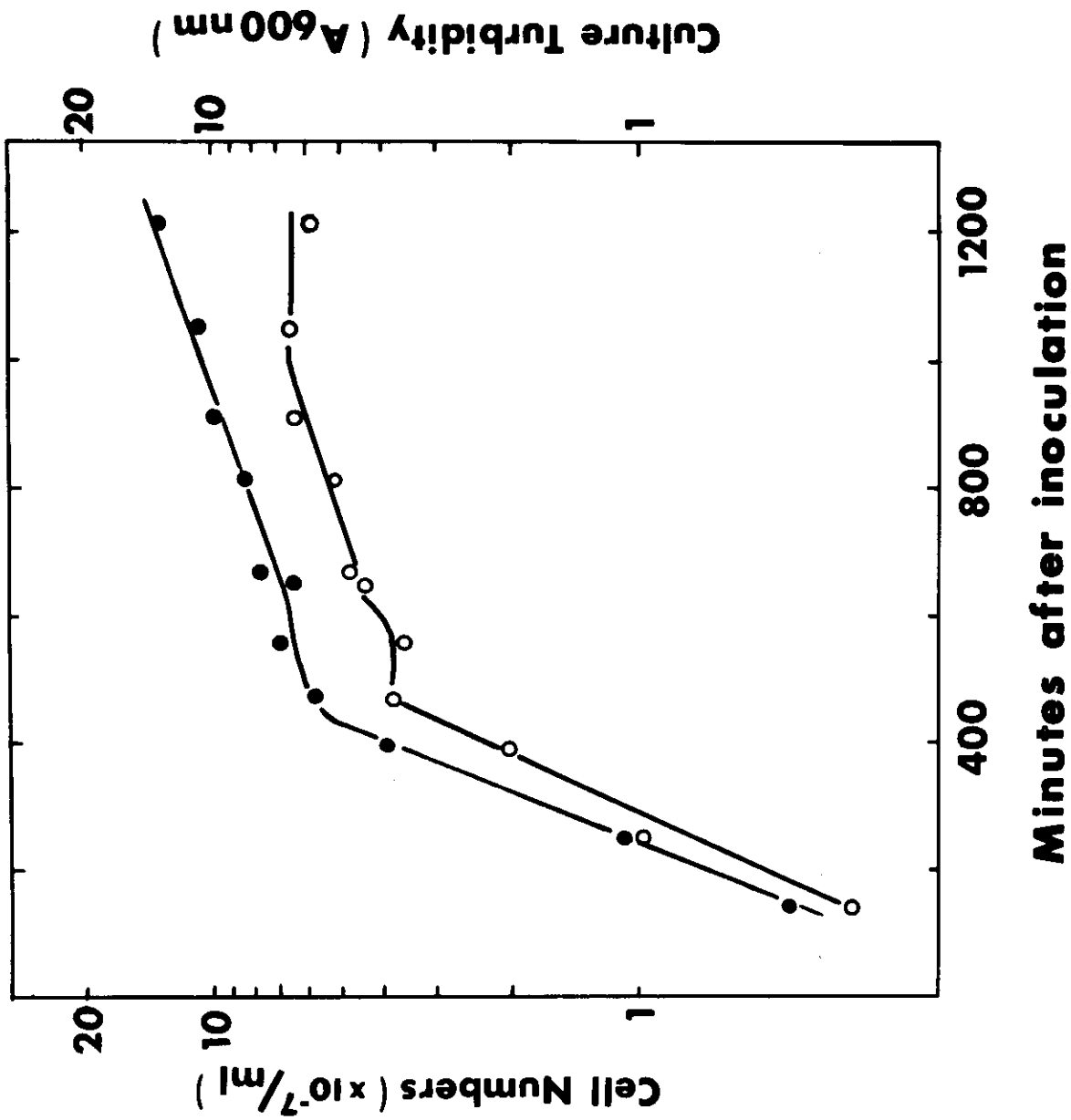

Culture Turbidity (A600nm)
20
10
1
Cell Numbers (x10^7/ml)
20
10
1
400
800
1200
Minutes after inoculation

Figure 2. Typical growth curve of yeast in semidefined medium. The medium contains: 0.8% glucose, 0.1% yeast extract and 0.67% Difco nitrogen base without amino acids. The two exponential growth phases have generation times of 80-87 minutes and 550-570 minutes.

Symbols: Turbidimetric readings at 600 nm (●); and cell numbers (0).


Culture Turbidity (A600nm)
20
10
1
Cell Numbers (x 10^7/ml)
20
10
1
400
800
1200
Minutes after inoculation

in the two exponential phases, respectively.

Typical growth curves after the transfer of cultures from glucose to ethanol medium or vice versa are shown in Figure 3. When a log phase glucose culture is transferred from glucose to ethanol medium, exponential growth is resumed after a 1 hour lag. A short lag of about 40 minutes is seen when ethanol phase cultures are transferred to glucose medium. Transfers from glucose medium to glucose medium and from ethanol medium result in continued growth at the pre-transfer rate without any detectable lag.

Analyses of Pyridine Nucleotides

NAD^+ Assay. Figure 4 shows typical standard curves of the NAD^+ assays. Under the conditions described in "Methods", the assay gives a linear response up to 120 pmoles of NAD^+ per assay. Control tubes without NAD^+ give values of less than 4 pmoles of NAD^+. Color development is totally dependent upon addition of alcohol dehydrogenase. Duplicate analyses were performed routinely on the extracts, and these rarely differed from each other by more than 5%. Because of the existence of phenazine ethosulfate in alkaline extraction media, the assays have a higher response in alkaline extraction media than in the acid extraction media.

Figure 3. Typical growth curves of yeast after changes of growth media. Log phase cultures, corresponding to 1-1.5 A_{600nm} turbidimetric units in glucose phase and 7.6-8.6 A_{600} nm units in ethanol phase were transferred from glucose to ethanol medium or vice versa as described in "Methods".

Symbols: Cultures before transfer in glucose phase (●) and in ethanol phase (▲). Cultures transferred to glucose medium (0) and ethanol medium (Δ). The arrow denotes the times of transfer.

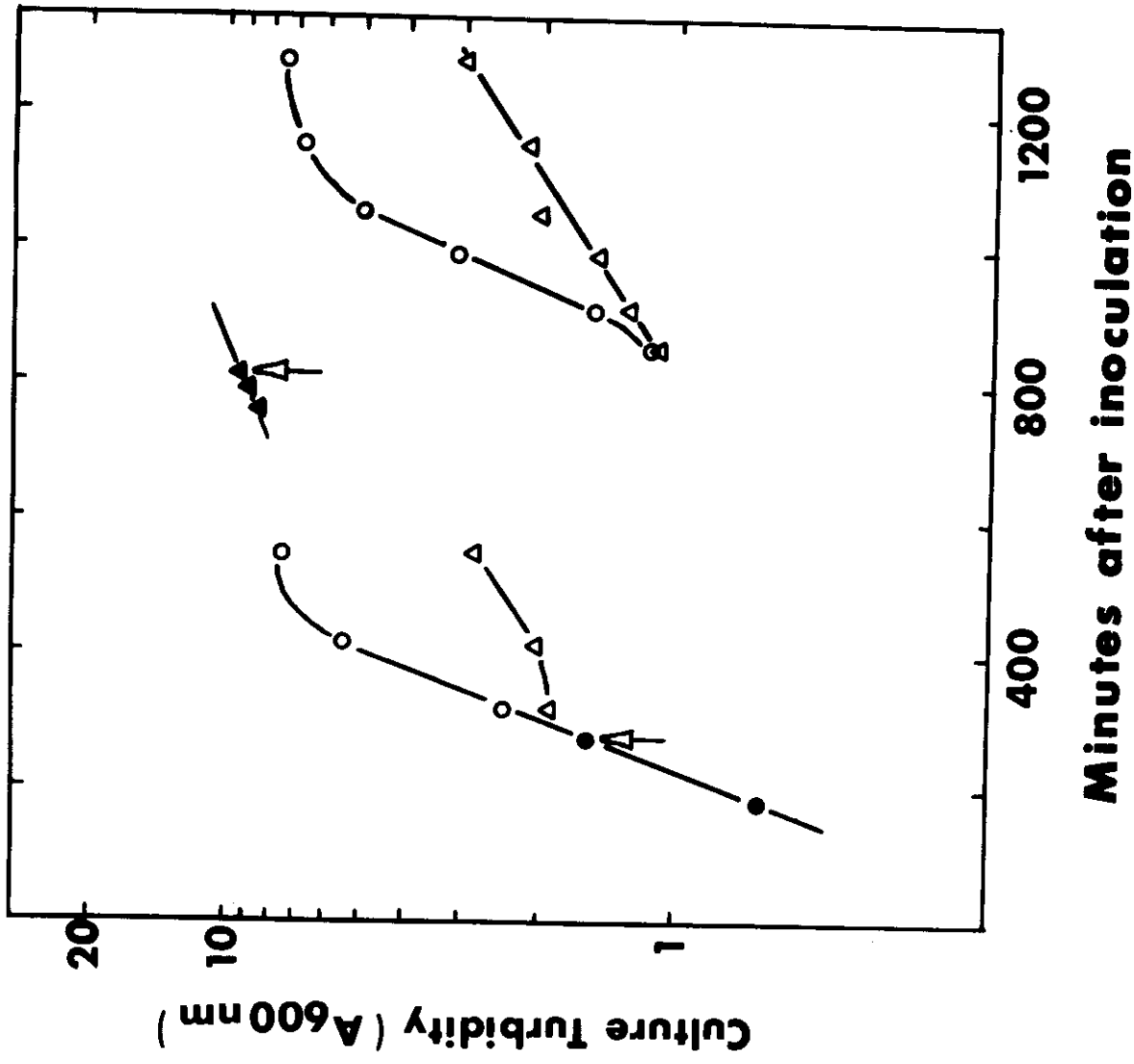
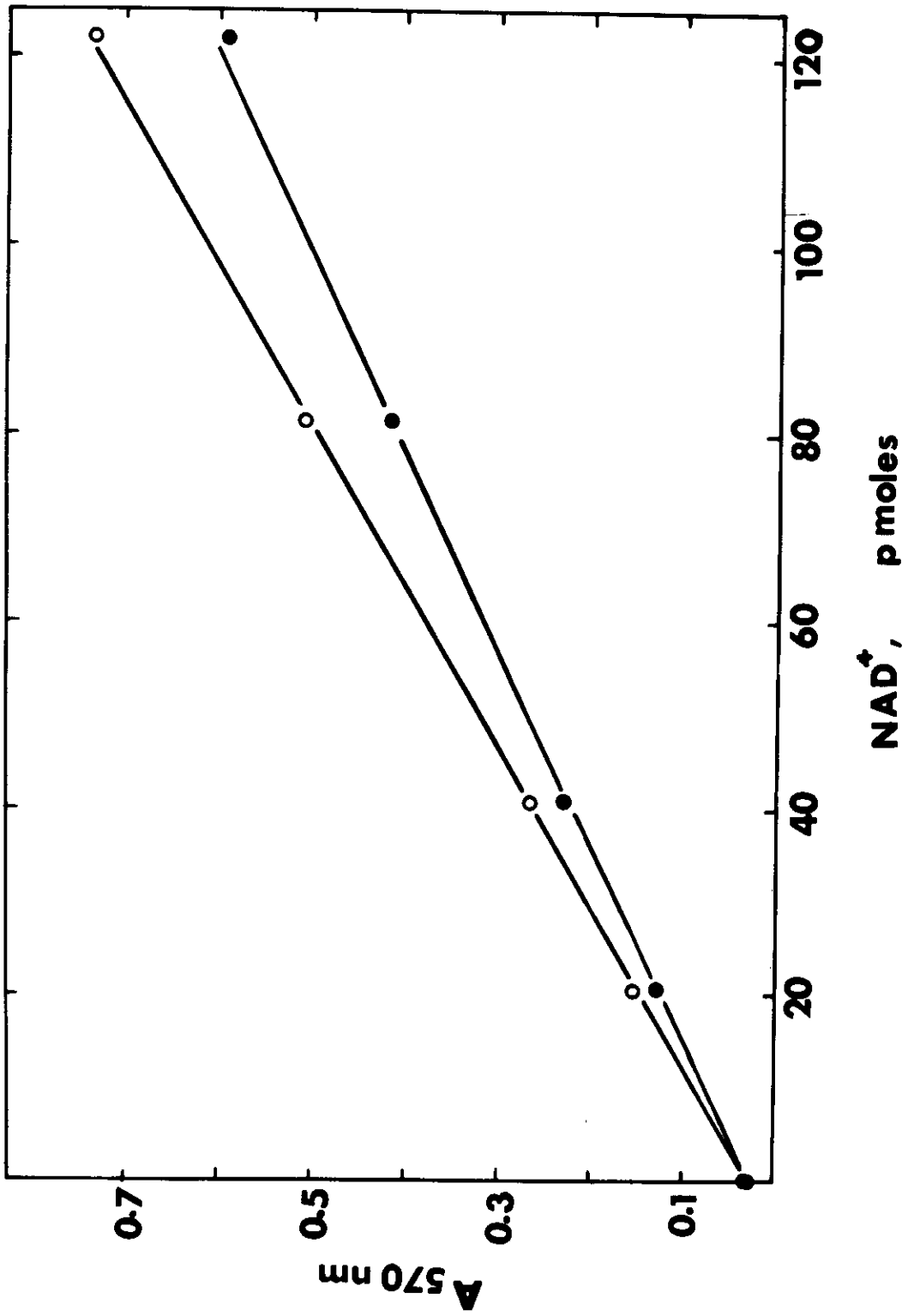

Culture Turbidity (A600nm)
20
10
1
400
800
1200
Minutes after inoculation

Figure 4. Typical standard curves of NAD^+ assays. The assay mixture (1.2 ml) contains: 0.1 M Na-Bicine pH 8.0, 0.5 M ethanol, 0.42 mM MTT tetrazolium, 1.66 mM phenzaine ethosulfate, 4.16 mM EDTA, 0.8 mg/ml bovine serum albumin and 0.6 ml of acid or alkaline extraction medium. Assays were initiated with 42 µg (100 µl) of alcohol dehydrogenase and were terminated with 0.5 ml of 12 mM iodoacetate after 30 minutes incubation at 30C. Absorbance was measured at 570 nm. Duplicate analyses routinely agree within 5%.

Symbols: Acid extraction medium (●); and alkaline extraction medium (0).


A 570 nm
0.7
0.5
0.3
0.1
20
40
60
80
100
120
NAD+, p moles

NADP$^+$ Assay. Figure 5 shows typical standard curves of the NADP$^+$ assays. Under the conditions described in "Methods", the assay gives a linear response up to 110 pmoles of NADP$^+$ per assay tube. Control tubes without NADP$^+$ give values of less than 7 pmoles of NADP$^+$. Color development is totally dependent upon the addition of isocitrate dehydrogenase. Duplicate analyses were done routinely, and the results rarely differed by more than 10%. The higher blank and higher response of the assays in alkaline extraction media was also observed because of the presence of phenazine ethosulfate.

Efficiency of Assaying of Pyridine Nucleotides. To test for the presence in the extracts of either activators or inhibitors of the cycling assay, known amounts of NAD$^+$ were added to the assay tubes. As shown in Table I, neither activation nor inhibition is observed in the extracts. Similar checks were made on the NADP$^+$ assay, and again neither activation nor inhibition was seen.

Efficiency of Extraction of Pyridine Nucleotides. Two different extraction methods were carried out for oxidized pyridine nucleotides. In earlier experiments, oxidized pyridine nucleotides were extracted by ice cold perchloric acid. Table II shows data on the recovery of NAD$^+$ and NADP$^+$ using this extraction method. It can be seen that 94 $\pm$ 7% of NAD$^+$ and 98 $\pm$ 8% of NADP$^+$ were recovered from extractions of pure

Figure 5. Typical standard curves of $NADP^+$ assays. The assay mixture (1.2 ml) contains: 0.1 M Na-Bicine pH 8.0, 10 mM D,L-isocitrate, 0.42 mM MTT tetrazolium, 1.66 mM phenazine ethosulfate, 0.8 mg/ml bovine serum albumin and 0.6 ml of acid or alkaline extraction medium. Assays were initiated with 0.25 mg (100 µl) of isocitrate dehydrogenase and were terminated with 0.5 ml of 2.5 mM p-hydroxymercuriobenzoate in 50 mM glycylglycine after 30 minutes of incubation at 30C. Absorbance was measured at 570 nm. Duplicate analyses routinely agree within 5%.

Symbols: Acid extraction medium (●); and alkaline extraction medium (O).

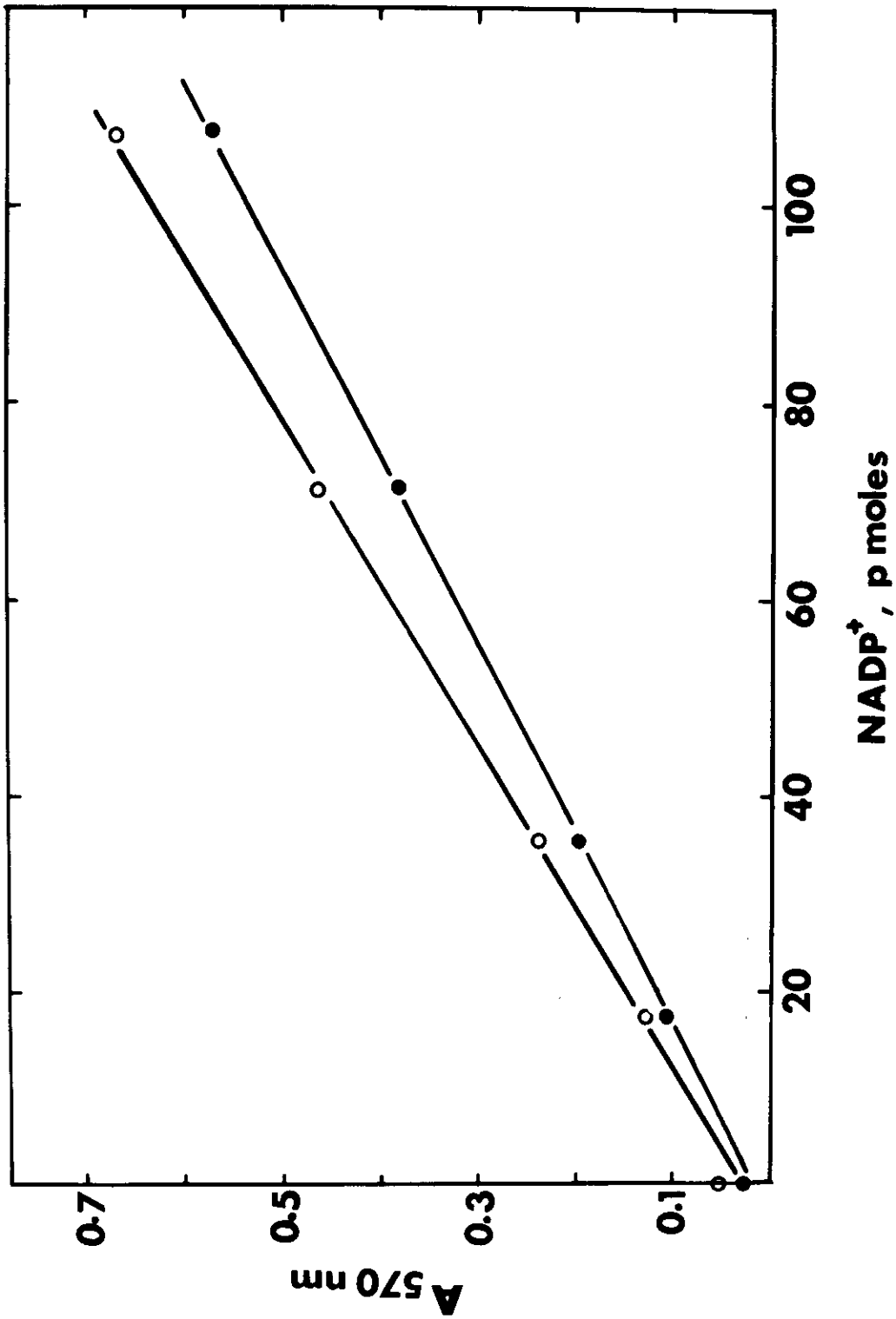

A 570 nm
0.7
0.5
0.3
0.1
20
40
60
80
100
NADP+, p moles

TABLE I

EFFECTS OF CELL EXTRACTS ON THE NAD^+ CYCLING ASSAY

	Pyridine Nucleotide* pmoles per assay tube	
	NAD^+	NADH
Amount measured in extracts	61	43
Amount of standard added	19.8	19.8
Amount measured in extract with standard added	80	62
% Recovery	99	98.7

* NAD^+ was determined by the enzymatic cycling assay as described in "Methods". Known amounts of NAD^+ were added to the acid or alkaline extracts of cells to test for activation or inhibition of the assay by the extract.

TABLE II

EFFICIENCY OF ACID EXTRACTION OF OXIDIZED PYRIDINE NUCLEOTIDES FROM *S. CEREVISIAE* BY COLD PERCHLORIC ACID

	Pyridine Nucleotide Measured	
	NAD^+	$NADP^+$
STANDARDS:		
No. of extractions	30	8
Range of standard extracted (pmol/ml extract)	339-5404	39-530
% Recovery	94 ± 7	98 ± 13
CELLS:		
No. of extractions	4	8
Range of nucleotide in cells (pmol/ml extract)		
1st extraction	1387-1711	66-196
2nd extraction	347-563	36-77
% of total nucleotide recovered:		
1st extraction	76 ± 2	66 ± 15
2nd extraction	24 ± 2	34 ± 15

standards. However, only 76 ± 2% of the total NAD^+ and 66 ± 15% of the total $NADP^+$ were extracted in the first extraction of yeast cells. A second extraction was necessary to obtain the total NAD^+ and $NADP^+$. The acid extraction procedure was modified by heating the cell pellets, which had been suspended in perchloric acid, at 60C for 10 minutes. As shown in Table III, heat treatment yielded extraction efficiencies of 95 ± 7% for standard NAD^+ samples and 85 ± 2% for standard $NADP^+$ samples. Also, 97 ± 5% of NAD^+ and 87 ± 5% of $NADP^+$ were recovered in the first extraction from cells. This procedure was utilized for the extraction of NAD^+ and $NADP^+$ from yeast cells.

In additional experiments, different amounts of oxidized pyridine nucleotides were added to the cell pellets and were extracted along with the cells. Comparisions of the amount of NAD^+ and $NADP^+$ obtained from separate and combined extractions are shown as the recovery ratio. It can be seen that the amounts of NAD^+ and $NADP^+$ from co-extractions of cells and standards is very similar to the amounts determined when cells and standards are extracted separately.

Table IV shows the efficiency of alkaline extraction of reduced pyridine nucleotides by sodium hydroxide. 95 ± 11% of NADH and 92 ± 5% of NADPH were recovered from extraction of NADH and NADPH standards, and 96 ± 1% of the total NADH and 86 ± 6% of the total NADPH recovered was obtained in the

TABLE III

EFFICIENCY OF ACID EXTRACTION OF OXIDIZED PYRIDINE NUCLEOTIDES FROM S. CEREVISIAE BY PERCHLORIC ACID WITH 60C HEATING

	Pyridine Nucleotide Measured	
	NAD^+	$NADP^+$
STANDARDS:		
No. of extractions	4	4
Range of standards extracted (pmol/ml extract)	3960-7810	651-841
% Recovery	95 ± 7	85 ± 2
CELLS:		
No.of extractions	12	12
Range of nucleotide in cells (pmol/ml extract)		
1st extraction	6600-8731	268-509
2nd extraction	96-255	55-69
% of total nucleotide recovered:		
1st extraction	97 ± 2	87 ± 5
2nd extraction	3 ± 2	13 ± 5
CELLS plus STANDARDS:		
No. of extractions	12	12
Range of nucleotide recovered (pmol/ml extract)	8910-15400	729-1100
Recovery ratio	0.93 ± 0.06	0.89 ± 0.14

TABLE IV

EFFICIENCY OF ALKALINE EXTRACTION OF REDUCED PYRIDINE NUCLEOTIDES FROM S. CEREVISIAE BY SODIUM HYDROXIDE

	Pyridine Nucleotide Measured	
	NADH	NADPH
STANDARDS:		
No. of extractions	8	10
Range of standards extracted (pmol.ml extract)	4704-5470	93-740
% Recovery	95 ± 11	92 ± 5
CELLS:		
No. of extractions	16	16
Range of nucleotide in cells (pmol/ml extract)		
1st extraction	1711-6000	255-840
2nd extraction	55-195	45-85
% of total nucleotide recovered:		
1st extraction	96 ± 1	86 ± 6
2nd extraction	4 ± 1	14 ± 6
CELLS plus STANDARDS:		
No. of extractions	16	16
Range of nucleotide recovered (pmol/ml extract)	6445-10200	477-1100
Recovery ratio	0.99 ± 0.02	0.92 ± 0.06

first extraction from yeast cells. When standards of NADH and NADPH were extracted together with cell pellets, the recoveries of these nucleotides from these co-extractions are similar to those obtained when cells and standards were extracted separately.

Cellular Contents of Protein and RNA as a Function of Growth

Duplicate aliquots of culture (5-10 $A_{600\ nm}$ units) were centrifuged and assayed for protein and RNA as described in "Methods". Figures 6 and 7 show the cellular levels of protein and RNA plotted as a function of the logarithm of culture turbidity in complex medium. The highest levels of protein and RNA were observed in glucose phase, and the contents of both protein and RNA decreased from late glucose phase to the stationary phase.

Growth Changes in the Cellular Levels of Pyridine Nucleotides

Duplicate aliquots of culture (5-10 $A_{600\ nm}$ units) were centrifuged and the oxidized and reduced pyridine nucleotides were extracted by perchloric acid and sodium hydroxide, respectively, as described in "Methods". Figure 8 shows the relative amounts of NAD and NADP as a function of growth in complex medium. Total NAD or NADP refers to the sum of the oxidized and reduced forms. The data are shown as the ratio

Figure 6. Total protein content of yeast grown in complex medium plotted as a function of culture turbidity. Aliquots of culture (5-10 $A_{600\ nm}$ units) were centrifuged, and cellular proteins were precipitated by 10% (w/v) TCA. The pellets were suspended in 0.1 M NaOH and assayed by the procedure of Lowry (Lowry et al., 1951). Each point represents the mean of a duplicate analysis that agreed within 5%.

Symbols: ●, and ▲ represent two sets of experimental data. "A" represents glucose phase growth; "B", transitional phase, "C", ethanol phase growth; and "D", stationary phase.

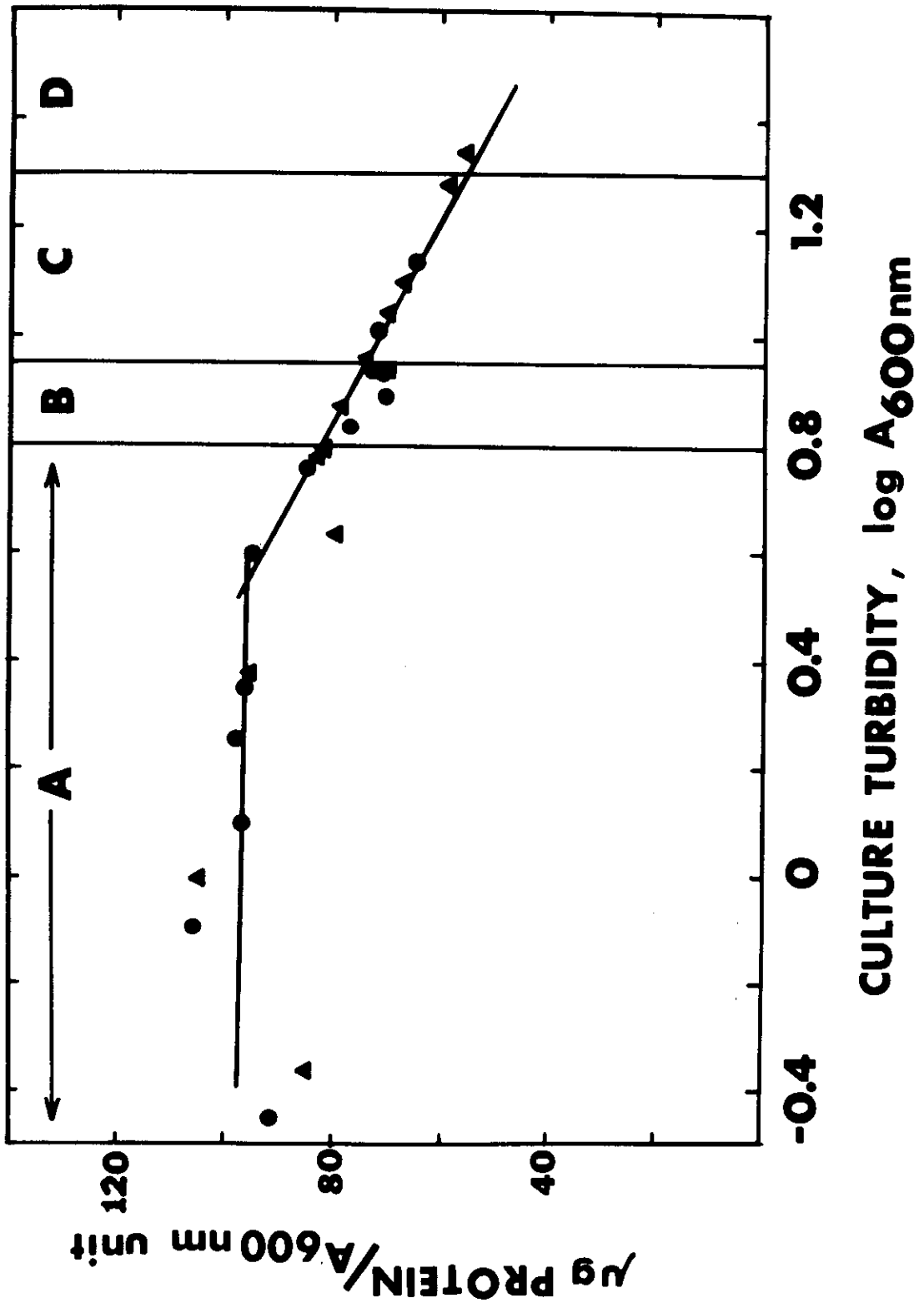

A
B
C
D
120
80
40
μg PROTEIN/A600 nm unit
-0.4
0
0.4
0.8
1.2
CULTURE TURBIDITY, log A600nm

Figure 7. Total RNA levels of yeast grown in complex medium plotted as a function of culture turbidity. Aliquots of culture (5-10 $A_{600\ nm}$ units) were centrifuged. RNA was extracted from the TCA precipitates with 0.3 M NaOH at 37C for 45 minutes. The supernatants were acidified with $HClO_4$ to a final concentration of 0.5 M. The resulting supernatant was read at $A_{260\ nm}$ ($A_{260\ nm}$ = 1 = 34 µg of RNA; Fleck and Munro, 1962). Duplicate analyses routinely agree within 5%.

Symbols: ●, and ▲ represent two sets of experimental data. "A" represents glucose phase growth; "B", transitional phase; "C", ethanol phase growth; and "D", stationary phase.

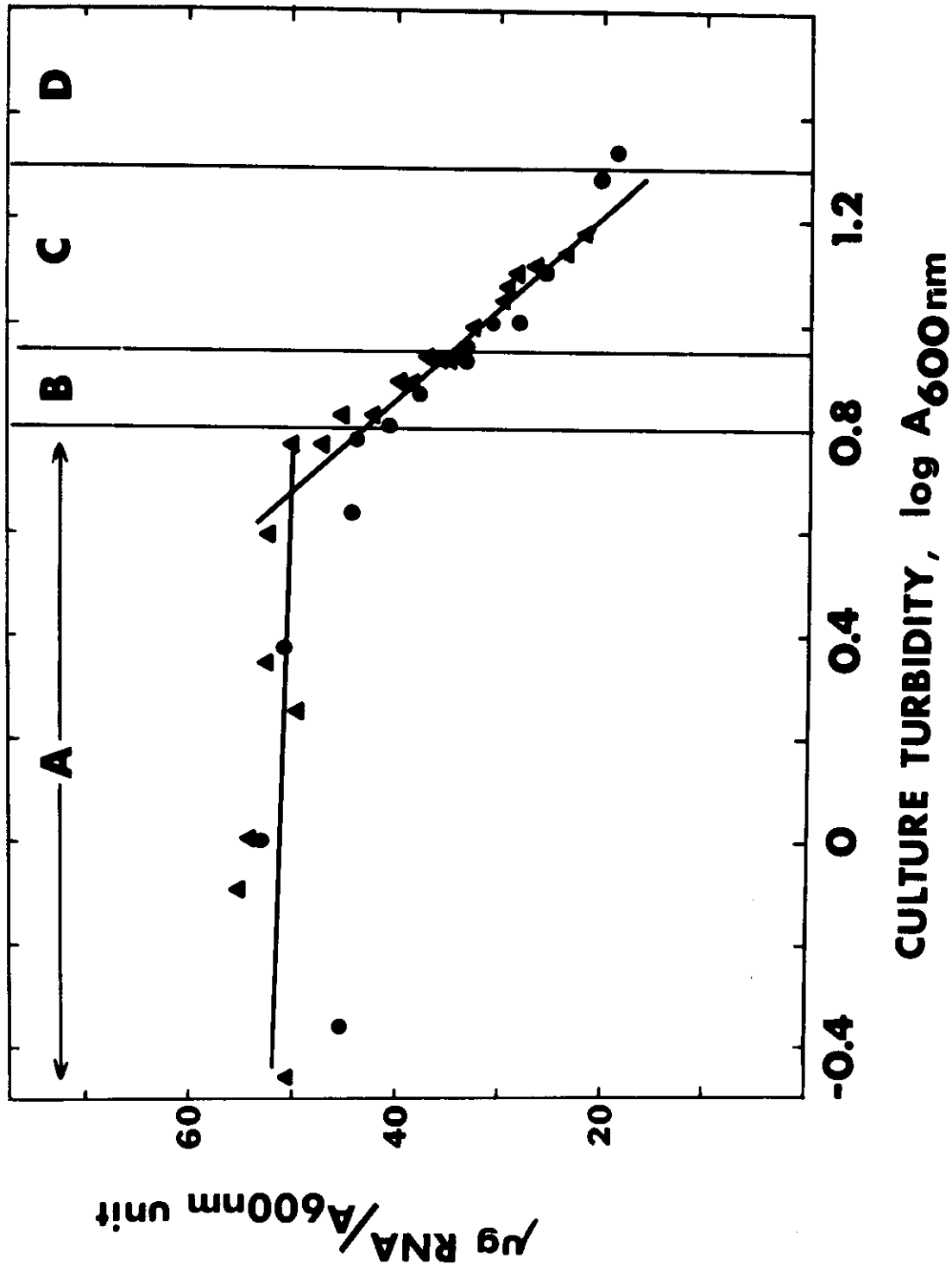
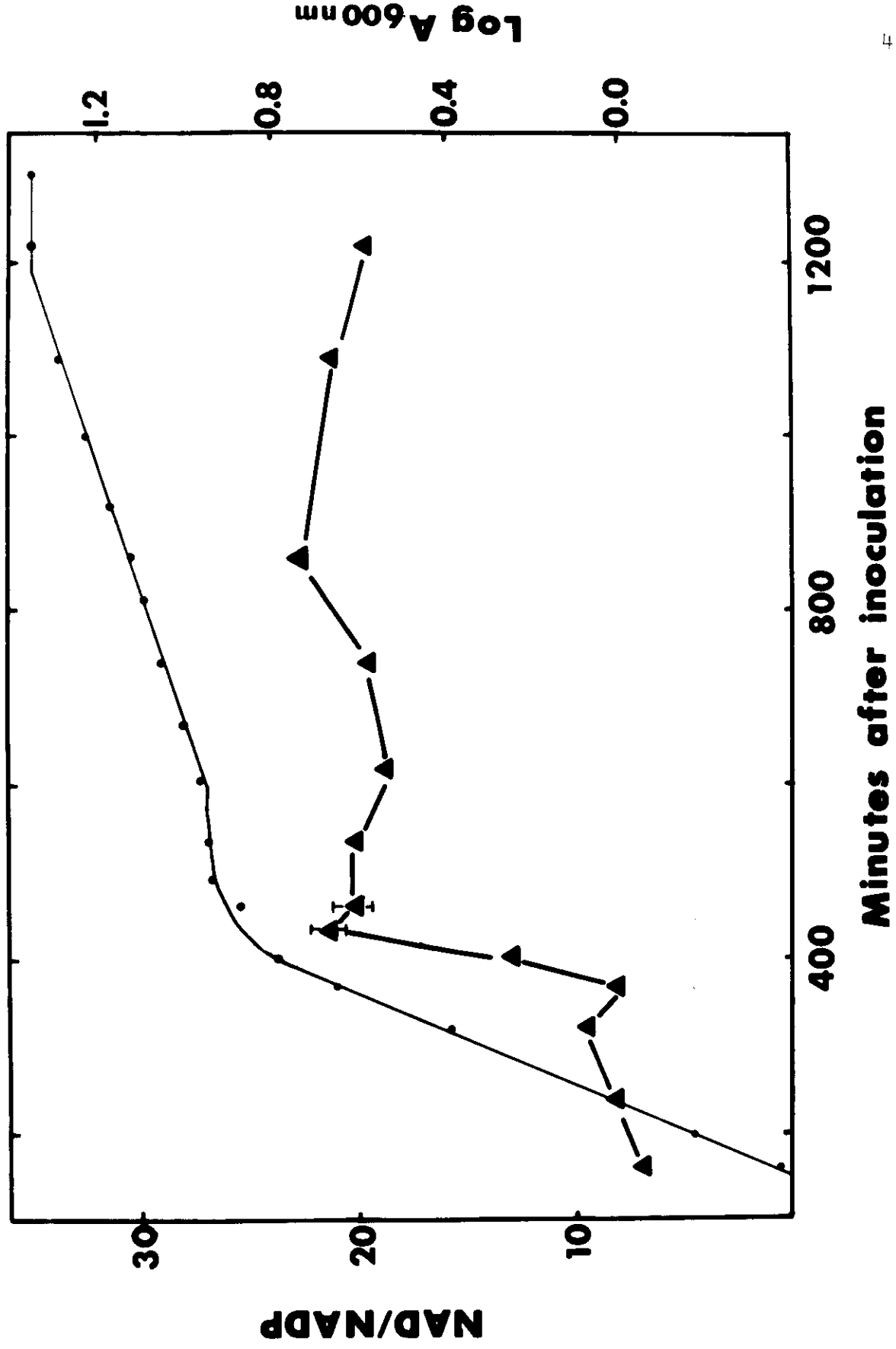

A
B
C
D
60
40
20
μg RNA/A600nm unit
-0.4
0
0.4
0.8
1.2
CULTURE TURBIDITY, log A600nm

Figure 8. Ratio of NAD nucleotides to NADP nucleotides as a function of growth. Aliquots of culture (5-10 A_{600nm} units) were centrifuged. The oxidized pyridine nucleotides (NAD^+ and $NADP^+$) were extracted from the cell pellets with 2 ml of 0.5 M hot perchloric acid; and the reduced pyridine nucleotides (NADH and NADPH) were extracted with 2 ml of 0.25 M sodium hydroxide as described in "Methods". Total NAD refers to the sum of NAD^+ plus NADH; and total NADP to the sum of $NADP^+$ plus NADPH. Duplicate analyses were performed. If the values agreed within 5%, they were presented as one point; if they differed by more than 5%, they were presented as the average value with a range bar. Culture turbidity was plotted as the log of turbidimetric readings at 600nm.

Symbols: Culture turbidity (●); NAD to NADP ratio (▲).


Log A600nm
1.2
0.8
0.4
0.0
NAD/NADP
30
20
10
400
800
1200
Minutes after inoculation

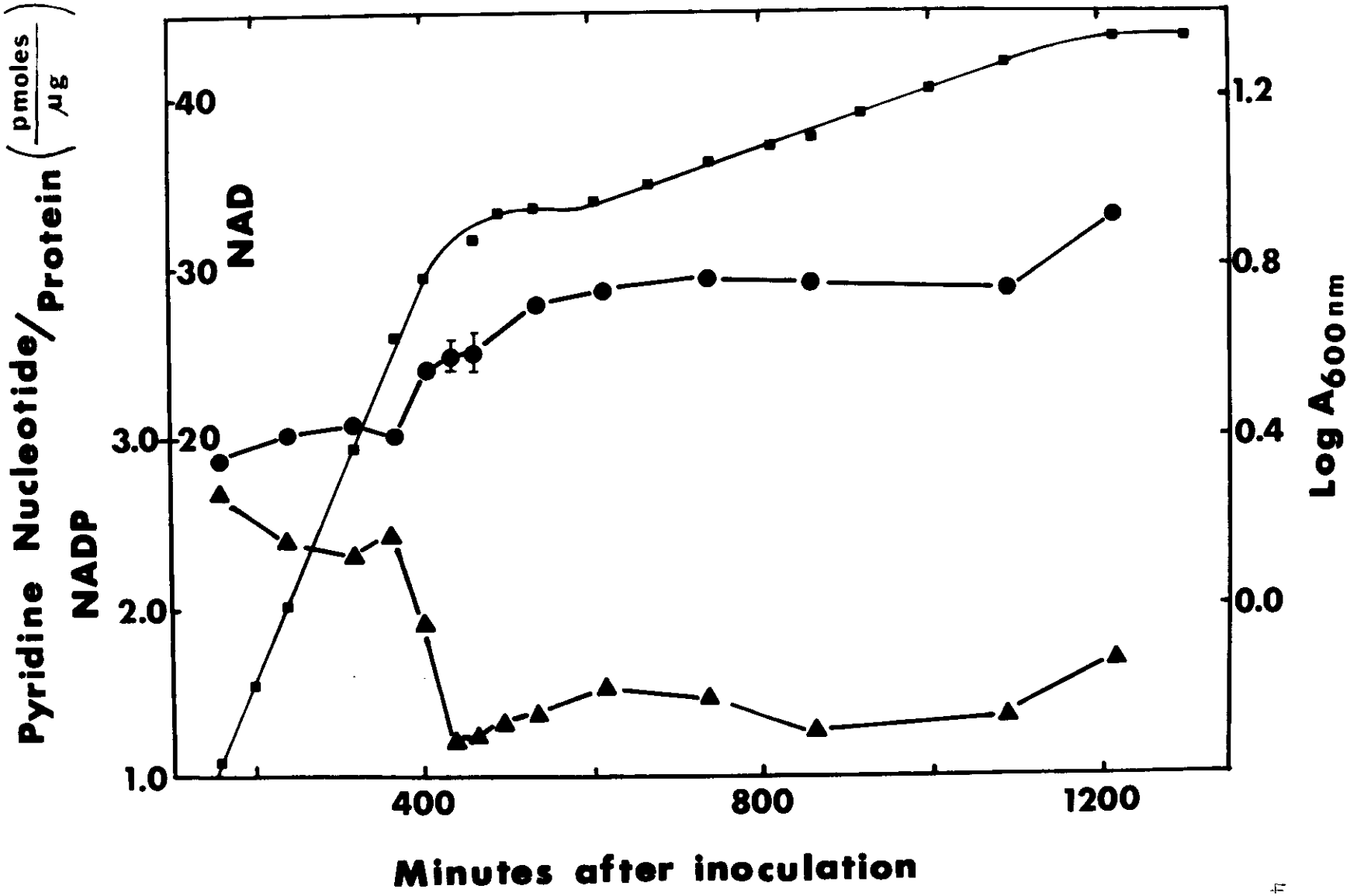
of NAD nucleotides to NADP nucleotides. The NAD/NADP ratio is relatively constant during growth on glucose with a mean value of 8.2 $\pm$ 0.79. This corresponds to 89% NAD and 11% NADP present in the cells. In the lag period, following exhaustion of glucose, a more than two fold increase in NAD/NADP ratio occurs, reaching a mean value of 20.6 $\pm$ 1.17. This higher NAD/NADP ratio is maintained during growth on ethanol.

Data on Figure 9 shows that this change in the relative amounts of NAD and NADP represents both an increase in the NAD/protein ratio and a decrease in the NADP/protein ratio. Approximately constant ratios of NAD/protein and NADP/protein are seen in the growth on glucose. At the end of the glucose phase, a marked increase in the NAD/protein ratio, and a sharp decrease in the NADP/protein are observed. The NAD/protein ratio rose by 40%, and NADP/protein ratio dropped 44% in the lag stage.

Since the oxidized and reduced forms are in rapid equilibrium with each other, centrifugation of cells prior to extraction might alter the redox state of the pyridine nucleotide pools (Stollar and Kaplan, 1961). In order to obtain estimates of the redox state of the NAD and NADP pools *in vivo*, aliquots of culture were subjected to a direct extraction with NaOH to rapidly inactivate all enzymatic activities and to extract reduced pyridine nucleotides. Since the ex-

Figure 9. Ratio of pyridine nucleotides (NAD and NADP) to protein. Duplicate aliquots of culture (5-10 A_{600nm} units) were centrifuged. The oxidized and reduced pyridine nucleotides were extracted by $HClO_4$ and NaOH, respectively. The protein concentration was determined by the procedure of Lowry. Duplicate analyses were performed. If the values agreed within 5%, they were presented as one point; if they differed by more than 5%, they were presented as the average value with a range bar. Culture turbidity was plotted as the log of turbidimetric readings at 600nm.

Symbols: Culture turbidity (■); NAD to protein ratio (●); and NADP to protein ratio (▲).


Pyridine Nucleotide/Protein (pmoles/µg)
NADP
NAD
3.0
2.0
1.0
40
30
20
400
800
1200
Minutes after inoculation
1.2
0.8
0.4
0.0
Log A600 nm

tracts derived from the direct alkaline extraction of cultures in complex media have appreciable absorbance in the absence of added enzyme in the $NADP^+$ assay, cells were grown in the semidefined medium instead of the complex medium. Table V shows the comparison of the growth rate and cellular levels of pyridine nucleotides of S. cerevisiae in complex and semidefined media. It can be seen that a similar increase in the NAD/NADP ratio is also observed in the semidefined medium in ethanol phase when compared to glucose phase.

Redox State of Pyridine Nucleotide Pools

By relating the amount of pyridine nucleotide in the direct alkaline extracts to the total, the redox state of the pools were estimated. These data for glucose and ethanol phases of growth in a semidefined medium are shown in Table VI. The NAD pool is approximately 50% reduced under these conditions during both glucose and ethanol phases. For the NADP pool, a difference is seen between the redox state of the two phases, with approximately 67% of the pool reduced in glucose phase, and 48% reduced in ethanol phase.

Thus we observe a correlation between a decreased reducing charge of the NADP pool and a decreased amount of total NADP between glucose and ethanol growth phases.

Medium Transfers

Table VII shows that a similar two fold increase of

TABLE V

COMPARISON OF PYRIDINE NUCLEOTIDE LEVELS IN SEMIDEFINED AND COMPLEX MEDIA

	Semidefined Medium		Complex Medium	
	Glucose growth	Ethanol growth	Glucose growth	Ethanol growth
Generation time (minutes)	80-87	550-570	68-72	370-390
TOTAL NAD (pmoles per A_{600nm} unit of culture)	1800 ± 510 (n = 10)	2200 ± 350 (n = 11)	1950 ± 140 (n = 12)	2050 ± 70 (n = 6)
TOTAL NADP (pmoles per A_{600nm} unit of culture)	171 ± 10.3 (n = 9)	126 ± 6.3 (n = 11)	234 ± 21.1 (n = 8)	100 ± 8.0 (n = 8)
NAD to NADP ratio	11.6 ± 1.24 (n = 7)	19.2 ± 2.17 (n = 9)	8.2 ± 0.79 (n = 6)	20.6 ± 1.17 (n = 8)

Standard deviations are shown. The number of extractions is shown in parenthesis. Each extraction was analyzed in duplicate.

TABLE VI

REDOX STATE OF PYRIDINE NUCLEOTIDES IN S. CEREVISIAE

Redox State*	Glucose Growth	Ethanol Growth
% NADH	53 ± 10 (n = 9)	52 ± 2 (n = 6)
% NADPH	67 ± 8 (n = 6)	48 ± 2 (n = 5)

* The reduced NADH and NADPH were determined by direct alkaline extraction of the cells in the culture medium. Total NAD is the sum of NAD^+ and NADH and total NADP is the sum of $NADP^+$ and NADPH as determined by acid and alkaline extractions, respectively. "n" is the number of extractions. Standard deviations are shown. Each extraction was analyzed in duplicate.

TABLE VII

RATIO OF NAD TO NADP AFTER TRANSFER

FROM GLUCOSE MEDIUM TO ETHANOL MEDIUM*

	Transfer from Glucose Medium to:	
	Glucose Medium	Ethanol Medium
	NAD/NADP RATIO	
Before Transfer	14	--
49 Minutes	17	30
159 Minutes	15	27

*Duplicate aliquots of a log phase culture (1-1.5 $A_{600\ nm}$ units) growing on glucose were filtered through membrane filters and transferred to media containing:

(1) 0.37% (80 mM) ethanol, 0.1% yeast extract and 0.67% Difco nitrogen base without amino acids, and

(2) 0.53% (35 mM) glucose, 0.1% yeast extract and 0.67% Difco nitrogen base without amino acids, as control.

NAD relative to NADP is seen 50 minutes after transferring a log phase culture from glucose to ethanol medium. The control shift is also shown. When cultures were transferred from ethanol to glucose, as shown in Table VIII, a slight decrease in the NAD/NADP ratio is observed in 21 minutes, and a two-fold decrease is observed after 129 minutes. Again the control shift is shown.

The redox states of cultures shifted to glucose, and cultures shifted to ethanol were measured. The data on Table IX shows the ratio of reducing charge in ethanol medium to reducing charge in glucose medium after glucose to ethanol, and ethanol to glucose shifts. It can bee seen that the NAD pools have approximately equal reducing charge, after shifts from glucose to ethanol medium or from ethanol to glucose medium as evidenced by ratios near 1; while the NADP pools have a lower reducing charge in ethanol medium than in glucose medium, no matter which direction the cultures were shifted. Again the changes in NAD/NADP ratio are correlated with the changes in the redox state of NADP pool.

Incorporation of L-(4,5-^{3}H)-Leucine in the Presence of Protein Synthesis Inhibitors

The incorporation of L-(4,5-^{3}H)-leucine into protein by exponentially growing yeast cultures in the presence and absence of protein synthesis inhibitors is shown in Figure 10. After

TABLE VIII

RATIO OF NAD TO NADP AFTER TRANSFER

FROM ETHANOL MEDIUM TO GLUCOSE MEDIUM*

	Transfer from Ethanol Medium to:	
	Glucose Medium	Ethanol Medium
	NAD/NADP Ratio	
Before Transfer	--	23
21 Minutes	19	21
129 Minutes	13	27

*Duplicate aliquots of a log phase culture (7.6-8.6 $A_{600\ nm}$ units) growing on ethanol were filtered through membrane filters and transferred to media containing:

(1) 0.8% (40 mM) glucose, 0.1% yeast extract and 0.67% Difco nitrogen base without amino acids, and

(2) 0.32% (70 mM) ethanol, 0.1% yeast extract and 0.67% Difco nitrogen base without amino acids, as control.

TABLE IX

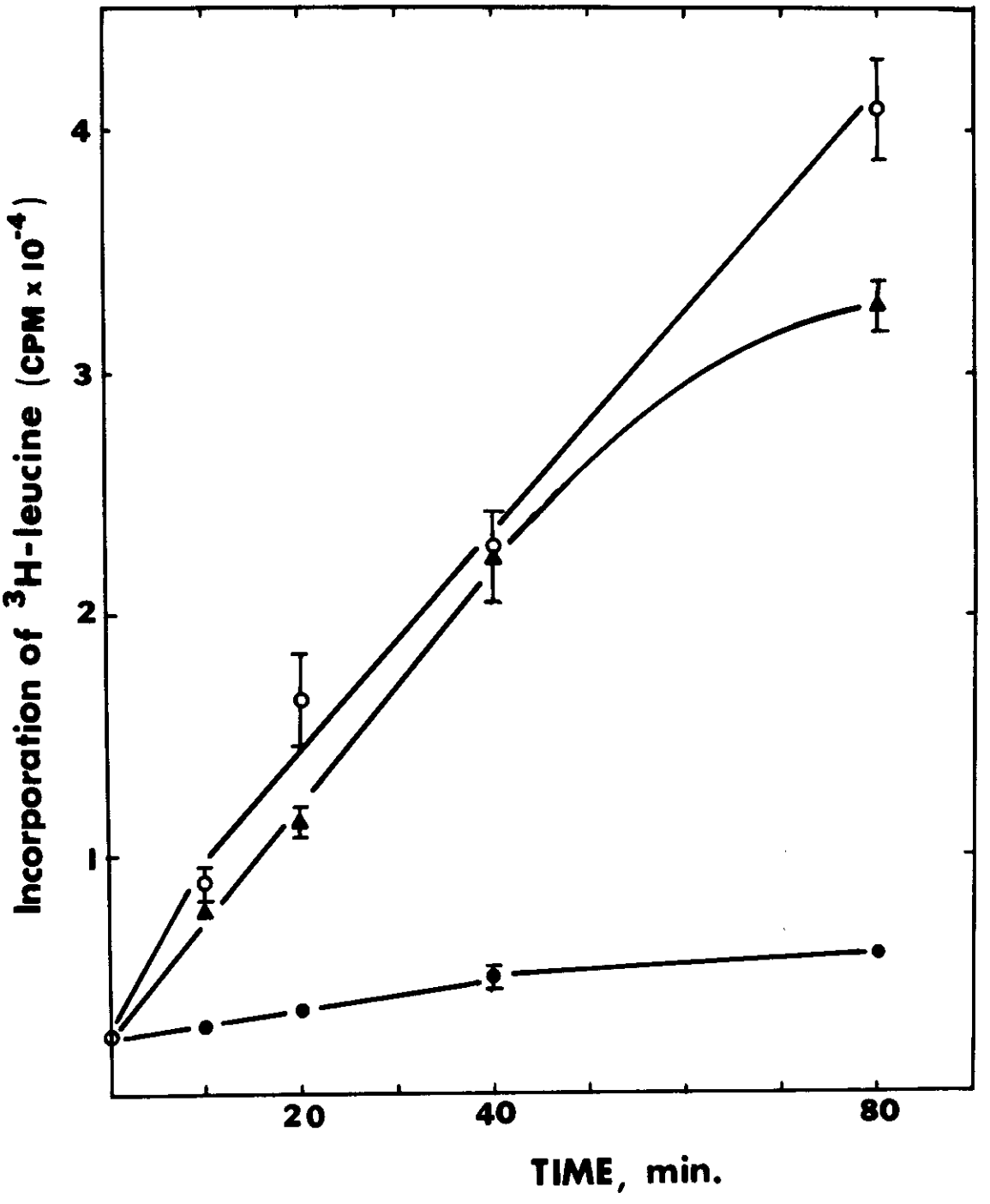
COMPARISON OF REDOX STATES AFTER MEDIA TRANSFERS

	Ratio of % Reduced * Pyridine Nucleotide: Ethanol Medium/Glucose Medium	
	NAD	NADP
Before Transfer	1.01	0.71
Glucose → Ethanol (n=6)	1.17 ± 0.26	0.64 ± 0.19
Ethanol → Glucose (n=10)	1.01 ± 0.12	0.62 ± 0.19

*The reduced pyridine nucleotides were extracted by direct alkaline extraction as described in "Methods." By relating the reduced nucleotides to the total, the redox states of the pools were estimated. Data shown above is the ratio of reducing charge in ethanol medium to reducing charge in glucose medium after glucose to ethanol, and ethanol to glucose shifts. Standard deviations are shown. The number of extractions is shown in parenthesis. Each extraction was analyzed in duplicate.

Figure 10. Incorporation of ^{3}H-leucine when protein synthesis inhibitors are present. 1.0 μCi/ml of ^{3}H-leucine (9.1 mM) and either 4 mg/ml medium of chloramphenicol or 2 μg/ml of cycloheximide were added at the same time to a log phase culture (1.2-1.5 A_{600nm} units/ml) in a semidefined medium. Analysis of the TCA insoluble material is described in "Methods". Duplicate analyses were performed. If the value agreed with 5%, they were presented as one point; if they differed by more than 5%, they were presented as the average value with a range bar.

Symbols: Medium in the presence of chloramphenicol (▲); in the presence of cycloheximide (●); and control (O).


Incorporation of ^{3}H-leucine (CPM x 10^{-4})
4
3
2
1
20
40
80
TIME, min.

80 minutes in the presence of 2 µg/ml of cycloheximide, which inhibits protein synthesis in the cytoplasm (Lamb et al., 1968), more than 80% of the protein synthesis is inhibited while about 20% inhibition occurs after the administration of 4 mg/ml of chloramphenicol.

Cultures Transferred in the Presence of Protein Synthesis Inhibitors

Table X shows the NAD/NADP ratio when cultures were transferred from glucose to glucose medium as control, and from glucose to ethanol medium in the presence of either chloramphenicol or cycloheximide. After 91 minutes, a two-fold increase in the NAD to NADP ratio occurs in the cultures transferred to ethanol whether or not these inhibitors are present.

TABLE X

EFFECT OF INHIBITORS OF PROTEIN SYNTEHSIS*

	Transfer from Glucose Medium to:	
	Glucose Medium	Ethanol Medium
	NAD/NADP Ratio	
Before Transfer	12	--
91 Min after transfer:		
No additions	14	26
Plus chloramphenicol	14	29
Plus cycloheximide	10	21

*Duplicate aliquots of a log phase culture (1-1.5 A_{600nm} units) growing on glucose were transferred from glucose medium to ethanol medium or glucose medium as described in "Methods". The new medium contained either 4 mg/ml of chloramphenicol or 2 µg/ml of cycloheximide.

DISCUSSION

Similar patterns of growth of yeast are observed in complex or semidefined media containing 0.8% glucose as carbon source although the growth rates are slower in the semidefined media (Figures 1 and 2, Table V). A linear correlation between cell numbers and culture turbidity at 600 nm is observed during periods of logarithmic growth (Figures 1 and 2). This is of interest since turbidimetric readings are much easier to obtain.

The enzymatic cycling assays for NAD^+ and $NADP^+$ can measure the amount of pyridine nucleotide from small amounts of culture accurately and reproducibly. The efficiency of acid and alkaline extractions of these nucleotides is such that 95% of the total NAD and 85 to 90% of the total NADP can be extracted from 2 to 8 X 10^7 cells (corresponding to 5-10 A_{600nm} units). This heating process during perchloric acid extraction destroys 5 to 10% of the $NADP^+$; however, it enhances the extraction efficiency of both NAD^+ and $NADP^+$ from the cell pellets by 20%. By increasing the amount of culture extracted from 5 to 10 A_{600nm} units to 20 or 30 A_{600nm} units, more than 95% of the total NAD, and 60 to 67% of the total NADP was recovered from cells. The recovery ratios shown in Tables III and IV indicate that neither inhibitors nor activators of the enzymatic cycling assay are

extracted from cells and that appreciable losses are not encountered during the workup of the samples.

The cellular content of RNA and protein of yeast decreases in the ethanol phase of growth (Figures 6 and 7). The decrease in RNA content is more rapid than the decrease in protein content. Cells examined under the microscope were visibly smaller during the ethanol phase of growth. These data are of interest with respect to pyridine nucleotide levels because if these levels are expressed relative to cell numbers or turbidimetric units are also dependent upon changes in cell size.

The data of Figure 8 show that the relative amounts of NAD and NADP are essentially constant during growth on glucose. However, a definite increase in the relative amount of NAD nucleotides occurs near the time of exhaustion of glucose from the culture medium. A more than two fold increase in the NAD/NADP ratio is maintained during subsequent growth on ethanol. This increase in the NAD/NADP ratio is the result of both an increase in the NAD/protein ratio and a decrease in the NADP/protein ratio (Figure 9). This two fold increase in NAD/NADP ratio is seen when log phase cultures are transferred from glucose to ethanol medium (Table VII). Likewise, the transfer of ethanol phase cultures to glucose medium results in a two fold decrease in the NAD/NADP ratio (Table VIII).

Under each of the experimental conditions in which the

NAD/NADP ratio is altered, a decreased reducing charge of the NADP pool is correlated with an increased NAD/NADP ratio (Figure 9 and Table VI). The NAD/NADP ratio change is observed in the presence of inhibitors of protein synthesis (Table X) suggesting that the ratio change involves a change in the regulation of pre-existing enzymatic activities.

NAD can be synthesized by a number of possible de novo and salvage pathways (Schemes I and II). The relative contributions of these possible pathways to the maintenance of cellular NAD levels under given physiological conditions are not known. However, it is of interest with respect to the increased levels of NAD observed during transition from the glucose growth phase to the ethanol growth phase that the activity of kynurenine 3-hydroxylase, which is a mitochondrial enzyme involved in the de novo biosynthesis of of quinolinic acid from tryptophan (Schott and Staudinger, 1971), is ten-fold higher in aerobic cultures than in anaerobic cultures. Since mitochondrial enzyme activities are glucose repressed (Perlman and Mahler, 1974), the increased levels of NAD observed during the ethanol phase of growth may be due to increased de novo synthesis of NAD under these conditions.

Clearly the mechanisms that regulate the relative amounts of NAD and NADP nucleotides are altered upon exhaustion or removal of glucose from the culture medium. Studies of pyridine nucleotide metabolism in E. coli have shown that NAD

and NADP are rapidly interconverted in vivo (Lundquist and Olivera, 1973). The relative amounts of NAD and NADP should be a function of the net rate of NAD biosynthesis and the rates of interconversion of NAD and NADP. The absolute levels of NADP should be controlled by the level of NAD and the rates of interconversions of NAD and NADP. These interpretations could be affected by non-mixing pools of free and bound nucleotide or by separate metabolic pools.

Since the levels of NAD increase upon exhausion of glucose from the culture medium, the sharp decrease in NADP nucleotide levels observed most probably represents a decreased rate of conversion of NADP to NAD.

NAD is converted to NADP by the action of NAD kinase. Cytoplasmic NAD kinase of yeast has been partially purified and studied (Apps, 1970). The enzyme has a Km of 0.32 mM for NAD^+ and 0.58 mM for ATP. NADH is a strict competitive inhibitor with NAD^+, and ATP shows positive homotropic effects on the activity (Apps, 1970). The activity could also be retarded by reduced levels of ATP, or NAD^+ kinase may be sensitive to the redox state of the NADP pool. Atkinson (1968) has proposed that the reducing charge of the NADP pool may be an important factor in regulating those processes that generate NADPH.

The enzymatic activities that convert NADP to NAD have not been studied in any detail; although a NADP phosphatase in yeast has been detected (Bernofsky and Utter, 1968). An

enhanced rate of conversion of NADP to NAD could occur in a number of ways. One possibility that would be consistent with the data presented here would be that NADP phosphatase activity is greater for $NADP^+$ than for NADPH; thus, the redox change that accompanies the exhaustion of glucose from the culture medium would provide increased levels of $NADP^+$ for conversion to NAD^+.

It is not known if any of the factors above play a major role in maintaining the levels of NAD and NADP in cells. The fact that reproducible changes in the NAD/NADP ratio and the reducing charge of the NADP pool occurs under a variety of conditions indicates that these ratios are important in cellular metabolism.

The *in vivo* changes in the regulation of pyridine nucleotide metabolism observed here will aid *in vitro* studies of the regulation of pyridine nucleotide metabolism. Isolation and characterization of NAD kinases and NADP phosphatases will facilatate the further study of the molecular mechanisms of the regulation of NAD and NADP levels.

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