Nicotinamide-Adenine Dinucleotide-Glycohydrolase Activity in Experimental Tuberculosis

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1. The specific NAD-glycohydrolase activity is increased 70 and 50% over the normal in lung and liver tissues respectively of tuberculous mice. 2. Concomitant with the increase in the NAD-glycohydrolase activity, the NAD-isonicotinic acid hydrazide-exchange activity also is increased in infection. The isonicotinic acid hydrazide analogue of NAD formed by the lung enzyme from tuberculous mice has been isolated and identified. 3. The increased NAD-glycohydrolase activity in infection has been shown to be of host-tissue origin and not due to the activation of the bacterial enzyme on growth of the organism *in vivo*. 4. In addition to NAD, NMN and NADP also participate in the exchange reaction with isonicotinic acid hydrazide catalysed by NAD glycohydrolase. The interference of the drug at the nucleotide level of metabolism is therefore suggested.

The enzyme NAD glycohydrolase (EC 3.2.2.5) is present in an inhibited state in crude cell-free extracts of the organism *Mycobacterium tuberculosis* H₃₇R_v, grown *in vitro*, and the enzyme has been purified after heat activation from this source (Gopinathan, Sirsi & Ramakrishnan, 1963; Gopinathan, Sirsi & Vaidyanathan, 1964a). The presence of this enzyme in an active state in lung-grown tubercle bacilli has been reported by Artman & Bekierkunst (1961a). An increase in the NAD-glycohydrolase activity in the tissues of tuberculous mice and guinea pigs has also been reported (Bekierkunst & Artman, 1962; Chaudhuri, Suter, Shah & Martin, 1963; Windman, Bekierkunst & Artman, 1964). The possible origin of this increased enzyme activity in infection could be either bacterial, as a result of activation of the bacterial enzyme on growth of the organism *in vivo*, or the host tissue itself. If the latter is the case, the tubercular process could be simulating other cellular degenerative processes such as treatment of Ehrlich ascites cells with nitrogen mustard or exposure of thymocytes to γ -ray irradiation (Green & Bodansky, 1962; Scaife, 1963). We wished to trace the origin of the increased NAD-glycohydrolase activity in infection by comparing its properties with those of the enzymes from normal animal tissue and the bacteria. A short communication on this has been published (Gopinathan, Sirsi & Vaidyanathan, 1964b).

MATERIALS AND METHODS

Chemicals. NAD, NADP and NMN were all from Sigma Chemical Co., St Louis, Mo., U.S.A.

The INH* used was a Dumex product.

Other chemicals were all of reagent grade.

Growth of bacteria and preparation of enzyme. The growth of the organism *M. tuberculosis* H₃₇R_v, and the preparation and purification of the enzyme were all carried out as described by Gopinathan *et al.* (1964a).

Animal infection and preparation of animal tissue enzyme. Eighteen normal healthy male albino mice, weighing 17–20 g., were infected with the virulent strain of *M. tuberculosis* H₃₇R_v (0.5 mg. wet wt. of bacilli/animal by intravenous injection) and were fed *ad libitum*. The course of infection was followed by body weight measurements and the animals were killed on the nineteenth day after infection (Fig. 1), when mortality started in the group (three animals died). The animals were killed by cervical dislocation. Post-mortem analysis revealed an advanced stage of tuberculosis of the lungs in all the animals. The lung and liver tissues were collected and pooled in chilled vessels. Homogenizations were carried out either in a Waring Blendor or in an MSE homogenizer for 2 min. at top speed, and the homogenates were centrifuged at 250 g for 15 min. and again at 13000 g for 20 min. In this treatment, the bacilli remain intact and are removed on centrifugation (Segel & Bloch, 1956; Artman & Bekierkunst, 1961b). The supernatants, free of bacilli, were used as enzyme source.

The lung and liver extracts from normal animals also were prepared in a similar way, but 5 ml. of water was used for suspension per animal tissue, instead of 10 ml. as for the infected tissues.

The fraction precipitated by between 20 and 75% saturated (NH₄)₂SO₄ was also used after dialysis wherever indicated.

NAD-glycohydrolase and NAD-INH-exchange activities of tissues from normal and tubercular mice. The NAD-glycohydrolase activity was determined as described by

* Abbreviation: INH, isonicotinic acid hydrazide.

Gopinathan *et al.* (1964a). Enzyme incubations were carried out for 15 min. at 37°.

The NAD-isonicotinic hydrazide-exchange reaction was determined by the method of Zatman, Kaplan, Colowick & Ciotti (1954a). The enzyme assay system contained (final vol. 0.8 ml.): potassium phosphate buffer, pH 7.5 (100 μ moles), NAD (0.5 μ mole) and enzyme (1.16–1.3 mg. of protein for the lung enzyme and 2.7–4.0 mg. of protein for the liver enzyme). The incubations were carried out for 30 min. at 37°, and 3.0 ml. of 0.1 N-NaOH was added to stop the reaction. The extinctions of the samples were read at 390 m μ in a Beckman model DU spectrophotometer.

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

The K_m values were determined by the Lineweaver-Burk graphical method.

RESULTS

NAD-glycohydrolase and NAD-INH-exchange activities of tissue extracts from normal and tubercular mice. The results are summarized in Table 1. The specific NAD-glycohydrolase activity, expressed as μ moles of NAD cleaved/min./mg. of protein, is increased 70 and 35% over the normal in lung and liver tissues respectively of the infected animal.

The specific NAD-INH-exchange activity, expressed as μ moles of INH analogue of NAD formed/min./mg. of protein, is over 50 and 18% in lung and liver tissues respectively of the infected animal. A molar extinction coefficient of 4.9×10^6 cm.²/mole is assumed for the INH analogue of NAD for calculation (Zatman, Kaplan, Colowick & Ciotti, 1954b).

Properties of the enzyme preparations. The properties of the NAD glycohydrolase from lungs of normal and infected animals were compared with those of the purified bacterial enzyme (purified up to the calcium phosphate-gel eluate stage; Gopinathan *et al.* 1964a).

The properties studied include the pH optima, substrate specificity, K_m values, effects of some inhibitors and NAD-INH-exchange activities of these enzyme preparations. The results are presented in Tables 2 and 3.

The bacterial enzyme was highly sensitive to inhibition by low concentrations of thiol poisons such as *p*-chloromercuribenzoate, mercuric chloride or *N*-ethylmaleimide, and this effect was reversed by GSH (Gopinathan *et al.* 1964a), whereas the animal-tissue enzymes (normal and infected) were only partially inhibited even at much higher concentrations of these inhibitors. On the other hand, nico-

Fig. 1. Change in average body weight with progress of infection. Eighteen normal healthy albino mice were infected with *M. tuberculosis* H₃₇R_v (0.5 mg. wet wt. of bacilli/animal by intravenous injection). The body weights of the animals were recorded and the animals were killed when mortality started in the group. Details were given in the Materials and Methods section.

Table 1. *NAD-glycohydrolase and NAD-INH-exchange activities of tissues from normal and tuberculous mice*

The NAD-glycohydrolase assay system contained (final vol. 0.6 ml.): potassium phosphate buffer, pH 6.5 (100 μ moles), enzyme (homogenate of normal or infected animal tissue) and NAD (0.25 μ mole). Incubations were carried out for 15 min. at 37° and the reactions stopped with 3.0 ml. of 1.0 M-KCN. The NAD-INH-exchange assay system contained (final vol. 0.8 ml.): potassium phosphate buffer, pH 7.5 (100 μ moles), enzyme (protein contents as given in the text), NAD (0.5 μ mole) and INH (5 μ mole). Incubations were carried out for 30 min. at 37° and the reactions terminated by the addition of 3.0 ml. of 0.1 N-sodium hydroxide.

Source of enzyme	NAD-glycohydrolase activity			NAD-INH-exchange activity		
	Sp. activity (μ moles of NAD hydrolysed/min./mg. of protein)		Percentage increase over normal	Sp. activity (μ moles of INH analogue of NAD formed/min./mg. of protein)		Percentage increase over normal
	Normal	Infected		Normal	Infected	
Lung	9.55	16.26	70	3.33	4.99	50
Liver	5.89	7.94	35	2.02	2.39	18

Table 2. *Effect of inhibitors on the NAD-glycohydrolase activity of the lungs from normal and tuberculous mice and of the bacteria*

The animal-tissue enzyme preparations used were the fractions precipitated by between 20 and 75% saturated $(\text{NH}_4)_2\text{SO}_4$. The assay system was the same as that described in Table 1, except that the inhibitor also was present in the reaction mixtures. With the bacterial inhibitor, preincubations were carried out for 15 min. at room temperature before the addition of substrate. The protein concentrations employed were 0.53 mg. of protein of the normal-lung enzyme and 0.87 mg. of protein for the infected-lung enzyme.

Inhibitor	Percentage inhibition		
	Normal-lung enzyme	Infected-lung enzyme	Bacterial enzyme
<i>p</i> -Chloromercuribenzoate (0.1 mM)	18	10	100
Mercuric chloride (0.01 mM)	5	5	100
<i>N</i> -Ethylmaleimide (1.0 mM)	14	7	100
Nicotinamide (1.0 mM)	55	50	0
Bacterial inhibitor (5–13 μg . of protein)	0	0	100

Table 3. *Properties of NAD-glycohydrolase activities of lungs from normal and tuberculous mice and of the bacteria*

The assay system was the same as that described in Table 1. For the pH optima and K_m determinations, the fractions precipitated by between 20 and 75% saturated $(\text{NH}_4)_2\text{SO}_4$ of the animal-tissue enzymes were used. The activities on NADP and NMN are expressed as the percentage activity of NAD hydrolysis, the latter being assumed to be 100 in individual cases.

Property	Normal-lung enzyme	Infected-lung enzyme	Bacterial enzyme
Substrate specificity			
NAD	100	100	100
NADP	58	63	100
NMN	52.5	51.5	—
NAD-INH exchange	+	+	—
pH optimum	6.0–7.5	6.0–7.5	6.5
K_m (NAD)	43.3 μM	66.7 μM	143 μM

tinamide in concentrations about 50–55% inhibitory for the animal-tissue enzymes had no effect on the bacterial enzyme.

Also, the inhibitor with which the NAD glycohydrolase is associated in crude cell-free extracts of *M. tuberculosis* (Gopinathan *et al.* 1964a), partially purified and devoid of enzyme activity (K. P. Gopinathan, M. Sirsi & C. S. Vaidyanathan, unpublished work), had no effect on the enzyme from the animal tissues, in amounts 2–3 times more than that needed for complete inhibition of the bacterial enzyme.

The enzyme from normal as well as infected animal tissue had a comparatively broad optimum at pH 6.0–7.5, whereas the bacterial enzyme exhibited a sharp optimum at pH 6.5.

If the cleavage of NAD is taken as 100%, the enzyme from tissues of the normal animal cleaved

Fig. 2. Dependence of analogue formation on NAD concentration. The assay system contained (final vol. 0.8 ml.): potassium phosphate buffer, pH 7.5 (100 μmoles), INH (5 μmoles), enzyme [20–75% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of the infected-lung homogenate: 1.7 mg. of protein] and various amounts of NAD solution (2.5 $\mu\text{moles/ml.}$) as indicated. Incubations were carried out for 30 min. at 37° and the reactions terminated by the addition of 3.0 ml. of 0.1 N-NaOH.

NADP and NMN at 58 and 52% respectively of the rates with NAD, and the enzyme from tissues of the infected animal also exhibited the same pattern of activity. The bacterial enzyme reacted with NAD and NADP at equal rates, whereas NMN was not hydrolysed at all.

The K_m values also were very close for the enzymes from normal and infected tissues, and was different from that for the bacterial enzyme.

The animal-tissue enzymes catalyse the NAD-INH-exchange reaction also, whereas the bacterial enzyme was inactive in this system.

The initial activation of the bacterial enzyme was achieved after heat treatment at 85° for 1 min. (Gopinathan *et al.* 1964a). Under this condition 75% of the activity of the infected-tissue enzyme was lost. A parallel loss in the NAD-glycohydrolase and the NAD-INH-exchange activities of the infected-lung enzyme on heat treatment at various temperatures was also observed.

Comparison of these results clearly establishes that the NAD-glycohydrolase activity in infected tissues had properties almost identical with those of the host enzyme and differs from that of the bacterial enzyme considerably.

NAD-INH-exchange reactions. As shown in Table 1, the enzyme from the animal tissue catalyses the exchange reaction between NAD and INH. Formation of the analogue was indicated by the appearance of a yellow colour in the reaction mixture on termination of the reaction with 0.1 N-sodium hydroxide (Zatman *et al.* 1954a). To prove conclusively that the appearance of the yellow colour was due to the formation of the INH ana-

logue of NAD, the dependence of the colour formation on INH, NAD and enzyme concentrations was shown. The results are given in Figs. 2, 3 and 4.

Isolation and identification of the analogue. Larger amounts of enzyme reaction mixtures were taken and the reactions terminated by the addition of trichloroacetic acid (final concn. 5%, w/v). To the protein-free supernatant 5 vol. of cold acetone was added, and the mixture was left for precipitation at 0° overnight. The precipitate was collected by centrifugation, washed once with cold acetone and then dissolved in a small amount of water.

Samples of this were spotted on Whatman no. 3MM filter paper and chromatograms were developed in ethanol-acetic acid (1:1, v/v) by the ascending technique. The dried chromatograms were examined under a Mineralight SL2537 lamp, and the dark spot appearing below authentic NAD was cut and eluted with 2.0 ml. of water (containing 0.1 ml. of 1.0 N-hydrochloric acid). To this 3.0 ml. of 0.1 N-sodium hydroxide was added and the ultraviolet absorption spectrum was taken. The spectrum showed a sharp absorption maximum near 260 m μ and a less prominent and broader absorption maximum near 380 m μ .

To another sample of the acetone precipitate was added the NAD glycohydrolase from *Aspergillus*

Fig. 3. Dependence of analogue formation on INH concentration. The conditions were as given in Fig. 2, except that the NAD concentration was fixed (0.5 μmole/0.8 ml.) and the amount of INH solution (50 μmoles/ml.) added varied as indicated.

Fig. 4. Dependence of analogue formation on enzyme concentration. The conditions were as given in Fig. 2, except that the NAD concentration was fixed (0.5 μmole/0.8 ml.) and the amount of enzyme solution (8.7 mg. of protein/ml.) added varied as indicated.

Fig. 5. Absorption spectrum of the INH analogue of NAD. The INH analogue of NAD was treated with NAD glycohydrolase from *A. niger* to hydrolyse any unchanged NAD still present; 3.0 ml. of 0.1 N-NaOH was then added and the absorption spectrum was taken (curve A). On acidification, the yellow colour and the absorption maximum near 380–385 mμ disappeared (curve B).

niger (Sarma, Rajalakshmi & Sarma, 1964), and the mixture was incubated for 1 hr. at 37° in potassium phosphate buffer, pH 7.0. The enzyme from *A. niger* cleaves only any unchanged NAD still present with the analogue. At the end of the incubation period, 3.0 ml. of 0.1 N-sodium hydroxide was added and the absorption spectra were taken (Fig. 5).

The yellow colour and the extinction at about 380 mμ disappear on the addition of hydrochloric acid and reappear on the addition of sodium hydroxide.

These properties tally well with those reported for the INH analogue of NAD (Zatman *et al.* 1954b).

Another finding was the participation of NMN or NADP also in the NAD-glycohydrolase-catalysed exchange reaction with INH by enzyme from tissues of normal as well as infected mice at varying rates. If the molar extinction coefficients for the INH analogue of these two compounds are the same as for the INH analogue of NAD, namely 4.9×10^6 cm.²/mole (Zatman *et al.* 1954b), NMN participates in the exchange reaction (90% of the NAD activity) to a much greater extent than does NADP (55% of the NAD activity), although in the hydrolysis reaction the rate of cleavage of NADP is faster than that of NMN.

DISCUSSION

An increase in the NAD-glycohydrolase activity of tuberculous-mouse tissues has been reported by Bekierkunst & Artman (1962). In the present paper we have also shown an increase in this enzyme

activity in lung and liver tissues of tuberculous mice, but our values (percentage increase over the normal) are lower than those reported previously. However, Bekierkunst & Artman (1962) expressed the specific activity of their preparation in terms of wet wt. of tissue, whereas we have expressed it on the basis of protein content. This type of difference was found for the values of the nicotinamide nucleotide contents of fatty livers, induced by choline or threonine deficiency, when expressed in terms of wet wt. of tissue or of mg. of nitrogen (Methfessel, Mudambi, Harper & Falcone, 1964). There is an appreciable increase in the weight of lung and liver tissues in tuberculosis, and we considered that it would be better to express the specific enzyme activity in terms of the protein content, because the amount of protein solubilized from the tissues also might vary (owing to damage of the tissue in infection).

In the present paper we have compared the properties of the increased NAD-glycohydrolase activity in lungs from tuberculous mice with those of the enzymes from normal-mouse lung and the bacteria. The results clearly show that the enzyme of tissues from infected mice had all the properties of the enzyme from normal animal tissue but differed from the bacterial enzyme. Therefore it is concluded that the increased enzyme activity in infection is derived from the host tissue and is not of bacterial origin.

Increase in the NAD-glycohydrolase activity even in the plasma of tuberculous guinea pigs has been reported by Windman *et al.* (1964). Lysozyme activity in the sera of guinea pigs and rabbits is also reported to be increased in experimental tuberculosis (Metzger & Szulga, 1963). Studies by Artman, Bekierkunst & Barkai (1964) on the sub-microsomal localization of NAD glycohydrolase has shown a 10% solubilization of the enzyme in experimental tuberculosis, which is otherwise associated with the particulate fraction (in normal animals), in addition to a total increase of this enzyme activity in infection. Shah, Martin & Fox (1964) have reported that NAD-glycohydrolase activity reached normal values in tuberculous guinea pigs after the administration of INH. Comparison of all the above-mentioned findings and the necrotic degeneration of tissues observed in tuberculosis lead us to suggest that the tubercular process also might lead to the release of enzyme that is otherwise bound to the particles. The lysosomes may be expected to undergo changes when autolysis and cell death occur, and the release of lysosomal enzymes occurs under a variety of conditions (Novikoff, 1961; de Duve, 1959).

Concomitant with the increase in the NAD-glycohydrolase activity, the NAD-INH-exchange activity also is increased in tissues from tuberculous

mice. The INH analogue of NAD formed has been isolated and identified. The formation of this type of an analogue of NAD is one of the postulated modes of action of this potent antitubercular drug, because this analogue, once formed, cannot participate in the dehydrogenase reactions wherein NAD functions as the coenzyme (Zatman *et al.* 1954a; Goldman, 1954). This is the first time that actual evidence for the formation of this type of analogue in tuberculous infection has been reported, and it gives support for the idea that the above mode of action of the drug occurs when it is administered to infected animals.

Our present observation of the participation of NMN and NADP also in the exchange reaction with INH catalysed by NAD glycohydrolase shows the possible interference of the drug even at the nucleotide level in metabolism.

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