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Nicotinamide–Adenine Nucleotides of *Mycobacterium tuberculosis* $H_{37}R_V$

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(*Received* 29 *October* 1962)

Although the prominent role of the nicotinamide–adenine nucleotides in biological systems has long been known, and their distribution in various tissues and subcellular particles has been studied in some detail, comparatively few reports relate to micro-organisms. Those micro-organisms that have been studied for their differential nicotinamide–adenine nucleotide concentrations are listed by Kaplan (1960). Among the mycobacteria, information is available only on the nicotinamide–adenine nucleotide concentrations of *Mycobacterium butyricum* (Kaplan, 1960) and *Mycobacterium phlei* (Weber & Swartz, 1960).

In this Laboratory, Indira & Ramakrishnan (1959, 1962), Ramakrishnan, Indira & Maller (1962) and Suryanarayana Murthy, Sirsi & Ramakrishnan (1962) have studied the dissimilation of glucose by *Mycobacterium tuberculosis* with special reference to virulence. As a result of their studies, which showed the presence of the glycolytic and oxidative enzymes in the organism, it was considered worth while to measure the relative concentrations of reduced and oxidized nicotinamide–adenine nucleotides in the organism since they are the limiting factors in oxidative metabolism.

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MATERIALS

NAD, $NADH_2$, NADP, $NADPH_2$, crystalline yeast alcohol dehydrogenase (alcohol–nicotinamide–adenine dinucleotide oxidoreductase, EC 1.1.1.1), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–nicotinamide–adenine dinucleotide phosphate oxidoreductase, EC 1.1.1.49) and glutathione reductase [dihydronicotinamide–adenine dinucleotide(phosphate)–glutathione oxidoreductase, EC 1.6.4.2] were all obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Glucose 6-phosphate (barium salt) was converted into the potassium salt before use and oxidized glutathione (GSSG) was prepared by the oxidation of reduced glutathione (GSH).

Neurospora nicotinamide–adenine dinucleotide nucleosidase (nicotinamide–adenine dinucleotide glycohydrolase, EC 3.2.2.5) was prepared by the method of Kaplan, Colowick & Nason (1951) from zinc-deficient *Neurospora crassa* and purified as far as 'step 3'.

The virulent *M. tuberculosis* strain $H_{37}R_v$ and its avirulent mutant $H_{37}R_a$ were grown on the synthetic liquid medium described by Youmans & Karlson (1947). The virulent strain was harvested on the fifteenth day and the avirulent strain on the twenty-first day.

METHODS

The assay procedures employed for nicotinamide–adenine nucleotides were essentially those described by Weber & Swartz (1960).

The spectrophotometric measurements were made on a Beckman model DU spectrophotometer and the fluorescence determinations were carried out on a Lumetron model 402 EF colorimeter. The enzymic incubations were all carried out in an electrically controlled thermostat.

Oxidized nicotinamide–adenine nucleotides. The cells were harvested by filtration, washed with ice-cold water and suspended in cold 5% (w/v) trichloroacetic acid and subjected to disruption for 10 min. at 0–3° in a 10 kcyc./sec. Raytheon sonic oscillator. The ultrasonic extract was centrifuged at 13000*g* in the cold for 30 min. and the supernatant used for the determination of oxidized nicotinamide–adenine nucleotides.

Reduced nicotinamide–adenine nucleotides. The cells were harvested by filtration and washed with ice-cold 5 mM-KCN. The washed cells were suspended in 0·1 M-sodium carbonate and treated ultrasonically at 0–3° for 10 min. The disrupted-cell suspension was transferred to a pre-heated tube kept in a boiling-water bath and maintained at 100° for 4 min. The contents were then transferred to a chilled centrifuge tube and centrifuged at 13000*g* for 30 min. The supernatant was used for the determination of reduced nicotinamide–adenine nucleotides.

The fluorimetric determinations were carried out on the same day as the preparation of extracts by the modified ethyl methyl ketone method of Carpenter & Kodicek (1950). NAD, NADP, $NADH_2$ and $NADPH_2$ were determined by specific enzymic fluorimetric methods applied to acid and alkali extracts as described by Ciotti & Kaplan (1957) and Jacobson & Astrachan (1957).

Assay of total oxidized nicotinamide–adenine nucleotides. Reaction mixtures contained 0·3 ml. of the trichloroacetic acid extract, sufficient 1·0 M-tris–NaOH buffer to bring the pH to 7·5, and 0·1 ml. each of 0·1 M-potassium phosphate buffer, pH 7·2, and *Neurospora* nicotinamide–adenine dinucleotide glycohydrolase, in a final volume of 1·0 ml. Parallel samples without nicotinamide–adenine dinucleotide glycohydrolase, and 6·5 μg. standards of NAD with and without nicotinamide–adenine dinucleotide glycohydrolase, were also included. Incubations were carried out at 37° for 30 min.

Assay of nicotinamide–adenine dinucleotide. Reaction mixtures contained 0·3 ml. of trichloroacetic acid extract, sufficient 1·0 M-tris–NaOH buffer to bring the pH to 7·5, 0·1 ml. of ethanol–tris (twice the strength of that described by Weber & Swartz, 1960) and 0·05 ml. of a solution (1 mg./ml.) of yeast alcohol dehydrogenase, in a final volume of 1·0 ml. Parallel samples without alcohol dehydrogenase, and 6·5 μg. standards of NAD with and without alcohol dehydrogenase, were also included. Incubations were carried out at 37° for 7·5 min.

Assay of nicotinamide–adenine dinucleotide phosphate. Reaction mixtures contained 0·3 ml. of trichloroacetic acid extract, sufficient 1·0 M-tris–NaOH buffer to bring the pH to 7·5, 0·1 ml. of glucose 6-phosphate (2·5 μmoles), 0·1 ml. of 0·1 M-magnesium chloride, 0·1 ml. of 0·1 M-glycylglycine buffer, pH 7·5, and 0·05 ml. of glucose 6-phosphate dehydrogenase (2 mg./ml.), in a final volume of 1·0 ml. Parallel samples without glucose 6-phosphate dehydrogenase, and samples of standard NADP with and without glucose 6-phosphate dehydrogenase, were also included. The reaction mixtures were incubated at 37° for 15 min.

Assay of dihydronicotinamide–adenine dinucleotide. Reaction mixtures contained 0·6 ml. of the alkali extract, sufficient 1·0 N-HCl to bring the pH to 7·5, 0·1 ml. of 0·1 M-potassium phosphate buffer, pH 7·5, 0·05 ml. of 0·1 M-acetaldehyde and 0·05 ml. of alcohol dehydrogenase (1 mg./ml.), in a final volume of 1·0 ml. Parallel samples without alcohol dehydrogenase, and standards of $NADH_2$ with and without alcohol dehydrogenase, were also included. Incubations were carried out at 37° for 7·5 min.

Assay of dihydronicotinamide–adenine dinucleotide phosphate. Reaction mixtures contained 0·6 ml. of the alkali extract, sufficient 1·0 N-HCl to bring the pH to 7·5, 0·1 ml. of GSSG solution (2·5 μmoles) and 0·05 ml. of glutathione reductase (1 mg./ml.), in a final volume of 1·0 ml. Parallel samples without glutathione reductase, and standards of $NADPH_2$ with and without glutathione reductase, were also included. Incubations were carried out at 37° for 10 min.

At the end of the incubation period 0·5 ml. of 5 N-HCl was added to each of the tubes and fluorimetric determinations were carried out.

Assay of nicotinamide–adenine dinucleotide-glycohydrolase activity of Mycobacterium tuberculosis. The nicotinamide–adenine dinucleotide-glycohydrolase activity of *M. tuberculosis* was tested by the method of Zatman, Kaplan & Colowick (1953) by incubating cell-free extracts of the bacteria, prepared by ultrasonic oscillation or by grinding with powdered glass (Suryanarayana Murthy *et al.* 1962), with 0·1 ml. of a solution of NAD (2·5 μmoles/ml.) and following the extinction of the cyanide addition product at 325 mμ or by determining the NAD concentration fluorimetrically. The amount of protein was determined by the biuret method (Gornall, Bardawill & David, 1957).

RESULTS

Nicotinamide–adenine dinucleotide-glycohydrolase activity. Spectrophotometric or fluorimetric examination of cell-free extracts of *M. tuberculosis* $H_{37}R_V$ prepared either by ultrasonic oscillation in 0·1 M-potassium phosphate buffer, pH 6·5 or 7·0, or in 0·05 M-tris–sodium hydroxide buffer, pH 8·0, or by grinding with powdered glass, failed to reveal any NAD-cleaving activity. However, heat treatment of the extracts enabled nicotinamide–adenine dinucleotide-glycohydrolase activity to be detected. The heat treatment was carried out by keeping 10 ml. of the crude extract in a boiling-water bath for 2 min., followed by immediate cooling in an ice bath. The denatured proteins were removed by centrifuging. Nicotinamide–adenine dinucleotide-glycohydrolase activity could be precipitated by 70% saturation of ammonium sulphate and was linear with time (ΔE_{325} was 0·122/hr. with 1·5 mg. of extract protein in a final volume of 3·0 ml.). This may be due to the presence of a heat-labile inhibitor in the crude extracts.

Dihydronicotinamide–adenine dinucleotide oxidase and dihydronicotinamide–adenine dinucleotide phosphate oxidase. These oxidase activities are summarized in Fig. 1. The dihydronicotinamide–adenine dinucleotide-oxidase activity was inhibited by 30 mM-potassium cyanide although 3 mM-potassium cyanide was without effect.

Concentrations of nicotinamide–adenine nucleotides in whole-cell extracts. The values obtained for the nicotinamide–adenine nucleotide concentrations are summarized in Table 1. Although the values obtained for total oxidized nicotinamide–adenine nucleotides by the nicotinamide–adenine dinucleotide-glycohydrolase assay and the reagent-omission method were in good agreement, the sum of the individual values of NAD and NADP was higher than that obtained by the other methods. Of the oxidized form of the nucleotides NAD was present at almost double the concentration of NADP, whereas the reverse was true for the reduced forms.

The total concentration of oxidized nicotinamide–adenine nucleotides of the avirulent strain of *M. tuberculosis* also was almost the same as that of the virulent strain, as indicated by the nicotinamide–adenine dinucleotide-glycohydrolase assay or the reagent-omission method (313 ± 16 μg./g. wet wt.). In this strain also about 75 % of the oxidized nicotinamide–adenine nucleotides was in the form of NAD (231 ± 9 μg./g.).

Effect of nicotinic acid and nicotinamide on the nicotinamide–adenine nucleotide content. The results of the presence of nicotinic acid and nicotinamide in the growth medium of *M. tuberculosis* $H_{37}R_V$ are shown in Fig. 2. Although a small increase in the concentrations of intracellular total oxidized nicotinamide–adenine nucleotides was observed when nicotinamide was added to the medium, the presence of nicotinic acid led to a decrease in their concentrations. Still higher concentrations of the compounds could not be tried as there was slight inhibition of growth.

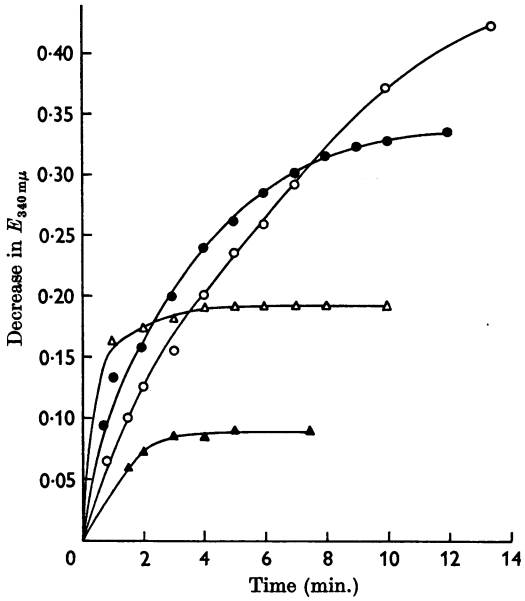

Fig. 1. Dihydronicotinamide–adenine dinucleotide oxidase and dihydronicotinamide–adenine dinucleotide phosphate oxidase of *M. tuberculosis*. The assay system (final vol. 3·0 ml.) contained 1·7 mg. of enzyme protein (prepared by ultrasonic oscillation), 0·25 μmole of $NADH_2$ or $NADPH_2$, 10 μmoles of $MgCl_2$, 250 μmoles of potassium phosphate buffer, pH 7·3, and the indicated amounts of cyanide. Experimental details are given in the text. △, Dihydronicotinamide–adenine dinucleotide phosphate oxidase; ●, dihydronicotinamide–adenine dinucleotide oxidase; ○, dihydronicotinamide–adenine dinucleotide oxidase in the presence of 3 mM-KCN; ▲, dihydronicotinamide–adenine dinucleotide oxidase in the presence of 30 mM-KCN.

DISCUSSION

Although the values for the concentrations of nicotinamide–adenine nucleotides in the same extract of *M. tuberculosis* agreed well, unpredictable variations did occur in different batches, especially with NADP. This agrees with our finding of a great variation in the endogenous reduction of NADP by different batches of cell-free extracts. The low $NADH_2$ concentrations of the organism are possibly due to the presence of a very powerful dihydronicotinamide–adenine dinucleotide oxidase in the cell-free extracts. Though the cells were washed

Table 1. *Oxidized and reduced nicotinamide–adenine nucleotides in whole-cell extracts of* Mycobacterium tuberculosis $H_{37}R_V$

Experimental details are given in the text. The alkali-omission method was carried out by substituting water for alkali in blank mixtures used for the development of fluorescence. Each result, expressed as μg./g. wet wt. of cells, is given as the mean ± S.E.M. of six experiments, each carried out with a different extract prepared from a different batch of cells. The values in parentheses indicate the ranges.

Total oxidized nicotinamide–adenine nucleotides									
NAD-glycohydrolase method	Alkali-omission method	NAD	NADP	$NADH_2$	$NADPH_2$	$\frac{NAD}{NADP}$	$\frac{NADPH_2}{NADH_2}$	$\frac{NADP}{NADPH_2}$	$\frac{NAD}{NADH_2}$
316 ± 11 (290–326)	315 ± 7 (300–336)	252 ± 4 (238–263)	129 ± 6 (119–152)	24 ± 2 (19–29)	54 ± 2 (54–58)	1·95	2·25	2·39	10·50

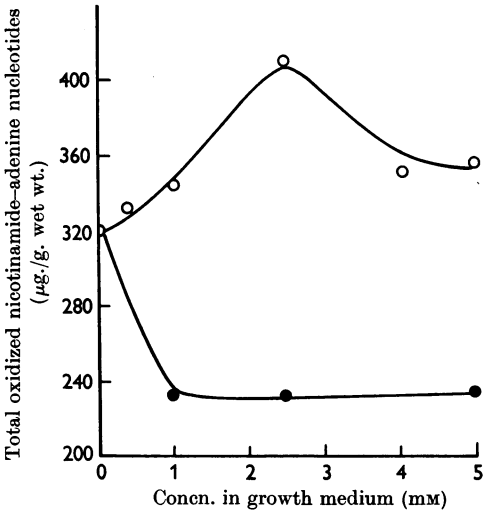

Fig. 2. Effect of the presence of nicotinamide and nicotinic acid in the growth medium on the total nicotinamide–adenine nucleotides of *M. tuberculosis*. Experimental details are given in the text. The total oxidized nicotinamide–adenine nucleotides were determined by the alkali-omission method. Each point represents the mean of two determinations. ○, Nicotinamide added to the growth medium; ●, nicotinic acid added to the growth medium.

with cyanide before the determination of reduced nicotinamide–adenine nucleotides, dihydronicotinamide–adenine dinucleotide oxidase was not inhibited by low concentrations of cyanide and the values obtained may be lower than the actual concentrations present. The inefficiency of cyanide in the inhibition of dihydronicotinamide–adenine dinucleotide oxidase also indicates the existence of pathways other than the cytochrome-linked chain for the terminal respiration of mycobacteria.

Unlike *M. butyricum*, which contains only NAD in the oxidized form (Kaplan, 1960), the $H_{37}R_V$ strain of *M. tuberculosis* possesses both NAD and NADP, similar to *M. phlei* (Weber & Swartz, 1960). The extracts of *M. tuberculosis* do not contain *N*-methylnicotinamide or any other similar quaternary pyridine compounds, as indicated by the agreement in the assay values obtained by the nicotinamide–adenine dinucleotide-glycohydrolase assay and by the reagent-omission method. The NADP content of $H_{37}R_V$ is much higher than that in many of the reported organisms or animal-tissue fractions. Perhaps this can account for the high NADP requirement for most of the dehydrogenases of the crude cell-free extracts of the organism (Suryanarayana Murthy *et al.* 1962). The elevated concentrations of $NADPH_2$ may be due to the presence of an active fatty acid-synthesizing system in the organism, since the preferential utilization of $NADPH_2$ for the synthesis of fatty acid and cholesterol by rat-liver slices has been reported (Foster & Bloom, 1961).

The inhibitory effect of nicotinic acid on the nicotinamide–adenine nucleotide concentrations is not well understood. Imsande & Pardee (1962) have shown that there is no increase in the concentrations of nicotinamide–adenine nucleotides when *Escherichia coli* is grown in a medium containing hyperphysiological concentrations of nicotinic acid, although the starting material for the synthesis of NAD in this organism is nicotinic acid (Imsande, 1961). However, the presence of the same concentrations of nicotinamide led to a small increase in the concentrations of the nicotinamide–adenine nucleotides of $H_{37}R_V$, although not comparable with the increase in their concentrations in rat tissues caused by the administration of this compound (Kaplan, Goldin, Humphreys, Ciotti & Stolzenbach, 1956). Nicotinic acid also, in lower concentrations, elevated the nicotinamide–adenine nucleotide concentrations of the rat tissues to the same extent as that caused by higher concentrations of nicotinamide. The inability of higher concentrations of nicotinic acid to elevate the nicotinamide–adenine nucleotide concentrations has been explained by a substrate-inhibition phenomenon of nicotinamide–adenine nucleotide synthesis (Langan, Kaplan & Shuster, 1959). Sarma, Rajalakshmi & Sarma (1961) and Joshi & Handler (1962) point out that nicotinamide is a breakdown product of NAD (by the action of nicotinamide–adenine dinucleotide glycohydrolase) which is converted into nicotinic acid by the enzyme nicotinamidase, and the acid then enters the biosynthetic pathway of NAD again, making the entire process cyclic.

The presence of nicotinamide–adenine dinucleotide glycohydrolase has also been sought in some of the *Mycobacterium* species. Kern & Natale (1958) have purified a heat-stable nicotinamide–adenine dinucleotide glycohydrolase and a heat-labile inhibitor for the enzyme from *M. butyricum*; but Zatman, Kaplan, Colowick & Ciotti (1954*a*, *b*) could not find any nicotinamide–adenine dinucleotide-glycohydrolase activity in many of the bacterial species tested, including *M. tuberculosis* 607.

The presence of nicotinamide–adenine dinucleotide glycohydrolase in the cell-free extracts of lung-grown *M. tuberculosis* $H_{37}R_V$ has been reported by Artman & Bekierkunst (1961), and its presence was believed to be due to the adsorption of the enzyme by the bacteria from the phagocytizing cell. From the present results, the enzyme is presumably present in the crude cell-free extracts of $H_{37}R_V$ together with an inhibitor, which is heat-labile, and in this respect *M. tuberculosis* resembles the *M. butyricum* strain. It is possible that growth *in vivo* results in the loss of the inhibitor.

The existence of nicotinamide–adenine dinucleotide glycohydrolase in an inhibited state in *M. tuberculosis* may be, as Swartz, Kaplan & Frech (1956) have pointed out for 'heat-activated' enzymes in general, a mechanism of cellular control of the action of the enzyme. The fact that the inhibitor of *M. tuberculosis* is subject to nutritional control (Artman & Bekierkunst, 1961), indicates that it may play a significant role in regulating the oxidative metabolism of the organism. In fact, these authors found that lactate dehydrogenase, a key enzyme in controlling the amount of glucose dissimilated by the anaerobic and aerobic pathways, was inhibited in lung-grown *M. tuberculosis* by the nicotinamide–adenine dinucleotide glycohydrolase present in the organism. In crude extracts of glycerol-grown *M. tuberculosis*, on the other hand, the presence of the nicotinamide–adenine dinucleotide-glycohydrolase inhibitor allows the lactate dehydrogenase to be fully active under the normal conditions of assay (Suryanarayana Murthy *et al.* 1962). The activity and inhibition of lactate dehydrogenase are likely to result in profound effects in tuberculosis infection and resistance to tuberculosis. Dubos (1950) found that 0·01 M-lactic acid destroyed tubercle bacilli within 2 days, and Hirsch (1952) showed that caseous areas become acidic in relation to the surrounding tissue. The heat-labile nicotinamide–adenine dinucleotide-glycohydrolase inhibitor in *M. tuberculosis* $H_{37}R_V$ may thus have a vital role to play in the survival and multiplication of these bacilli in host tissues.

SUMMARY

1. In an effort to understand the role played by the nicotinamide–adenine nucleotides in the oxidative metabolism of *Mycobacterium tuberculosis*, a study of these coenzymes has been carried out in this organism.

2. The concentrations of the oxidized and reduced nicotinamide–adenine nucleotides have been determined in the virulent strain of *M. tuberculosis* $H_{37}R_V$. NAD is present in the organism in almost double the concentration of NADP, whereas the reverse is true for the corresponding reduced compounds.

3. The total concentrations of the oxidized nicotinamide–adenine nucleotides of the virulent and avirulent strains are almost the same, and in the avirulent strain also the concentration of NAD is substantially higher than that of NADP.

4. The concentrations of nicotinamide–adenine nucleotides in the organism are increased by the addition of nicotinamide and decreased by the addition of nicotinic acid to the growth medium.

5. The presence of a powerful dihydronicotinamide–adenine dinucleotide oxidase, a less powerful dihydronicotinamide–adenine dinucleotide phosphate oxidase, and a heat-stable nicotinamide–adenine dinucleotide glycohydrolase that is associated with a heat-labile inhibitor, are reported in the cell-free extracts of the organism. The significance of this inhibitor is discussed in relation to the survival and multiplication of tubercle bacilli in host tissues.

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