Nicotinamide–Adenine Dinucleotide Glycohydrolase of *Mycobacterium tuberculosis* $H_{37}R_v$

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(*Received* 2 *September* 1963)

The enzyme NAD glycohydrolase (EC 3.2.2.5) catalyses an exchange reaction between NAD and added free nicotinamide or other structurally related compounds, in addition to the hydrolytic cleavage of oxidized nicotinamide–adenine nucleotides (Zatman, Kaplan & Colowick, 1953; Zatman, Kaplan, Colowick & Ciotti, 1954). This type of exchange reaction results in the formation of a structural analogue of NAD, wherein the nicotinamide part of the coenzyme is replaced by the added compound. The potent antitubercular drug isonicotinic hydrazide is a good exchanger in the NAD–NAD-glycohydrolase system (Zatman *et al.* 1954), and the isonicotinic hydrazide analogue of NAD once formed cannot function as the coenzyme in many dehydrogenase reactions (Goldman, 1954). The formation of such an analogue has been put forward as one of the possible modes of action of the drug.

However, this type of exchange reaction is catalysed by the enzyme derived from only certain mammalian tissues, whereas the enzymes from *Neurospora crassa* or *Mycobacterium butyricum* fail to do so (Zatman *et al.* 1954; Kern & Natale, 1958). The mammalian-tissue enzyme is usually associated with the particulate fraction, and has been solubilized and purified from pig brain (Windmueller & Kaplan, 1962) and ox spleen (Alivisatos & Woolley, 1956). A soluble enzyme from bull semen, purified extensively, has been shown to catalyse, in addition to the hydrolytic cleavage of NAD and NADP, the imidazolysis of NMN, NAD and NADP (Abdel Latif & Alivisatos, 1962).

Gopinathan, Sirsi & Ramakrishnan (1963) reported the presence of NAD glycohydrolase in an inhibited state in cell-free extracts of the organism *Mycobacterium tuberculosis*. The present study deals with a partial purification of the enzyme, after its activation by heat treatment, from cell-free extracts and its general properties.

MATERIALS AND METHODS

Chemicals. NAD, NADP, NMN, $NADH_2$, $NADPH_2$, GSH, tris and trypsin (twice-recrystallized, salt-free) were all from Sigma Chemical Co., St Louis, Mo., U.S.A. Acetylpyridine–adenine dinucleotide, acetylpyridine–hypoxanthine dinucleotide and nicotinamide–hypoxanthine dinucleotide were all from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

The antitubercular drugs used were: isonicotinic hydrazide (Dumex), streptomycin sulphate (Glaxo Laboratories) and *p*-aminosalicylic acid (Rhodia).

The other chemicals were of reagent grade. Alumina C_γ and calcium phosphate gels were prepared according to standard methods (Colowick, 1955).

Growth of bacterial cells and preparation of the enzyme. The virulent strain *M. tuberculosis* $H_{37}R_v$ and its avirulent mutant $H_{37}R_a$ were grown on the synthetic liquid medium described by Youmans & Karlson (1947). Samples (75 ml.) of medium were distributed in 250 ml. Pyrex conical flasks and the organisms were grown as surface cultures. The virulent strain was harvested on the fifteenth day, and the avirulent strain on the twenty-first day, by filtration under sterile conditions through Whatman no. 1 filter-paper circles (24 cm. diam.). The cells were washed with ice-cold distilled water. Approx. 1·25 g. wet wt. of bacterial cells was obtained from each flask. The cells were suspended in 50 mM-potassium phosphate buffer, pH 7·0 (10 ml./g. wet wt.), and subjected to ultrasonic disintegration in a 10 kcyc./sec. Raytheon ultrasonic oscillator for 40 min. The disintegrated cell suspensions were centrifuged at 10 000*g* in a Servall centrifuge for 45 min. and the supernatants were used as crude cell-free extracts.

Enzyme activity. The NAD-glycohydrolase activity was determined by following the disappearance of NAD by the cyanide-addition method (Zatman *et al.* 1953). The incubation system, in a final volume of 0·6 ml., contained 100 μ-moles of potassium phosphate buffer, pH 6·5, approx. 0·25 μmole of NAD and the enzyme. The reaction was carried out for 1 hr. at 37° and was stopped by the addition of 3·0 ml. of 1·0 M-potassium cyanide. The extinction of the NAD–cyanide complex at 325 mμ was measured in a Beckman model DU spectrophotometer. Blanks were included where NAD was added at the end of the incubation period, just before the addition of cyanide.

One unit of enzyme is defined as that amount which will cleave 1 μmole of NAD/min. at 37°, and the specific activity is given as μm-moles of NAD cleaved/min./mg. of protein.

Heat treatment. In the preliminary studies before the temperature optimum for the activation was determined, the heat treatment was carried out with 0·5 ml. samples containing 0·1 ml. of 0·1 M-potassium phosphate buffer, pH 7·0, and the enzyme, and the samples were kept in a water bath at the required temperature for 1 min. and immediately chilled in ice.

Purification of the enzyme. Step 1: heat treatment. Samples (25 ml.) of the crude *M. tuberculosis* $H_{37}R_v$ extracts (6·5–7·0 mg. of protein/ml.) in 100 ml. Pyrex conical flasks were kept in a boiling-water bath to reach 85° and maintained at this temperature for 1 min. The flasks were immediately chilled in ice–salt mixture and the turbid solution was used as the crude enzyme. All the subsequent steps were carried out at 0–5°.

Step 2: ammonium sulphate precipitation. To 100 ml. of the heat-treated crude extracts solid ammonium sulphate was added to give 20% saturation, the mixture was kept for 15 min. and the precipitate was centrifuged off at 10 000*g* in a Servall centrifuge. The supernatant was brought to 50% saturation by the further addition of ammonium sulphate. The precipitate was collected by centrifugation at 10 000*g* for 15 min., dissolved in 10 mM-potassium phosphate buffer, pH 7·0, and dialysed against 2 l. of the same buffer for 16–18 hr.

Step 3: isoelectric precipitation. The pH of the 20–50% saturated ammonium sulphate fraction, after dialysis, was lowered to 5·5 with cold N-HCl and the residue after centrifugation was discarded. The pH of the supernatant was further lowered to 2·5 with N-HCl and immediately centrifuged at 13 000*g* for 10 min. in a Lourdes refrigerated centrifuge. The precipitate was dissolved in 50 mM-potassium phosphate buffer, pH 7·0.

Step 4: adsorption on calcium phosphate gel. The pH of the above fraction was lowered to 5·5 with cold N-HCl, and then sodium acetate buffer, pH 5·5, and calcium phosphate gel [gel:protein ratio, 4:1 (dry wt. of gel)] were added so that the final molarity of the adsorption medium was 25 mM with respect to phosphate and 10 mM with respect to acetate. The mixture was kept for 15 min. with occasional stirring and centrifuged at 13 000*g* for 10 min.

The sedimented gel was treated successively with 8·0 and 4·0 ml. of 100 mM-potassium phosphate buffer, pH 7·0, for 15 min. each, with occasional stirring, and centrifuged at 13 000*g* for 10 min. The two eluates were mixed.

Step 5: second ammonium sulphate fractionation. To the gel eluate was added a saturated solution of ammonium sulphate [pH adjusted to 7·0–7·5 with aq. ammonia (sp.gr. 0·88)] to give 25% saturation. The precipitate was discarded after centrifugation and the supernatant was brought to 45% saturation with the same ammonium sulphate solution. The precipitate was dissolved in 10 mM-potassium phosphate buffer, pH 7·0, and dialysed against 2 l. of the same buffer for 14 hr. This step was carried out mainly to eliminate the high concentrations of phosphate from the preceding step besides concentrating the enzyme preparation.

The protein contents were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of rat- or mouse-tissue extracts. The animals were killed by a single blow on the neck or by decapitation and the tissue was removed, washed once with 100 mM-potassium phosphate buffer, pH 7·3, and then homogenized in the same buffer (10 ml./g. of tissue). The resulting suspension was centrifuged and the supernatant was used.

Preparation of the Neurospora *and* Aspergillus *enzymes.* NAD glycohydrolase was prepared from zinc-deficient *Neurospora crassa* by the method of Kaplan, Colowick & Nason (1951) and purified as far as 'step 3'. The enzyme from *Aspergillus niger* was prepared by the method of Rajalakshmi, Sarma & Sarma (1963).

Tryptic digestion was carried out by incubating 1·0 ml. of the crude cell-free extracts with 100 μg. of trypsin for 1 hr. at 37° at pH 7·0. Samples were tested for enzyme activity before and after heat treatment.

RESULTS

Activation of nicotinamide–adenine dinucleotide-glycohydrolase activity. Maximal NAD-glycohydrolase activity was obtained with cell-free extracts kept at 85° for 1 min. There was no activation after 1 min. at 60°, and only about 35% of the activity could be observed after 5 min. at 60°. The effect of heating the crude cell-free extracts at 85° for various times is given in Fig. 1.

After heat treatment at 100° for 1 min. only 70% of the activity was observed. The activity was greater when heat treatment (at 85° for 1 min.) was carried out in potassium phosphate buffer, pH 7·0, than in sodium acetate buffer, pH 5·5, or tris–hydrochloric acid buffer, pH 8·5. Added GSH (1·0 mM) before heat treatment had little effect on the enzyme activity.

Other methods of activation were tried. Dialysis of the crude cell-free extracts did not activate them, and the activation could be achieved after heat treatment of the dialysed extract. Ammonium sulphate precipitation (20–50% saturated fraction), isoelectric precipitation with cold N-hydrochloric acid (pH 5·5 supernatant, pH 2·5 precipitate), acetone precipitation [50–80% (v/v) fraction], ethanol precipitation [40–75% (v/v) fraction], protamine sulphate precipitation (super-

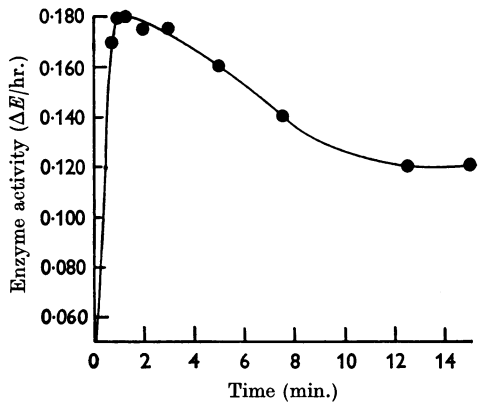

Fig. 1. Effect of heating crude extracts of *M. tuberculosis* NAD glycohydrolase at 85° for various times. The heat treatment was carried out with 0·5 ml. samples containing 0·1 ml. of 0·1 M-potassium phosphate buffer, pH 7·0, and the crude cell-free extracts (0·4 ml., containing 2·4 mg. of protein) by keeping in a water bath maintained at 85°, followed by immediate cooling. The enzyme reaction was started by the addition of 0·1 ml. of NAD (0·25 μmole) and assays were carried out as described in the text.

natant), adsorption on calcium phosphate or alumina Cγ gels (adsorption medium, 0·1 M-potassium phosphate, pH 7·0) or tryptic digestion (see the Materials and Methods section) did not result in activation of the enzyme. However, these fractions showed enzyme activity after heat treatment at 85° for 1 min. The passage of 3·5–4·0 ml. (24–28 mg. of protein) of the crude cell-free extract over a diethylaminoethylcellulose or a Sephadex G-25 column (1 cm. × 12 cm.) and subsequent elution from the column with potassium phosphate buffer, pH 7·0, of increasing molarity also did not yield an active enzyme preparation.

Table 1. *Fractionation of the heat-activated nicotinamide–adenine dinucleotide glycohydrolase of* Mycobacterium tuberculosis $H_{37}R_v$

Experimental details are given in the text.

Fraction	Sp. activity (μm-moles of NAD/min./mg. of protein)	Recovery (%)
Crude heat-treated extract	0·675	100
20–50% satd. $(NH_4)_2SO_4$ fraction	3·52	55
pH 2·5 precipitate	4·70	60
Ca_3PO_4-gel eluate	6·85	40
25–45% satd. neutral $(NH_4)_2SO_4$ fraction	—	20

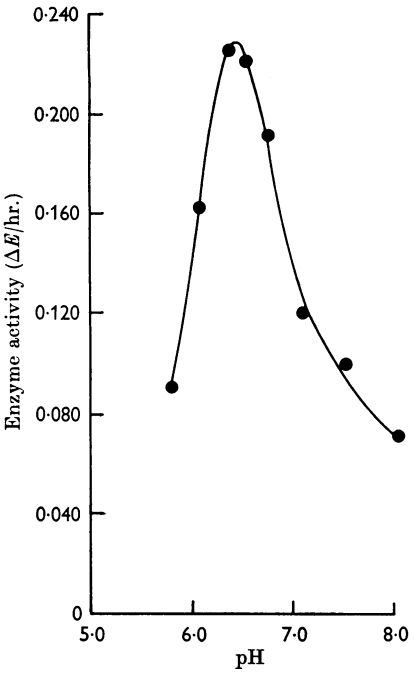

Fig. 2. Effect of pH on NAD-glycohydrolase activity. The reaction mixtures contained potassium phosphate buffer (20 μmoles), purified enzyme (0·1 ml., containing 300 μg. of protein) and NAD (0·25 μmole) in a final volume of 0·6 ml. Incubations were carried out for 1 hr. at 37° and the reactions were stopped with 3·0 ml. of 1·0 M-KCN.

Incubation of the crude cell-free extracts at pH 1·0 for 1 hr. at 37° did not activate the enzyme, although under similar conditions the enzyme from *M. butyricum* is activated (Kern & Natale, 1958); but the precipitated proteins, after dissolution in potassium phosphate buffer and heat treatment (at 85° for 1 min.), showed a weak activity.

The enzyme activity could be precipitated from the crude extracts by 5% (w/v) trichloroacetic acid, and the washed precipitate, when dissolved in potassium phosphate buffer, showed enzyme activity on heat treatment.

Preincubation studies with rat- or mouse-tissue homogenates. An active NAD glycohydrolase has been reported in lung-grown tubercle bacilli (Artman & Bekierkunst, 1961); the activation of this enzyme in crude cell-free extracts from cells grown *in vitro*, by incubation with some of the animal-tissue homogenates, was therefore tried. Incubation of the crude bacterial extracts with rat-liver homogenates for up to 24 hr. at 2° or for up to 6 hr. at 37° did not result in the activation of the bacterial enzyme. Results were the same on incubating the cell-free extracts (0·2 ml.) with mouse-brain, -liver, -lung, or -spleen homogenates (0·1 ml.) for 1 hr. at 37°. However, there was no inhibition of the NAD-cleaving activity of the animal-tissue homogenates.

Purification of nicotinamide–adenine dinucleotide glycohydrolase. In the light of the above results, the enzyme was partially purified after its activation by heat treatment. The results are summarized in Table 1.

Substrate specificity. The purified enzyme cleaved NAD and NADP at equal rates, but the enzyme was inactive towards NMN, acetylpyridine–adenine dinucleotide, acetylpyridine–hypoxanthine dinucleotide, nicotinamide–hypoxanthine dinucleotide and the reduced forms $NADH_2$ and $NADPH_2$.

Although the enzyme activity was initiated only after keeping at higher temperatures, the optimum temperature for the cleavage of NAD was 35–37°. The enzyme reaction was linear with time for up to 90 min. Linearity of the enzyme reaction with enzyme and substrate concentrations was also observed.

The effect of substrate concentration on the enzyme activity was tested in the ranges 0·13–0·76 μmole/0·6 ml. for NAD and 0·13–0·77 μmole/0·6 ml. for NADP. The K_m values, calculated by the Lineweaver–Burk graphical method, were 0·33 mM for NAD and 0·31 mM for NADP.

Optimum pH. The enzyme had a sharp pH optimum at pH 6·5 (Fig 2) and differs in this respect from the NAD glycohydrolases of *Neuro-*

spora or *M. butyricum*, which have broad pH optima between 5 and 8.

Maximal activity was observed in potassium phosphate buffer, pH 6·5, and almost equal activity was observed in sodium arsenate or tris–acetate buffers, pH 6·5. Only 50 % of the activity was observed in potassium phthalate buffer, pH 6·5. In each case, 50 μmoles of the buffer in 0·6 ml. of reaction mixture was used.

Effect of inhibitors and antitubercular drugs. The effects of some inhibitors and antitubercular drugs on the enzyme activity are summarized in Table 2.

The inhibitions by *p*-chloromercuribenzoate and mercuric chloride were completely reversed by higher concentrations of GSH.

Cysteine, GSH and EDTA had no effect on the enzyme activity.

Products of enzyme action. Larger concentrations of enzyme and NAD were used and incubations were carried out for 2 hr. at 37°. The reactions were stopped by the addition of perchloric acid, and the neutralized deproteinized reaction mixtures were chromatographed in ethanol–acetic acid (1:1, v/v) or ethanol–0·1 M ammonium acetate (pH adjusted to 5 with hydrochloric acid) (7:3, v/v) solvent systems by the ascending method. The dried chromatograms under ultraviolet light (Mineralight SL 2537 lamp was used) revealed one quenching spot corresponding to authentic ADP-ribose in R_F, and another quenching spot corresponding to authentic nicotinamide in its R_F and reaction with cyanogen bromide and benzidine or *p*-aminobenzoic acid.

Studies on the inhibitor. The presence of an excess of free inhibitor was observed in the crude cell-free extracts since it inhibited the activated enzyme when mixed with it. Experiments were carried out

Table 2. *Effect of some inhibitors and antitubercular drugs on the partially purified nicotinamide–adenine dinucleotide glycohydrolase of* Mycobacterium tuberculosis $H_{37}R_v$

The reaction mixtures, in a final volume of 0·6 ml., contained 100 μmoles of potassium phosphate buffer, pH 6·5, enzyme (0·1 ml., containing 300 μg. of protein), 0·25 μmole of NAD and the inhibitor. Incubations were carried out for 1 hr. at 37° and the reactions stopped with 3·0 ml. of 1·0 M-KCN.

Substance used	Final concn. (mM)	Inhibition (%)
Iodoacetate	1·00	0
Sodium arsenite	1·00	0
Sodium azide	1·00	0
Sodium fluoride	1·00	0
p-Chloromercuribenzoate	0·01	100
	0·001	23
Mercuric chloride	0·01	100
	0·001	7
Streptomycin	1·00	0
Isonicotinic hydrazide	1·00	0
p-Aminosalicylic acid	1·00	0
Nicotinamide	10·00	0
	50·00	30
Nicotinic acid	10·00	0
	50·00	10
	10·00	0

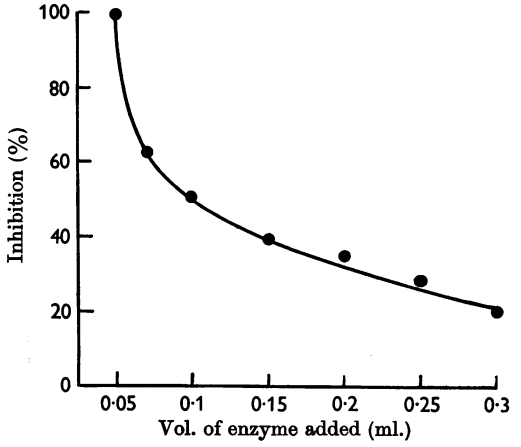

Fig. 3. Effect of varying the enzyme concentration on NAD-glycohydrolase activity. The reaction mixtures contained samples of enzyme (gel eluate; 1·6 mg. of protein/ml.), 0·1 ml. (15 μg. of protein) of diluted crude cell-free extracts and 100 μmoles of potassium phosphate buffer, pH 6·5. After incubation for 15 min. at 28°, 0·1 ml. of NAD (0·25 μmole) was added to start the reaction, and incubation was continued for 1 hr. at 37°. The reactions were stopped with 3·0 ml. of 1·0 M-KCN.

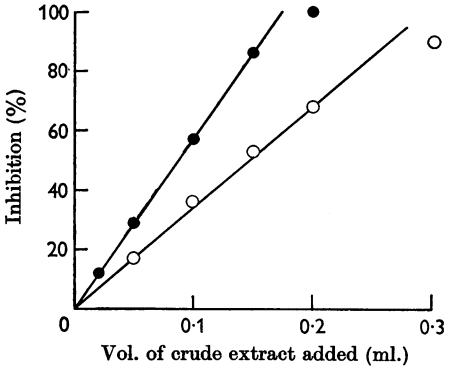

Fig. 4. Effect of varying the inhibitor concentration on NAD-glycohydrolase activity. Two enzyme concentrations were chosen [160 μg. (●) and 480 μg. (○) of protein] which differed threefold and various amounts of crude extract (180 μg. of protein/ml.) were added. Preincubations were for 15 min. at 28° in the presence of 100 μmoles of potassium phosphate buffer, pH 6·5, and NAD was added to start the reaction. The procedure was then as for Fig. 3.

with increasing concentrations of the enzyme with a fixed concentration of the crude extract and vice versa, in parallel to those described by Kern & Natale (1958). The results are presented in Figs. 3 and 4.

The enzyme–inhibitor complex formed is undissociable.

Specificity of the inhibitor. Preincubation of the crude cell-free extracts with the purified NAD glycohydrolase from *Neurospora* for up to 24 hr. at 2° or 6 hr. at 37° did not cause any inhibition of the enzyme. Also, there was no inhibition of the enzyme from *A. niger*. The crude cell-free extracts from the avirulent strain *M. tuberculosis* $H_{37}R_a$ inhibited the partially purified enzyme from the virulent strain to the same extent.

DISCUSSION

The presence of the enzyme NAD glycohydrolase in an inhibited state in cell-free extracts of the organism made the study of the enzyme interesting, because it has been shown to be present in an active state in the lung-grown tubercle bacilli (Artman & Bekierkunst, 1961). The activity observed after heat treatment of the cell-free extracts of cells grown *in vitro* was comparatively weak, and other methods of activation were tried. However, in all the methods employed, the enzyme and the inhibitor were still associated and activation could be achieved only after heat treatment of the particular fraction. These results suggest that the enzyme and inhibitor are firmly bound or in the form of a complex. The low purification achieved and the weak activity of the enzyme suggest a partial denaturation of the enzyme itself during heat treatment, although it is more heat-stable than the inhibitor.

NAD glycohydrolase is especially important in *M. tuberculosis* because the enzyme from other sources catalysed the exchange reaction between NAD and isonicotinic hydrazide, the potent antitubercular drug. The presence of the enzyme in the organism thus raised the hope of finding the mechanism of action for the drug, as postulated by previous workers (Goldman, 1954; Zatman *et al.* 1954). However, the enzyme failed to show any analogue formation from NAD and isonicotinic hydrazide even in the presence of 0·5 M-ethanol and 0·02 M-nicotinamide in 0·1 M-glycine–sodium hydroxide buffer, pH 9·5 (Zatman *et al.* 1953). In this respect the enzyme from *M. tuberculosis* resembles the enzyme from *M. butyricum*, but differs from the latter in its weak activity, sharp pH optimum and equal reactivity towards NAD and NADP.

Alivisatos (1959) suggested the participation of the histidyl moieties of NAD glycohydrolase (solubilized and purified from ox spleen) in its catalytic action. The partially purified enzyme from *M. tuberculosis* $H_{37}R_v$ was very sensitive to *p*-chloromercuribenzoate and mercuric chloride, which completely inhibited the enzyme activity; the inhibition was prevented by higher concentrations of GSH. The enzyme thus appears to be of sulphydryl nature. However, added GSH or cysteine had no effect on enzyme activity, and iodoacetate, arsenite or azide failed to show any inhibition of the enzyme activity.

The inhibitor of the enzyme from *M. tuberculosis* appears to be specific for the enzyme from this source only, suggested by the lack of inhibition of the rat- or mouse-tissue NAD-cleaving activity even after prolonged incubation for up to 24 hr. at 2° or 6 hr. at 37°. The inhibitor is also inactive towards the partially purified enzyme from *N. crassa* or *A. niger*. The crude extracts from the avirulent strain *M. tuberculosis* $H_{37}R_a$ inhibited to the same extent partially purified enzyme from the virulent strain *M. tuberculosis* $H_{37}R_v$, and *M. tuberculosis* $H_{37}R_a$ extracts also showed an equal NAD-glycohydrolase activity on heat treatment. The enzyme activity thus appears to have no bearing on the virulence of the strain. The crude cell-free extracts contain an excess of free inhibitor because they are able to cause complete inhibition of the purified enzyme. The formation of the enzyme–inhibitor complex appears to be irreversible from the results obtained from studies on mixing the enzyme and inhibitor. The inhibition follows a linear relationship with the inhibitor concentration.

The presence of the enzyme in an active state in lung-grown *M. tuberculosis* $H_{37}R_v$ cells suggests the loss of its inhibitor during growth *in vivo*. However, in our studies, no activation of the bacterial enzyme could be achieved on incubating the cell-free extracts of the organism (containing the enzymes in the inhibited state) with the animal-tissue homogenates even for prolonged periods. Recent reports (Bekierkunst & Artman, 1962; Chaudhuri, Suter, Shah & Martin, 1963) point out that the NAD-glycohydrolase activity is increased several-fold in the tissues of tubercular mice and guinea pigs. Whether this increased enzyme activity in infection is of bacterial or of host-tissue origin is not established. An increase in NAD-glycohydrolase activity has been found in tissue damage, caused by nitrogen mustard in Ehrlich ascites cells (Green & Bodansky, 1962) or exposure of thymocytes to γ-ray irradiation (Scaife, 1963). A rapid lowering of 50 % of the NAD content of the liver after the administration of dimethylnitrosamine (Stirpe & Aldridge, 1961) and an increased turnover of NAD in mouse liver after the administration of azaserine (Narrod, Bonavita, Ehrenfeld & Kaplan, 1961) have also been reported. The tubercular process may thus simulate a condition caused by the

above agents in the necrotic destruction of tissues with a simultaneous enhancement of the NAD-glycohydrolase activity.

SUMMARY

1. The optimum conditions for the heat-activation of the enzyme nicotinamide–adenine dinucleotide glycohydrolase present in an inhibited state in the crude cell-free extracts of the virulent strain of *Mycobacterium tuberculosis* $H_{37}R_v$ have been described.

2. The heat-activated enzyme was partially purified by ammonium sulphate fractionation, isoelectric precipitation, and calcium phosphate-gel adsorption.

3. The optimum conditions of temperature, pH, substrate and enzyme concentrations are presented, and the K_m values for both the substrates, namely NAD and NADP, are given. The enzyme differs from the NAD glycohydrolases reported from other micro-organisms in having a sharp pH optimum and equal reactivity towards NAD and NADP.

4. The effect of various inhibitors and anti-tubercular agents on the partially purified enzyme has been studied.

5. The inhibitor with which the enzyme is associated in the crude cell-free extracts appears to be specific for the enzyme from this source. However, the inhibitor from the avirulent strain *M. tuberculosis* $H_{37}R_a$ was inhibitory for the partially purified enzyme from the virulent strain to an equal extent.

6. Evidence for the presence of an excess of free inhibitor in the crude cell-free extracts is presented and the complex formed between the enzyme and its inhibitor is shown to be undissociable.

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Regulation of Enzyme Synthesis in an Enucleate Cell

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(*Received* 7 *October* 1963)

A currently popular theory proposes that the regulation of enzyme synthesis in the cell is controlled by the flow of a special type of short-lived RNA from the gene to the site at which the enzyme is synthesized (Monod & Jacob, 1961; Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961). It has been suggested that the amount of enzyme formed bears some relationship to the amount of RNA synthesized on the gene concerned (Hiatt, Gros & Jacob, 1963); and since it is supposed that this RNA is rapidly destroyed after transfer to the cytoplasm, its continued production is held to be necessary for continued synthesis of the enzyme.

The evidence in support of this theory is indirect and rests heavily on experiments carried out on micro-organisms, especially *Escherichia coli*. How-