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Concentrations of Nicotinamide Nucleotide Coenzymes in Micro-Organisms

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SUMMARY

The results are presented of a survey of NAD and NADP concentration in twenty micro-organisms. They fell into three categories with respect to NAD concentration. (i) High NAD (> 4·5 μmoles/g. dry weight) in obligate anaerobes, members of the Lactobacillaceae and *Saccharomyces cerevisiae*. (ii) Medium NAD (1·0–3·0 μmoles/g. dry weight) in faculative anaerobes, photosynthetic bacteria and *Nocardia globerula*. (iii) Low NAD (< 0·9 μmoles/g. dry weight) in obligate aerobes.

Different categories were not found with respect to NADP concentration. Consistent differences in NAD concentration due to conditions of aeration were not found, but growth on different substrates frequently led to changes in NAD concentration. The concentration of NAD in *Streptococcus faecalis* grown on gluconate was only 6% of the concentration in glucose-grown organisms. In *Pseudomonas oxalaticus* NAD concentrations when grown on formate or oxalate were 13 and 8 times, respectively, greater than the concentration in acetate-grown organisms.

NADP concentrations in *Leuconostoc mesenteroides* and *Streptococcus faecalis* were increased 2- to 5-fold by aeration. In *Nocardia globerula, Streptomyces griseus* and *Bacillus megaterium* the concentrations of NAD found after growth in complex media were 3–5 times greater than the amounts found after growth on minimal media. This effect was not observed with *Pseudomonas fluorescens*.

INTRODUCTION

Although there is a substantial amount of information on the concentrations of nicotinamide nucleotide coenzymes in animal tissues (see Greenbaum, Clark & McLean, 1965), relatively little is known of the concentrations in micro-organisms. Kaplan (1960) published, for NAD^+ and $NADP^+$ concentrations in eight species of micro-organism, figures which show certain tendencies. NAD^+ is almost always present in larger quantities than $NADP^+$. The concentrations of coenzyme in the four faculative anaerobes (*Escherichia coli, Aerobacter aerogenes, Photobacterium fischeri, Proteus vulgaris*) are all of the same order and are a little below the figure for *Saccharomyces cerevisiae*. The amounts found in the strict aerobes (*Pseudomonas fluorescens, Azotobacter agile, Mycobacterium butyricum*) are much lower than those found in the faculative anaerobes.

Takebe & Kitahara (1963) estimated the concentrations of nicotinamide

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nucleotide coenzymes in a variety of micro-organisms, mainly lactic acid bacteria. They did not observe the striking differences between strict aerobes and facultative anaerobes reported by Kaplan (1960), but they did find very high values for NAD among the lactic acid bacteria. They also found that there were significant differences between homofermentative and heterofermentative species; in general, NAD concentrations were higher and NADP concentrations lower in homofermentative species. Takebe & Kitahara (1963) also found that conditions of aeration and variations in substrate affected the NAD concentrations in *Escherichia coli*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* when growing on complex media.

The work of Kaplan (1960) and of Takebe & Kitahara (1963) suggested that there might be significant differences in nicotinamide nucleotide coenzyme concentrations between bacteria of different physiological types, and that the concentrations of these coenzymes might be affected by nutritional and cultural conditions. The present results are derived from a survey of NAD and NADP concentrations in a variety of micro-organisms made to confirm and extend the work of Kaplan (1960) and of Takebe & Kitahara (1963), and to find whether any physical or biochemical factors influence the intracellular concentrations of these enzymes.

METHODS

Micro-organisms, media and cultural conditions

Unless stated otherwise, cultures of all the micro-organisms used in this study were taken from the collection kept in this department.

Bacillus cereus (obtained from the Department of Bacteriology, University of Sheffield) was maintained on slopes of PMG agar (g./l.: peptone, 10·0; Marmite, 3·0; glucose, 10·0; NaCl, 5·0; agar, 10·0) and grown on the following medium: (g./l.) Difco peptone, 2·0; Marmite, 1·0; NaCl, 10·0; glucose, 10·0 or sodium acetate trihydrate, 10·0.

Bacillus megaterium was maintained on slopes of PM agar (g./l.: peptone, 10·0; Marmite, 3·0; NaCl, 5·0; agar, 10·0) and grown on the following medium (Holland, 1961; g./l.): NH_4Cl, 1·0; KH_2PO_4, 0·3; K_2HPO_4, 0·7; Na_2SO_4, 0·1; L-glutamate, 0·5; glucose, 10·0; (mg./l.) $MgSO_4.7H_2O$, 40; $MnSO_4.7H_2O$,5; $CaCl_2.2H_2O$, 2; $FeSO_4.7H_2O$, 2.

Bacillus subtilis obtained from the Department of Bacteriology, University of Sheffield, was maintained on PM agar slopes and grown on the acetate medium used for *B. cereus*.

Chlorobium thiosulphatophilum (National Collection of Industrial Bacteria) NCIB 8327 was maintained and grown on the thiosulphate medium of Larsen (1952).

Clostridium pasteurianum strain W-5 (originally obtained from Dr R. S. Wolfe, University of Illinois, Urbana, U.S.A.) was maintained by weekly transfer on 10 ml. tubes of potato broth medium (Jensen & Spencer, 1947). For estimation of coenzymes, *C. pasteurianum* was grown on 500 ml. quantities of the ammonium sulphate medium of Carnahan & Castle (1958) as modified by Lovenberg, Buchanan & Rabinowitz (1963), but with glucose (7·5 g./l.) instead of sucrose.

Clostridium welchii strain SR 12 was maintained on Robertson's meat medium (Oxo Ltd., London, E.C.4) and grown on the following medium: (g./l.) Robertson's

meat medium, 2 tablets; Oxoid tryptone, 20·0; Oxoid Lab Lemco, 2·0; glucose 10·0; (mg./l.) pyridoxine HCl, 10.

Escherichia coli strain 4071 was maintained on PM agar slopes and grown on the following media: *a*. Complex: (g./l.) Oxoid tryptone, 10·0; Difco yeast extract, 10·0; K_2HPO_4, 0·5; glucose, 3·0; *b*. Minimal: (g./l.) $MgSO_4.7H_2O$, 0·2; NH_4Cl, 2·0; Na_2HPO_4, 6·0; KH_2PO_4, 3·0; NaCl, 3·0. In experiments where growth was limited by substrate, the amounts of substrate added were: glucose, 570 mg./l., or succinic acid (neutralized by addition of NaOH) 750 mg./l. To ensure that oxygen did not become limiting, these amounts were calculated from the aerobic growth yields for *E. coli* determined by Whitaker (1961) to give a final $E_{610\,m\mu}$ of 0·6. In aerobic experiments where growth was not limited by substrate, or in anaerobic experiments, the glucose concentration was 10 g./l. *c*. Defined complete medium (S. R. Elsden, personal communication). The amino acid composition of this medium is based on the amino acid composition of *Escherichia coli* determined by Roberts *et al.* (1955). The medium contains in mg./l. medium *b* (above): aspartate, 206; lysine HCl, 199; methionine, 79; threonine, 86; isoleucine, 94; glutamate, 242; proline, 83; arginine HCl, 177; serine, 100; glycine, 93·5; cysteine HCl, 41; alanine, 176; valine, 100; leucine, 161; tyrosine, 59·6; phenylalanine, 84; tryptophan, 33; histidine HCl, 28·8; adenine, 81; guanine, 109; cytosine, 53·5; uracil, 47; thymine, 11·4; thiamin HCl, 0·5; pyridoxine HCl, 0·5; calcium pantothenate, 0·5; riboflavin, 0·5; nicotinic acid, 1·0; *p*-aminobenzoic acid, 0·1; biotin, 0·001; folic acid, 0·01.

Leuconostoc mesenteroides was maintained on PMG agar slopes, and grown on the medium of Takebe & Kitahara (1963) with glucose as substrate.

Leucothrix mucor (obtained from Dr T. Brock, Department of Bacteriology, University of Indiana, Bloomington, Indiana, U.S.A.) was maintained and grown on the media of Brock (1964).

Micrococcus denitrificans (Verhoeven strain; obtained from Dr J. G. Morris, Department of Biochemistry, University of Leicester) was maintained on the medium described by Kornberg, Collins & Bigley (1960) and grown on the medium of Kornberg (1958) with glycerol (5·0 ml./l.) as carbon source, and with $(NH_4)_2SO_4$ (5·0 g./l.) as nitrogen source instead of ammonium acetate.

Nocardia globerula (NCIB 8852) was maintained on PM agar slopes and grown on: *a*, the complex medium of Krebs & Bellamy (1960) or, *b*, the defined medium of Mencher & Heim (1962).

Peptostreptococcus elsdenii strain LC 1 was maintained and grown on the media described by Walker (1958).

Pseudomonas fluorescens strain KB 1 was maintained on PM agar slopes and grown on the following media: *a*, minimal: (g./l.) $(NH_4)_2SO_4$, 0·5; KH_2PO_4, 10·0; nitrilotriacetic acid, 1·0; substrate (glucose or sodium succinate), 5·0; stock salt solution (Bauchop & Elsden, 1960), 1·0 ml. The pH was adjusted to 7·0 with NaOH. *b*, Complex: (g./l.) peptone, 10·0; Marmite, 3·0; NaCl, 5·0; succinic acid, 15·0; NaOH to pH 6·6.

Pseudomonas oxalaticus strain OX 1 was maintained on PM oxalate (5 mM) agar slopes. It was grown on the medium of Johnson, Jones-Mortimer & Quayle (1964) with 100 mM formate, oxalate or acetate as substrate.

Pseudomonas saccharophila (obtained from Dr M. Doudoroff, Department of Bacteriology, University of California, Berkeley, California, U.S.A.), was maintained

and grown on the following medium: (g./l.) KH_2PO_4, 2·32; Na_2HPO_4, 6·25; NH_4Cl, 1·0; $MgSO_4.7H_2O$, 0·5; ferric ammonium citrate, 0·1; $CaCl_2$, 0·01; starch, 2·5. (M. Doudoroff, personal communication).

Rhodospirillum rubrum strain 1.1.1 and *Rhodopseudomonas palustris* strain 2.1.7 (both obtained from Dr J. Lascelles, Department of Biochemistry, University of Oxford) were maintained and grown on the malate + glutamate media of Lascelles (1959).

Saccharomyces cerevisiae was maintained on PMG agar slopes and grown at 30° on the following media: *a*, PMG; *b*, minimal: (g./l.) KH_2PO_4, 1·0; $(NH_4)_2SO_4$, 0·5; glucose, 10·0; nitrilotriacetic acid, 1·0; inorganic salt solution B (Barton-Wright, 1946), 10·0 ml.; vitamin solution (Wickerham, 1951), 10·0 ml.

Streptococcus faecalis (National Collection of Type Cultures) NCTC 6782 was maintained on PMG agar slopes. Cultures for estimating coenzymes were grown on the complex medium of Takebe & Kitahara (1963) with either glucose (20·0 g./l.) or gluconate (3·0 g./l.) as substrate.

Streptomyces griseus NCTC 7807 was maintained on PMG agar slopes and grown on: *a*, PMG; *b*, the medium of Mencher & Heim (1962).

Three cultures of each organism studied were always grown, harvested and extracted simultaneously. With some exceptions, it was found that the yield of bacterial paste from 300 ml. medium contained enough coenzyme for measurement under the conditions described below. Apart from the exceptions mentioned below, cultures were harvested 12–15 hr after inoculation. The lactic acid bacteria were grown at 37°, the photosynthetic bacteria at 26°–29°; all others at 30°.

Anaerobic cultures of *Clostridium pasteurianum*, *C. welchii*, *Escherichia coli*, *Leuconostoc mesenteroides*, *Peptostreptococcus elsdenii*, *Saccharomyces cerevisiae* and *Streptococcus faecalis* were grown in 500 ml. florence flasks. The obligate anaerobes were grown under an atmosphere of H_2, the facultative anaerobes under N_2.

Aerobic cultures of *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae* and *Streptococcus faecalis* and of *Escherichia coli* in which substrate was not limiting, as well as all cultures of *Bacillus cereus*, *B. subtilis*, *B. megaterium*, *Pseudomonas fluorescens* and *P. saccharophila*, were grown in penicillin flasks which were agitated on a shaker with a rocking action. The rate of shaking was 40 strokes/min., with an amplitude of 20 cm.

Cultures of *Escherichia coli* grown aerobically on limited substrate, of *Pseudomonas fluorescens* grown on complex medium, and all cultures of *P. oxalaticus*, *Micrococcus denitrificans*, *Streptomyces griseus* and *Nocardia globerula*, were grown in 2 l. conical flasks, shaken at 200 strokes/min through a 1 in. circle in a Gyrotory incubator-shaker (Model G-25; New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.). With *P. oxalaticus* grown on formate or oxalate, where growth was limited by increasing pH value as the substrate was used, 600 ml. medium were used to give sufficient bacteria.

Rhodospirillum rubrum and *Rhodopseudomonas spheroides* were grown for 36 hr in 1 l. Roux bottles in the light as described by Hoare (1963).

Leucothrix mucor was grown in 1 l. conical flasks on a Griffin wrist-action shaker (Griffin & George Ltd., Wembley, Middlesex).

Reagents

NAD, NADP (sodium salt), glucose-6-phosphate (disodium salt), alcohol dehydrogenase (yeast), glucose-6-phosphate dehydrogenase (yeast), and glutamic dehydrogenase (bovine liver) were brought from Boehringer Corporation (London) Ltd., London, W. 5. 2-Oxoglutaric acid was obtained from Koch-Light Laboratories, Colnbrook, Buckinghamshire. All other reagents were Analar, or of comparable purity.

Extraction of nicotinamide coenzymes

The procedure finally adopted, based on the methods of Bassham *et al.* (1959) and Takebe & Kitahara (1963), was as follows. The bacteria were harvested in a refrigerated centrifuge (15–20 min. at 5400*g*) and washed once with one-fifth of the original culture volume of ice-cold 50 mM phosphate buffer (pH 7·0; $KH_2PO_4 + KOH$ mixture). The bacterial pellet was resuspended in 7–10 ml. 0·1M tris buffer, pH 8·2. Samples of this suspension were then taken to determine dry weight of organism. In the case of *Escherichia coli*, *Pseudomonas oxalaticus* and *Micrococcus denitrificans*, where curves relating extinction at 610 mμ (E_{610}) to dry weight already existed, dry weight was determined from the E_{610} reading of a diluted sample, measured in a Unicam SP 600 spectrophotometer. In the other cases, the bacteria in the sample were washed twice with water by centrifugation, resuspended in water, transferred to a tared weighing bottle and dried at 105° to constant weight.

The oxidized forms of the coenzymes were extracted by treatment with acid, the reduced forms by treatment with alkali. Acid and alkaline extraction was performed by adding HCl (1·5 ml. 0·33 N) or NaOH (1·5 ml. 0·33 N), respectively, to the suspensions, (3·0 ml.) in 20 × 150 mm. tubes. After incubation at 50° for 10 min., the extract was cooled to 0° and neutralized cautiously with either 0·45 ml. of N-NaOH or 0·45 ml. of N-HCl. In order to avoid local high concentrations, the acid or alkali was added at about 10 μl./sec., while the extract was stirred vigorously with a length of flexible polyethylene rod (3 mm. diam.) attached to an overhead stirrer. The neutralized extracts were centrifuged at 23,000*g* for 15 min. to remove insoluble material.

The oxidized forms of the coenzymes, extracted with acid, were then estimated by one of the methods described below. Before estimation, the reduced forms of both coenzymes (extracted with alkali) were oxidized enzymically by adding 10 μl. 2-oxoglutarate + NH_4Cl solution (containing 146 mg. 2-oxoglutarate and 53·5 mg. NH_4Cl/ml., adjusted to pH 7·0 with N-NaOH) and 10 μl. glutamic dehydrogenase (stock enzyme, diluted 5-fold with 0·1M phosphate buffer, pH 7·5) to each sample. After 15 min. at room temperature, 0·1 ml. 5 N-HCl was added to each tube, and the extracts incubated 15 min. at 50° to destroy enzyme. The extract was then neutralized by adding 0·09 ml. 5 N-NaOH, with the precautions described above and, when necessary, clarified by centrifuging for 15 min. at 23,000*g*.

The reliability of the extraction procedure was established by submitting standard solutions of oxidized and reduced forms of both coenzymes to the extraction procedure and measuring the recovery of the coenzymes spectrophotometrically (see below). The % recoveries were as follows: NAD^+, 98; NADH, 96; $NADP^+$, 98; and NADPH, 94. Further, it was shown that any reduced coenzyme present was

completely destroyed by acid extraction, and that the oxidized forms were destroyed during alkaline extraction.

Effect of extraction conditions on recovery of coenzymes. In preliminary experiments with *Saccharomyces cerevisiae*, by the procedure of Takebe & Kitahara (1963) it was found that the period of extraction was critical. Unless the extraction was carried out with extreme care, significant amounts of NAD were destroyed (Fig. 1). Since adequate regulation, both of temperature and of time of exposure of the bacterial, suspensions to heat under conditions 'near boiling' was difficult, the possibility of extracting the coenzymes at lower temperatures was investigated, and 50° was finally chosen as the extraction temperature. A time course of the release of NAD and NADP from *S. cerevisiae*, using the conditions of acid extraction described above, is shown in Fig. 2. Release of NAD(P) was complete in 8 min.; 10 min. was chosen as the standard time of extraction. There was no detectable loss of NAD(P) when the extraction time was extended to 15 min.

Some preliminary experiments were also done on the optimum concentration of acid for NAD extraction. The results are given in Table 1. The acid concentration chosen (equivalent to 0·5 ml. N-HCl in 4·5 ml. extract) was that giving maximum extraction at minimum acid concentration. The concentration of NaOH used in the alkaline extraction was chosen arbitrarily.

Table 1. *Effect of acid concentration on extraction of NAD from Saccharomyces cerevisiae*

N-HCl added (ml.)	Final HCl concentration (mN)	Final pH value	NAD recovered (μmoles/g. dry wt.)
1·0	222	0·8	3·74
0·5	111	1·2	3·79
0·25	55·5	1·7	3·59
0·15	33·3	2·8	1·74

Suspensions of *Saccharomyces cerevisiae* (3 ml.) in a final volume of 4·5 ml. were extracted and the NAD assayed spectrophotometrically as described under 'Methods'.

Estimation of nicotinamide coenzymes

Spectrophotometric method. The coenzymes were reduced enzymically (NAD with alcohol dehydrogenase, NADP with glucose-6-phosphate dehydrogenase), and the amount of coenzyme in the sample calculated from the increase in E_{340} and the molar extinction coefficient for NAD(P)H ($6{\cdot}22 \times 10^3$; P-L Biochemicals, 1961). The spectrophotometer used was a Unicam SP 500 (Unicam Instruments, Cambridge, England). The cuvettes used were glass, with a 1 cm. light-path.

The NAD assay system consisted of 1·0 ml. extract, 2·0 ml. pyrophosphate + semicarbazide buffer (Bonnichsen, 1962) + 10 μl. M-ethanol. After determination of the initial E_{340}, 10 μl. alcohol dehydrogenase (stock enzyme, diluted 10-fold with pyrophosphate + semicarbazide buffer) was added. The reaction was usually complete in 5 min.; the final E_{340} value determined 10 min. after addition of the enzyme.

The NADP assay system consisted of 1–3 ml. extract, 50 mM tris buffer (pH 8·3), to 3·0 ml. + 10 μl. 0·1 M-glucose-6-phosphate. After determination of the initial E_{340} value, 10 μl. glucose-6-phosphate dehydrogenase (stock enzyme, diluted 10-fold in

0·4 M triethanolamine buffer (pH 7·6; Hohorst, 1962) was added. In this case, the reaction was a little slower; the final E_{340} value was determined 15 min. after the addition of enzyme.

Fluorimetric assay. NAD and NADP were also assayed fluorimetrically by following the increase in fluorescence produced by the reduction of $NAD(P)^+$. The assay systems were the same as those used in the spectrophotometric assays, except that the total volume in the assay tubes was increased to 5·0 ml. by the addition of buffer and the amount of extract used was decreased to 0·02–0·1 ml. The fluorimeter used was that described by Dalziel (1962). Readings were taken directly from

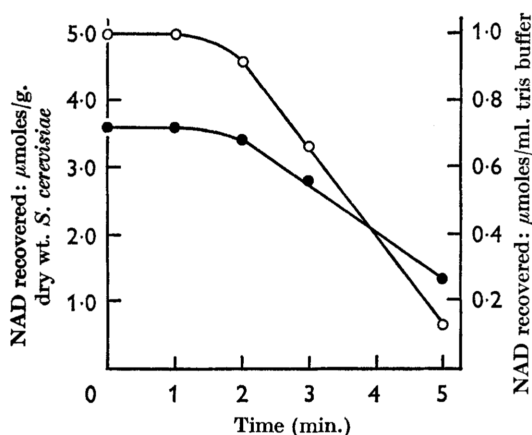

Fig. 1

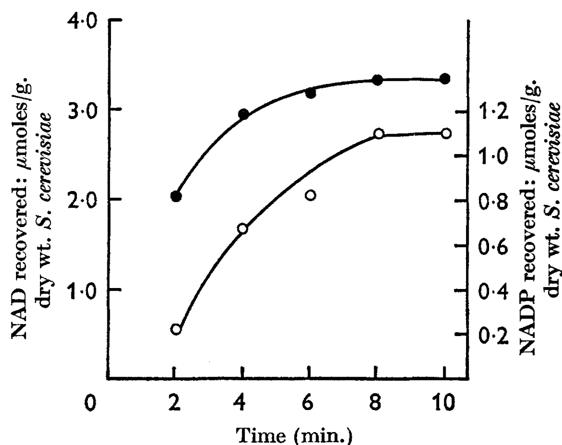

Fig. 2

Fig. 1. Destruction of NAD during extraction in boiling water bath. 3 ml. samples of 0·72 mM-NAD in tris buffer, pH 8·2; (○—○—○): 3 ml. samples of a suspension (25·0 mg. dry wt./ml.) of *Saccharomyces cerevisiae* in tris buffer, pH 8·2 + 1·5 ml. 0·33 N-HCl heated in boiling water bath. (●—●—●). Extracts neutralized and NAD assayed spectrophotometrically.

Fig. 2. Progress curve of extraction of NAD and NADP from *Saccharomyces cerevisiae* at 50°. 3 ml. samples of a suspension (25·0 mg. dry wt./ml.) of *S. cerevisiae* in tris buffer, pH 8·2 + 1·5 ml. 0·33 N-HCl extracted at 50°. Extracts neutralized and NAD (●—●—●) and NADP (○—○—○) assayed spectrophotometrically.

the millivoltmeter on the Vibron amplifier; the recorder was used only to indicate when the reaction was complete. The extracts varied considerably in their quenching properties and in inherent fluorescence. Increase in fluorescence was related to the quantity of NAD(P) present by addition of a known quantity of $NAD(P)^+$ as an internal standard.

RESULTS

Although the oxidized and reduced forms of the coenzymes were isolated and estimated separately, no significance was attached to the relative proportions of the two forms. The quantities of NAD(P) quoted refer to the sums of oxidized and reduced forms.

Survey of coenzyme concentrations. The results of a survey of NAD and NADP concentrations in various micro-organisms are given in Table 2. The bacteria studied apparently fall into three groups with respect to NAD concentration. No

Table 2. *Summary of NAD and NADP concentrations in various microorganisms*

Micro-organism	Medium	Conditions	NAD (μmoles/g. dry wt. organism)	NADP (μmoles/g. dry wt. organism)	No. of cultures assayed	Assay method
Streptococcus faecalis	Complex, glucose	Aerobic	10·59 ± 0·35	0·54 ± 0·05	6	S
S. faecalis	Complex, glucose	Anaerobic	10·06 ± 0·18	0·11 ± 0·02	6	S
Clostridium pasteurianum	Minimal, glucose	Anaerobic	8·91 ± 0·56	0·87 ± 0·08	6	S
Peptostreptococcus elsdenii	Complex, lactate	Anaerobic	7·25 ± 0·78	0·36 ± 0·11	6	S
Saccharomyces cerevisiae	Complex, glucose	Aerobic	8·26 ± 0·77	0·37 ± 0·07	6	S
S. cerevisiae	Complex, glucose	Anaerobic	6·70 ± 1·35	1·12 ± 0·05	6	S
Leuconostoc mesenteroides	Complex, glucose	Anaerobic	5·62 ± 1·35	0·30 ± 0·07	6	S
L. mesenteroides	Complex, glucose	Aerobic	4·90 ± 0·33	0·82 ± 0·10	6	S
Clostridium welchii	Complex, glucose	Anaerobic	4·91 ± 0·45	0·11 ± 0·01	6	S
Escherichia coli	Minimal, glucose (excess)	Anaerobic	2·67 ± 0·21	0·58 ± 0·21	6	S
E. coli	Minimal, glucose (excess)	Aerobic	2·44 ± 0·14	0·24 ± 0·07	6	S
E. coli	Minimal, glucose (limited)	Aerobic	2·56 ± 0·19	0·84 ± 0·21	6	S
E. coli	Complete (defined), glucose	Aerobic	1·98 ± 0·39	0·48 ± 0·07	3	F
Rhodospirillum rubrum	Malate + glutamate	Light, anaerobic	1·99 ± 0·04	0·43 ± 0·04	3	S
Nocardia globerula	Glucose + glycine + glutamate	Aerobic	1·96 ± 0·25	0·07 ± 0·03	6	S
Micrococcus denitrificans	Minimal, glycerol	Aerobic	1·33 ± 0·25	0·41 ± 0·22	6	S
Bacillus cereus	Complex, glucose	Aerobic	1·14 ± 0·35	0·12 ± 0·06	6	F
Chlorobium thiosulphatophilum	Thiosulphate	Light, anaerobic	1·11 ± 0·16	Not detected	3	S
Rhodopseudomonas palustris	Malate + glutamate	Light, anaerobic	1·09 ± 0·04	0·84 ± 0·24	3	S
Streptomyces griseus	Glucose + glycine + glutamate	Aerobic	0·89 ± 0·18	0·08 ± 0·05	6	S
Bacillus megaterium	Minimal, glucose	Aerobic	0·56 ± 0·14	0·11 ± 0·06	6	F
Pseudomonas saccharophila	Minimal, starch	Aerobic	0·40 ± 0·06	0·79 ± 0·01	3	S
Leucothrix mucor	Peptone	Aerobic	0·36 ± 0·08	0·10 ± 0·06	3	F, S
Bacillus subtilis	Complex, acetate	Aerobic	0·35 ± 0·14	0·70 ± 0·12	6	F
Pseudomonas fluorescens	Minimal, glucose	Aerobic	0·19 ± 0·08	0·04 ± 0·01	6	F, S

Extracts were prepared and coenzyme content assayed as described under Methods.
F = fluorimetric assay; S = spectrophotometric assay.
Values are presented in the form Mean ± standard deviation.

similar tendencies were observed with respect to NADP. The strict anaerobes and lactic acid bacteria had high concentrations of NAD (above 4·5 μmoles/g. dry weight). The faculative anaerobes had concentrations of between 1·0 and 3·0 μmoles/g. dry weight and the strict aerobes had, with one exception (*Nocardia globerula*), less than 1·0 μmole/g. dry weight. The three photosynthetic bacteria have NAD concentrations similar to those of the faculative anaerobes. *Saccharomyces cerevisiae*, which is metabolically a facultative anaerobe, had much higher concentrations of NAD than the bacteria of this physiological type.

Table 3. *Effect of anaerobiosis on NAD(P) concentrations*

Extracts were prepared and coenzyme concentrations measured spectrophotometrically as described under Methods. All cultures (six of each organism) were grown with glucose as substrate.

		Aerobic		Anaerobic	
		NAD	NADP	NAD	NADP
		(μmoles/g. dry wt. organism)		(μmoles/g. dry wt. organism)	
Organism	Medium				
Streptococcus faecalis	Complex	10·59 ± 0·35	0·54 ± 0·05	10·06 ± 0·18	0·11 ± 0·02
Leuconostoc mesenteroides	Complex	4·90 ± 0·33	0·82 ± 0·10	5·62 ± 1·35	0·30 ± 0·07
Escherichia coli	Minimal	2·44 ± 0·14	0·24 ± 0·07	2·67 ± 0·21	0·58 ± 0·21
Saccharomyces cerevisiae	Minimal	4·92 ± 1·27	0·27 ± 0·08	6·47 ± 1·12	0·31 ± 0·05
S. cerevisiae	Complex	8·26 ± 0·77	0·37 ± 0·07	6·70 ± 1·35	1·12 ± 0·05

Table 4. *Effect of complex medium on NAD(P) concentrations*

All cultures were grown aerobically. Extracts were prepared and coenzyme levels assayed as described under Methods. Coenzymes were assayed fluorimetrically in *Bacillus megaterium* (minimal medium). In all other cases they were assayed spectrophotometrically.

	Minimal medium		Complex medium	
	NAD	NADP	NAD	NADP
	(μmoles/g. dry wt. organism)			
Organism				
Nocardia globerula	1·96 ± 0·25	0·07 ± 0·03	5·23 ± 0·52	0·55 ± 0·10*
Streptomyces griseus	0·89 ± 0·18	0·08 ± 0·05	2·28 ± 0·10	0·18 ± 0·06*
Bacillus megaterium	0·56 ± 0·14	0·11 ± 0·06	1·52 ± 0·40	0·24 ± 0·11†
Pseudomonas fluorescens	0·27 ± 0·07	0·03 ± 0·01	0·07 ± 0·02	0·03 ± 0·01‡

* Grown on PMG medium; † grown on minimal medium + 0·1 % yeast extract; ‡ grown on PM-succinate.

Effect of conditions of growth on coenzyme concentration. The results of studies on the effect of anaerobiosis on NAD(P) concentration are summarized in Table 3. These results indicate that NAD concentration might differ between cultures grown aerobically and anaerobically, but no particular trend is discernible. NADP concentrations in *Streptococcus faecalis* and *Leuconostoc mesenteroides* were higher when the bacteria were grown aerobically than when they were grown anaerobically. This may reflect a greater use of the pentose phosphate pathway under aerobic conditions.

It was found with *Escherichia coli* that the addition of nicotinic acid (1·5 mg./l.) to minimal medium led to an increase in both NAD and NADP (NAD from

2·44 μmoles/g. to 9·44 μmoles/g.; NADP from 0·24 μmoles/g. to 0·45 μmoles/g.). With *Bacillus megaterium* the addition of 0·1 % yeast extract to minimal medium led to a 2-fold increase in NAD and NAD(P) (Table 4). NAD in *Nocardia globerula* and *Streptomyces griseus* was increased 2- to 3-fold on complex media, compared with the amounts found on minimal medium (Table 4). These increased amounts were not found when *Pseudomonas fluorescens* was grown on a complex medium. The effects on NAD(P) of growing *Saccharomyces cerevisiae* on complex and minimal media are shown in Table 3. Concentrations of NAD and NADP were generally lower when the organism was grown on a minimal medium than when it was grown on a complex one. The effects of growing *E. coli* under different conditions are shown in Table 2. It is not possible to detect any differences in NAD(P) concentration between *E. coli* grown in a completely defined medium with excess glucose and on a minimal medium with excess glucose, or between growth on a minimal medium with excess or with limiting glucose.

Table 5. *Effect of energy source on NAD(P) concentration*

Organisms were grown aerobically as described under Methods. *Escherichia coli* was grown on limiting substrate. Extracts were prepared and coenzymes measured as described under Methods. S = spectrophotometric assay; F = fluorimetric assay.

Organism	Substrate	NAD (μmoles/g. dry wt. organism)	NADP (μmoles/g. dry wt. organism)	No. of cultures assayed	Assay method
Streptococcus faecalis	Glucose	10·59 ± 0·35	0·54 ± 0·05	6	S
S. faecalis	Gluconate	0·66 ± 0·28	0·24 ± 0·01	3	S
Bacillus cereus	Glucose	1·14 ± 0·35	0·12 ± 0·06	6	F
B. cereus	Acetate	1·66 ± 0·28	1·42 ± 0·36	6	F
Pseudomonas fluorescens	Glucose	0·19 ± 0·08	0·04 ± 0·01	6	F, S
P. fluorescens	Succinate	0·27 ± 0·07	0·03 ± 0·01	6	F, S
Escherichia coli	Glucose	2·56 ± 0·19	0·84 ± 0·21	6	S
E. coli	Succinate	2·13 ± 0·26	0·70 ± 0·22	9	S
P. oxalaticus	Formate	3·59 ± 0·34	0·50 ± 0·25	6	S
P. oxalaticus	Oxalate	2·20 ± 0·37	0·87 ± 0·18	6	S
P. oxalaticus	Acetate	0·27 ± 0·07	0·41 ± 0·12	6	S

Some effects of substrate on NAD(P) concentrations are shown in Table 5. The NAD in gluconate-grown *Streptococcus faecalis* was about 16 times less than that found in glucose-grown organisms. The NAD found in gluconate-grown organisms is characteristic of a strict aerobe. When *Pseudomonas oxalaticus* was grown on acetate the NAD concentration was low, as would be expected of a strict aerobe; growth on formate results in a 13- to 14-fold increase, and on oxalate in an 8-fold increase in NAD over the concentration found in acetate-grown *P. oxalaticus*. The differences in NAD concentrations between glucose-grown and acetate-grown *Escherichia coli* or *P. fluorescens* are not significant. Only in the case of *Bacillus cereus*, where growth on acetate caused a 12-fold increase in NADP, was there any difference in NADP concentrations.

DISCUSSION

One of the objects of the work described here was to confirm and extend the work of Kaplan (1960) and Takebe & Kitahara (1963). The concentrations of NAD and NADP found by these authors are compared with those found in the present work in Table 6. In the case of the lactic acid bacteria, agreement between the values for NAD concentration of Takebe & Kitahara (1963) and those reported here is close. The amounts of NAD found by Takebe & Kitahara (1963) in *Saccharomyces cerevisiae* and *Escherichia coli* are lower, while their figures for *Bacillus subtilis* and *Pseudomonas fluorescens* are higher. The higher values might reflect the fact that these authors grew all their cultures on complex media. Comparison with the results of Kaplan (1960) is less easy, because his NAD(P)$^+$ concentrations are expressed as μg./g. wet weight, and because his bacterial extracts contained variable proportions of an unknown material other than NAD$^+$ or NADP$^+$ which reacted as oxidized nicotinamide nucleotide in his assay system; in addition, the method of extraction and assay was not described. Nevertheless, the concentrations of NAD found by Kaplan in four facultative anaerobes are of the same order as those reported here, as are the values for the strict aerobes. There is also good agreement between Kaplan and Takebe & Kitahara for the NAD concentration in *Saccharomyces cerevisiae*. In general, agreement between the different values reported for NADP concentrations is poor.

Table 6. *Comparison of NAD(P) concentrations with results of other authors*

	Present paper		Takebe & Kitahara (1963)*		Kaplan (1960)*	
	NAD	NADP	NAD	NADP	NAD	NADP
	μmoles/g. dry wt. organism					
Streptococcus faecalis	10·57	0·11	10·06	0·20	.	.
Leuconostoc mesenteroides	5·62	0·32	4·86–5·39	0·53–0·70	.	.
Saccharomyces cerevisiae	6·70	1·12	2·68–3·20	0·40–1·13	2·9	0·4
Escherichia coli	2·67–1·98	0·24–0·84	1·65	0·20	1·4	0·2
Aerobacter aerogenes	.	.	2·04	0·05	2·0	0·2
Achromobacter fischeri	.	.	.	.	2·2	0·3
Proteus vulgaris	.	.	.	.	1·9	0·2
Pseudomonas fluorescens	0·19	0·04	1·35	0·26	0·2	0·3
Bacillus subtilis	0·35	0·70	1·50	0·17	.	.
Azotobacter agile	.	.	.	.	0·6	0
Mycobacterium butyricum	.	.	.	.	0·5	0

* The values of Takebe & Kitahara (1963) were originally expressed as mg./g. dry wt. organism. Those of Kaplan (1960) were expressed as μg./g. wet weight. They have been converted to dry weight values on the assumption that the dry weight content of wet bacterial paste is 20 %.

These observations have been extended to show that the strict anaerobes and the Lactobacillaceae (which, though microaerophilic, have a strictly fermentative metabolism), the faculative anaerobes, and the strict aerobes, fall into distinct classes with respect to NAD concentration. There are two exceptions to this generalization. *Saccharomyces cerevisiae*, a faculative anaerobe, had NAD concentrations more typical of a strict anaerobe, and *Nocardia globerula*, a strict aerobe, had NAD concentrations as high as many facultative anaerobes.

The high NAD concentration found in the strict anaerobes seems to be associated with the absence of a haem-linked oxidation system. It was shown by Takebe, Shirakawa & Kitahara (1964) that in *Leuconostoc mesenteroides* at least 96% of the NAD remained in the supernatant fluid after a sonic extract had been centrifuged at 100,000*g* for 60 min. If the very small amount of NAD found here in strict aerobes was closely associated with the intracytoplasmic membrane, as the respiratory enzymes are in *Azotobacter agilis* (Pangborn, Marr & Robrish, 1962), and if most oxido-reduction reactions occurred close to the membrane, then the turnover of NAD could be very rapid and the effective concentration of NAD could be as high as it is in strict anaerobes.

Another object of the present work was to determine whether the concentration of NAD or NADP might vary with the conditions of cultivation. Takebe & Kitahara (1963) showed that NADP concentrations were generally higher in members of the Lactobacillaceae with a heterolactic fermentation pattern than those with a homolactic pattern. They suggested that this might be a reflexion of the greater utilization of the NADP-linked dehydrogenases of the pentose phosphate pathway in these organisms. Although NADP concentrations are in general at the limit of detection by the method used, it is possible to show that in *Streptococcus faecalis* growing on glucose there is a 3- to 5-fold increase in NADP in aerated cultures over the value in cultures grown under nitrogen. It is possible that under aeration there is an increased utilization of the pentose phosphate pathway in *Streptococcus faecalis*. This explanation cannot account for the similar increase in NADP found in *Leuconostoc mesenteroides*. In this organism the glucose-6-phosphate dehydrogenase reacts with both NAD and NADP, and 6-phosphogluconate dehydrogenase with NAD only (De Moss, 1955) and it has been shown (Kemp & Rose, 1964) that the hydrogen carrier for the reductive reactions of fermentation is NAD. It has not been possible to show any other consistent effects of aeration versus anaerobiosis or of minimal medium versus complex medium on NAD(P) concentrations.

The nature of the medium has been shown to have a considerable effect on NAD concentration. The addition of nicotinate (1·5 mg./l.) to a minimal medium caused a 4-fold increase in the NAD concentration in *Escherichia coli* and the addition of 0·1% yeast extract to a minimal medium led to a 2- to 3-fold increase in NAD concentration in *Bacillus megaterium*. In *Nocardia globerula* and *Streptomyces griseus* NAD after growth on complex media was 2–3 times higher than after growth on a minimal medium; NAD in *Pseudomonas fluorescens* was, in contrast, scarcely affected.

The nature of the carbon source may also affect NAD concentration. The dramatic decrease in NAD in gluconate-grown *Streptococcus faecalis* as compared with the value when the organism was grown on glucose was probably connected with the change-over from use of the glycolytic pathway in glucose-grown organisms to the ketogluconate pathway in gluconate-grown organisms (Gibbs, Sokatch & Gunsalus, 1955; Sokatch & Gunsalus, 1957).

The increased NAD concentration in *Pseudomonas oxalaticus* grown on formate may be explained by the fact that on formate the organism gains all its energy from the NAD-linked oxidation of formate, and that NADH is the reductant used in the reductive pentose phosphate cycle from which the biosynthetic pathways of the organism diverge (Professor J. R. Quayle, personal communication). The concentration

of NAD in oxalate-grown *P. oxalaticus* was some 8 times the value for acetate-grown organisms. During growth on oxalate the organism is still dependent on NAD-linked formate oxidation for its entire energy supply (Johnson *et al.* 1964), but the reducing agent in the early stages of biosynthesis is reduced NADP (Quayle, Keech & Taylor, 1961). This may account for the slightly higher concentration of NADP in oxalate-grown *P. oxalaticus*. In cases where growth on a different substrate did not induce a new catabolic pathway, as with *P. fluorescens* or *Escherichia coli* growing on glucose or succinate, NAD(P) concentration did not change.

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