Phytochemistry, 1969, Vol. 8, pp. 1147 to 1154. Pergamon Press. Printed in England.

STUDIES ON NAD GLYCOHYDROLASE IN *RUMEX ACETOSA* TUMOUR TISSUE CULTIVATED *IN VITRO*

S. K. SRIVASTAVA, S. B. MAINI and C. V. RAMAKRISHNAN

Department of Biochemistry, M.S. University of Baroda, Baroda, India

(*Received* 18 *September* 1968, *in revised form* 8 *January* 1969)

Abstract—Tumour tissue of *Rumex acetosa* seems to possess two NAD glycohydrolases. Enzyme-I localized in the 480 × *g* fraction can be solubilized by 5% NaCl while Enzyme-II localized in the 5090 and 20,000 × *g* fraction can be solubilized by 1% triton X-100. Both enzymes have been partially purified. They show an optimum pH of 4·0 and 4·5 respectively. Both of them act on NAD and NADP but not on NMN. They also act on deamino-NAD and thionicotinamide-AD but at a reduced rate. The K_m for NAD is different for the two enzymes. Both are inhibited by nicotinamide. The inhibition is competitive in case of the former, whereas non-competitive in the latter. Enzyme-II requires a heat-stable and dialysable cofactor. Mg^{2+}, Co^{2+} and Mo^{2+} are able to replace this cofactor requirement.

INTRODUCTION

THE LOW level of pyridine nucleotides in malignant tissues[1–8] is explained in terms of either a preferential utilization of the adenine moiety for the synthesis of nucleic acids[9] or a rapid cellular division characteristic of neoplastic tissue.[4,10] Deletion of the enzymes involved in biosynthesis[11,12] or the elevation of the enzyme NAD glycohydrolase[13–17] involved in the degradation of pyridine nucleotides has also been implicated.

In a previous communication[18] we reported the presence of the enzyme NAD glycohydrolase in tumour tissue of *Rumex acetosa* and its absence in the corresponding normal tissue. The level of total oxidized pyridine nucleotide was found to be low in tumour tissue. Further studies carried out on this enzyme in tumour tissue suggest the possibility of the presence of two separate enzymes differing in certain characteristic properties and localization. The results of these investigations are reported in this paper.

[1] C. J. KENSLER, K. SUGIURA and C. P. RHOADS, *Science* **91**, 623 (1940).
[2] L. A. JEDEIKIN and S. WEINHOUSE, *J. Biol. Chem.* **213**, 271 (1955).
[3] G. E. GLOCK and P. MCLEAN, *Biochem. J.* **65**, 413 (1957).
[4] R. K. MORTON, *Australian J. Sci.* **24**, 260 (1961).
[5] P. MANDEL, M. WINTZEREITH, N. KLEIN PETE and L. MANDEL, *Nature* **198**, 1000 (1963).
[6] L. A. JEDEIKIN, A. J. THOMAS and S. WEINHOUSE, *Cancer Res.* **16**, 867 (1956).
[7] M. V. NARURKAR, U. S. KUMTA and M. B. SAHASRABUDHE, *Br. J. Cancer* **11**, 482 (1957).
[8] G. E. GLOCK and P. MCLEAN, *Biochem. J.* **61**, 388 (1955).
[9] M. B. SAHASRABUDHE, *Nature* **182**, 163 (1958).
[10] R. K. MORTON, *Nature* **181**, 540 (1958).
[11] M. V. BRANSTER and R. K. MORTON, *Biochem. J.* **63**, 640 (1956).
[12] M. SHIMOYAMA, K. YAMAGUCHI and R. K. GHOLSON, *Cancer Res.* **27**, 578 (1967).
[13] W. ERBE, J. PREISS, R. SEIFERT and H. HILZ, *Biochem. Biophys. Res. Commun.* **23**, 392 (1966).
[14] H. G. WILLIAMS ASHMAN and E. P. KENNEDY, *Cancer Res.* **12**, 415 (1952).
[15] S. GREEN and O. BODANSKY, *J. Biol. Chem.* **237**, 1752 (1962).
[16] S. GREEN and O. BODANSKY, *J. Biol. Chem.* **238**, 2119 (1963).
[17] V. S. WARAVDEKAR and C. C. GRIFFIN, *Exptl Cell Res.* **33**, 450 (1964).
[18] S. B. MAINI, S. K. SRIVASTAVA and C. V. RAMAKRISHNAN, *Indian J. Biochem.* **3**, 169 (1966).

RESULTS AND DISCUSSION

The data presented in Table 1 show that the enzyme is present only in the fractions settling down at $480 \times g$ and $5090 \times g$, suggesting that the enzyme is particulate bound. The activity of the $480 \times g$ fraction seems to be higher at pH 4·0 while that of the $5090 \times g$ at pH 4·5.

TABLE 1. LOCALIZATION OF ENZYME ACTIVITY IN *Rumex* TUMOUR TISSUE

Fraction	Enzyme activity (%) at pH 4·0	Enzyme activity (%) at pH 4·5
1. Whole homogenate	100	100
2. $480 \times g$	77	69
3. $5090 \times g$	15	19
4. $20{,}000 \times g$	3	6
5. Supernatant	0	0

Of the various grinding media and procedures tried to solubilize the enzyme, only 5% NaCl or KCl solubilized the enzyme from acetone powder; even with these reagents, 40 per cent of the enzyme activity remained associated with the residue.

An attempt was made to solubilize the enzyme from the NaCl-treated residue using deoxycholate and triton X-100 (Table 2). The deoxycholate treatment resulted in 20 per cent activity in the residue and none in the supernatant. When fresh or boiled supernatant was added to the residue the original activity was restored. The dialysed supernatant could not restore the activity. These results suggest that the enzyme localized in the residue requires a heat-stable and dialysable factor and deoxycholate is only able to solubilize this factor but not the enzyme from the NaCl-treated acetone powder residue. On the other hand triton X-100 was able to solubilize both the enzyme and the factor, When the triton treated-supernatant was treated with calcium phosphate gel the enzyme was adsorbed, whereas the dialysable cofactor remained in the supernatant. Thus it was possible to separate the enzyme from its cofactor.

Data on the purification of the enzyme from the NaCl supernatant of the acetone powder (Enzyme-I) and from the supernatant obtained by triton X-100 treatment (Enzyme-II) are given in Table 3. Enzyme-I can be concentrated 27-fold and Enzyme-II 18-fold by calcium gel treatment with a recovery of 66 and 51 per cent respectively. The fact that the calcium gel supernatant is required for the activity of Enzyme-II but not for Enzyme-I shows that these two may differ in their cofactor requirements.

The data on the kinetic studies carried out on Enzyme-I and II are given in Figs. 1 and 2 and Tables 4–6. Enzyme-I has an optimum pH of 4·0 whereas Enzyme-II has an optimum of pH 4·5 (Fig. 1). This is different from that reported for the enzymes isolated from other sources[19–25] which either have a wide pH range varying from 3–10 or 6–7·5. The activity

[19] N. O. KAPLAN, S. P. COLOWICK and A. NASON, *J. Biol. Chem.* **191**, 473 (1951).
[20] S. G. A. ALIVISATOS, S. KASHKET and O. F. DENSTEDT, *Can. J. Biochem. Physiol.* **34**, 46 (1956).
[21] H. MCILWAIN and R. RODNIGHT, *Biochem. J.* **44**, 470 (1949).
[22] L. J. ZATMAN, N. O. KAPLAN and S. P. COLOWICK, *J. Biol. Chem.* **200**, 197 (1953).
[23] H. G. WINDMUELLER and N. O. KAPLAN, *Biochim. Biophys. Acta* **56**, 388 (1962).
[24] A. A. ABDEL-LATIF and S. G. A. ALIVISATOS, *J. Biol. Chem.* **237**, 500 (1962).
[25] K. P. GOPINATHAN, M. SIRSI and C. S. VAIDYANATHAN, *Biochem. J.* **94**, 446 (1965).

TABLE 2. ENZYME ACTIVITY OF ACETONE POWDER FRACTIONS TREATED WITH SODIUM CHLORIDE, SODIUM DEOXYCHOLATE AND TRITON X-100

Treatment	Enzyme activity (%)
1. Acetone powder (10 mg/ml ground with 5% NaCl) homogenate	100
2. Supernatant (S_1)	57
3. Residue (R_1)	46
A. Residue (R_1) (treated with 1% deoxycholate in 0·1 M phosphate buffer, pH 7·0) homogenate	100*
B. Supernatant of homogenate A (S_2)	0
C. Residue of homogenate A (R_2)	20
D. C+B (1:1)	102
E. C+boiled B (1:1)	123
F. C+dialysed B (1:1)	22
G. C boiled+B fresh (1:1)	0
A. Residue (R_1) (treated with 1% triton X-100) homogenate	100*
B. Supernatant of homogenate A (S_3)	80
C. Residue of homogenate A (R_3)	18
D. Calcium gel supernatant of supernatant (S_3)	0
E. Calcium gel eluate of supernatant (S_3)	21
F. E+D	
0·1 ml	38
0·2 ml	53
0·3 ml	78
0·4 ml	78
G. E+dialysed D (0·3 ml)	28
H. E+boiled D (0·3 ml)	75
I. E boiled+D (0·3 ml)	0

* The enzyme activity in the residue (R_1) is taken as 100 per cent.

TABLE 3. PURIFICATION OF NAD GLYCOHYDROLASE FROM *Rumex* TUMOUR TISSUE

Procedure	Volume (ml)	Total units*	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Enzyme-I						
1. Acetone powder suspension in 5% NaCl	50	51·50	42·10	1·22	—	100
2. Supernatant	45	35·55	7·11	5·00	4	69
3. Calcium phosphate gel eluate	45	34·20	1·04	33·00	27	66
Enzyme-II						
4. Suspension of residue 1 in 1% triton X-100	25	18·00	34·80	0·51	—	100
5. Supernatant	22	11·22	5·83	1·92	4	62
6. Calcium phosphate gel eluate (+gel supernatant as cofactor)	22	9·24	0·99	9·33	18	51

* One unit=1 μmole NAD breakdown in 1 hr under the assay conditions.

of both the enzymes increases proportionately with enzyme concentration (up to 0·3 ml) and period of incubation (up to 30 min). The activity is also found to increase proportionately with increase in substrate concentration until about 45 per cent of the substrate disappears and the observed decrease in velocity above this level could be due to failure to measure the initial rates.

The K_m for NAD is 4×10^{-4} M for Enzyme-I and $1{\cdot}7 \times 10^{-4}$ M for Enzyme-II. Both the enzymes act on NAD and NADP (Table 4) and thus resemble the enzymes of human erythrocytes[26] and pig brain[23] and differ from those of rabbit erythrocytes[27] and bull seminal

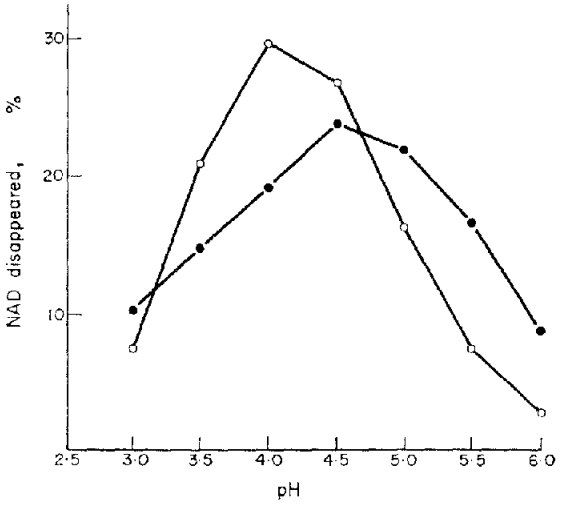

FIG. 1. OPTIMUM pH OF *Rumex* TUMOUR NAD GLYCOHYDROLASE.
Citrate-phosphate buffer was used from 3·0 to 6·0 pH range. Enzyme-I (—⊙—); Enzyme-II (—●—). Enzyme, 0·2 ml; NAD, 0·3 μmole; period of incubation, 30 min at 37°.

vesicular fluid,[28] where there is evidence for the presence of separate NAD and NADP glycohydrolases. The two enzymes have no affinity for NMN and in this respect they differ from the enzyme isolated from lung tissue.[25]

The heat treatment seems to have differential effects on the two enzymes (Table 5). Enzyme-II is completely inactivated in 10 min at 60° whereas Enzyme-I retains 27 per cent of the activity even after 20 min.

Studies carried out on the effect of nicotinamide, nicotinic acid and isonicotinic acid hydrazide on enzyme activity (Table 6) show that nicotinamide inhibits both the enzymes, nicotinic acid produces slight inhibition at higher levels and isonicotinic acid hydrazide produces high inhibition in case of Enzyme-II. The inhibition of nicotinamide in case of Enzyme-I seems to be competitive as it can be reversed by the addition of excess NAD, whereas in case of Enzyme-II it seems to be non-competitive (Fig. 2).

[26] E. C. G. HOFMANN and F. NOLL, *Acta. Biol. Med. Ger.* **6**, 1 (1961).
[27] E. C. G. HOFMANN, *Biochem. Z.* **329**, 428 (1957).
[28] E. C. G. HOFMANN and S. RAPOPORT, *Z. Physiol. Chem.* **304**, 157 (1956).

TABLE 4. EFFECT OF DIFFERENT COMPOUNDS AS SUBSTRATES ON ENZYME ACTIVITY

Substrate*	Substrate disappeared (%)	
	Enzyme-I	Enzyme-II
NAD	38	45
NADP	40	37
Deamino-NAD	18	18
Thionicotinamide-AD	25	30

* Compounds were used in a concentration of 0·3 and 0·15 μmoles for Enzyme-I and Enzyme-II respectively. NMN, 3-benzoicpyridine-AD, pyridine-3-aldehyde-AD, *N*-methylnicotinamide-AD and 3-acetylpyridine-AD did not act as substrates for either enzyme fraction.

TABLE 5. HEAT STABILITY OF THE ENZYME AT 60° FOR DIFFERENT PERIODS*

Time (min)	Inhibition (%)	
	Enzyme-I	Enzyme-II
5	26	86
10	50	100
15	60	100
20	72	100
25	89	100
30	100	100

* The two enzymes were kept at 60° for different periods, cooled and were then assayed at 37°.

Adenine compounds seem to inhibit both the enzymes (Table 6), inhibition being higher for Enzyme-II in all cases except adenine itself. It is interesting that ATP and ADP inhibit more than AMP. The fact that higher concentrations of inorganic phosphate and pyrophosphate, equivalent to that present in ATP, do not inhibit shows that the adenine moiety itself is responsible for inhibition rather than the phosphate.

The fact that Enzyme-II requires a heat-stable and dialysable factor suggested the possibility of the involvement of a metal ion. In order to confirm this, the activity of calcium phosphate gel eluate of Enzyme-II was tested in presence of 5 μmoles of various metals without the addition of cofactor containing gel supernatant. The data reported in Table 7 show that of the various metals tested Mo^{2+}, Mg^{2+} and Co^{2+} are able to restore the activity to the same level as that of supernatant containing cofactor. In a separate experiment different concentrations of these three metals were tried and the results (Table 8) show that all the three metals increase the activity of Enzyme-II, reaching an optimum at a concentration of 1·5–2·0 μmoles.

TABLE 6. EFFECT OF VARIOUS COMPOUNDS ON ENZYME ACTIVITY

Additions*	Concentration (μmoles)	Inhibition (%) Enzyme-I	Inhibition (%) Enzyme-II
Nicotinamide	10	46	39
	20	55	44
	30	67	56
	40	79	58
	50	87	63
Nicotinic acid	25	0	13
	50	30	30
Isonicotinic acid hydrazide	1	28	44
	2	28	80
Adenine	1·25	25	25
	2·50	40	39
AMP	1·25	25	44
	2·50	48	56
ADP	1·25	43	81
	2·50	57	88
ATP	1·25	57	81
	2·50	72	93

* Addition of inorganic phosphate or pyrophosphate (10 μmoles) had no inhibiting effect on either enzyme.

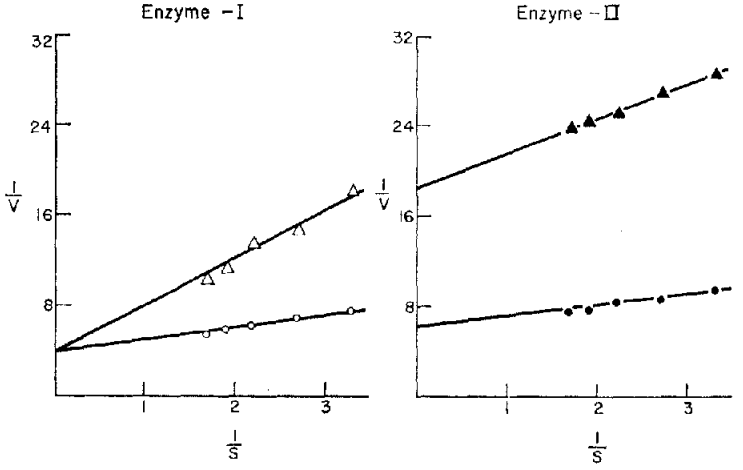

FIG. 2. LINEWEAVER–BURK PLOT FOR THE NICOTINAMIDE INHIBITION OF NAD GLYCOHYDROLASE ACTIVITY.

Enzyme-I without nicotinamide (—⊙—); with 30 μmoles of nicotinamide (—△—). Enzyme-II without nicotinamide (—●—); with 30 μmoles of nicotinamide (—▲—).

Since the data discussed above showed the possibility of the presence of two enzymes an attempt was made to find out the localization of these enzymes. The fractions obtained by centrifuging the tissue homogenate, prepared according to the method of Gentile[29] at 480,

[29] A. C. GENTILE, *Nature* **188**, 851 (1960).

TABLE 7. EFFECT OF VARIOUS METAL IONS ON THE ACTIVITY OF ENZYME-II IN THE ABSENCE OF ADDED COFACTOR

	Concentration of the metal ion (μmoles)	NAD disappeared (μmoles)
Enzyme	0	0·02
Enzyme + supernatant (containing cofactor)	0	0·08
Enzyme + Mo^{2+}	5	0·06
Enzyme + Mg^{2+}	5	0·06
Enzyme + Co^{2+}	5	0·06
Enzyme + Ni^{2+}	5	0·03
Enzyme + Ca^{2+}	5	0·02
Enzyme + Zn^{2+}	5	0·02

TABLE 8. EFFECT OF CONCENTRATION OF Mg^{2+}, Mo^{2+}, AND Co^{2+} ON THE ACTIVITY OF ENZYME-II

Concentration of the metal ion (μmoles)	NAD disappeared (μmoles) in presence of		
	Mg^{2+}	Mo^{2+}	Co^{2+}
0·5	0·05	0·04	0·04
1·0	0·05	0·05	0·05
1·5	0·06	0·05	0·06
2·0	0·06	0·06	0·06
4·0	0·06	0·06	0·05

5090 and 20,000 × *g*, were examined for the reversal of nicotinamide inhibition of NAD glycohydrolase by adding different amounts of NAD. The data reported in Table 9 show that the inhibition observed in fraction 480 × *g* can be reversed whereas that of the other fractions cannot be reversed by increasing NAD concentration. This suggests the localization of Enzyme-I in the 480 × *g* fraction and that of Enzyme-II in the 5090 × *g* fraction.

TABLE 9. REVERSAL OF NICOTINAMIDE INHIBITION IN 480, 5090 AND 20,000 × *g* FRACTIONS OF *Rumex* TUMOUR TISSUE HOMOGENATE

	Enzyme activity (%) in fractions		
	480 × *g*	5090 × *g*	20,000 × *g*
Control	100	100	100
Control + Nicotinamide*	58	45	60
Control + Nicotinamide + NAD (μmoles)			
0·075†	76	50	60
0·150	93	50	64
0·225	99	45	62
0·300	106	45	66

* 50 μmoles was used.

† Shows the amount of excess NAD added over what was already present in the assay system.

EXPERIMENTAL

Materials

Rumex tumour tissue was maintained *in vitro* as described elsewhere.[18]

Chemicals

Most chemicals used were best obtainable commercial grade. 3-Benzoicpyridine-AD, pyridine-3-aldehyde-AD, thionicotinamide-AD, *N*-methyl nicotinamide-AD, 3-actyl pyridine-AD were gifts from Professor N. O. Kaplan of Brandeis University, Waltham, Massachusetts, U.S.A., and isonicotinic acid hydrazide (INH) was a gift from Sarabhai Chemicals, Baroda, India.

Preparation of Acetone Powder

3–4-week-old tissue was ground with 4 vol. of chilled acetone for 2 min in a mortar kept in crushed ice. It was filtered quickly by suction. The residue was twice treated similarly and then was allowed to air-dry until free from acetone and stored in an air-tight bottle at 0–4°.

Preparation of Cell Fractions for Localization Studies

The method of Gentile[29] was used for the preparation of the tissue homogenate and the collection of fractions settling down at different speeds. The fractions settling down when centrifuged at $480 \times g$ for 10 min, $5090 \times g$ for 20 min and $20{,}000 \times g$ for 30 min and supernatant were used for enzyme assay.

Enzyme Assay

The method of enzyme assay was similar to that used by Sarma *et al.*[30] and has been described elsewhere.[18] A unit of enzyme activity is defined as the amount of enzyme required to break down 1 μmole of NAD in 1 hr under the assay conditions.

Treatment of Acetone Powder with Deoxycholate

Acetone powder was ground with 5% NaCl (10 mg/ml) for 15 min in a mortar kept in ice and centrifuged at $10{,}000 \times g$ for 15 min at 0–4°. The supernatant (S_1) was used for partial purification of Enzyme-I. The residue (R_1) was treated with 1% deoxycholate in 0·1 M phosphate buffer, pH 7·0 (volume of deoxycholate used was the same as that of sodium chloride solution), and centrifuged exactly in the same way as mentioned above. The residue (R_2) and the supernatant (S_2) obtained were tested for enzyme activity.

Treatment of Acetone Powder with Triton X-100

The residue (R_1), obtained when acetone powder was treated with 5% NaCl, was treated with 1% triton X-100 in water and centrifuged exactly in the same way as mentioned for deoxycholate treatment. The supernatant (S_3), obtained by centrifuging at $10{,}000 \times g$ for 15 min at 0–4°, was used for partial purification of Enzyme-II.

Partial Purification of Enzyme-I and Enzyme-II

All the operations reported below were carried out at 0–4°. For the purification of Enzyme-I, the supernatant (S_1) obtained when acetone powder was treated with 5% NaCl was used. The supernatant was dialysed for 2 hr against glass distilled water. Calcium phosphate gel (dry weight 22 mg/ml) was centrifuged at $10{,}000 \times g$ for 10 min to remove the supernatant and to the residue 3 vol. of dialysed enzyme was added. The mixture was stirred for 15 min and then centrifuged at $10{,}000 \times g$ for 15 min. The adsorbed enzyme was eluted with 1 vol. of 20% $(NH_4)_2SO_4$ in two batches.

For the purification of Enzyme-II, the supernatant (S_3) obtained by treating the residue (R_1) with 1% triton X-100 was used. The supernatant was placed on calcium phosphate gel, as above. The adsorbed enzyme was eluted from the gel with 1·0 M phosphate buffer, pH 7, and dialysed against glass distilled water for 4 hr.

Protein content of the enzyme preparation was estimated by the method of Lowry *et al.*[31]

Acknowledgement—This research project has been financed by the United States Department of Agriculture under PL 480 as per their grant No. FG-In-178.

[30] D. S. R. SARMA, S. RAJALAKSHMI and P. S. SARMA, *Biochim. Biophys. Acta* **81**, 311 (1964).
[31] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).