Grisolia, S., Moore, K., Luque, J., and Grady, H. (1969), *Anal. Biochem. 31*, 235.
Grisolia, S., and Tecson, J. (1967), *Biochim. Biophys. Acta 132*, 56.
Harrison, W. H., Boyer, P. D., and Falcone, A. B. (1955), *J. Biol. Chem. 215*, 303.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.
Ito, N., and Grisolia, S. (1959), *J. Biol. Chem. 234*, 242.
Jacobs, R. J., and Grisolia, S. (1966), *J. Biol. Chem. 241*, 5926.
Joyce, B. K., and Grisolia, S. (1960), *J. Biol. Chem. 235*, 2278.
Khorana, H. G., Tener, G. M., Wright, R. S., and Moffatt, J. G. (1957), *J. Amer. Chem. Soc. 79*, 430.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randell, R. J. (1951), *J. Biol. Chem. 193*, 265.
Markham, R., and Smith, J. D. (1952a), *Biochem. J. 52*, 552.
Markham, R., and Smith, J. D. (1952b), *Biochem. J. 52*, 558.
Mokrasch, L. C., Davidson, W. D., and McGilvery, R. W. (1956), *J. Biol. Chem. 222*, 179.
Mortimer, D. C. (1952), *Can. J. Chem. 30*, 653.
Najjar, V. A., and Pullman, M. E. (1956), *Science 119*, 631.
Nelson, N. (1944), *J. Biol. Chem. 153*, 375.
Pizer, L. I. (1962), *Enzymes 6*, 179.
Pizer, L. I., and Ballou, C. E. (1959), *J. Biol. Chem. 234*, 1138.
Rodwell, V. W., Towne, J. C., and Grisolia, S. (1956), *Biochim. Biophys. Acta 20*, 394.
Rodwell, V. W., Towne, J. C., and Grisolia, S. (1957), *J. Biol. Chem. 228*, 875.
Rutter, W. J. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 1249.
Sasaki, R., Sugimoto, E., and Chiba, H. (1966), *Arch. Biochem. Biophys. 115*, 53.
Smith, I. (1960), *in* Chromatographic and Electrophoretic Techniques, Smith, I., Ed., Vol. I, New York, N. Y., Interscience Publishers, p 279.
Somogyi, M. (1945), *J. Biol. Chem. 160*, 62.
Sugino, Y., and Miyoshi, Y. (1964), *J. Biol. Chem. 239*, 2360.
Sutherland, E. W., Posternak, T. Z., and Cori, C. T. (1949), *J. Biol. Chem. 179*, 501.
Torralba, A., and Grisolia, S. (1966), *J. Biol. Chem. 241*, 1713.
Towne, J. C., Rodwell, V. W., and Grisolia, S. (1956), *J. Biol. Chem. 226*, 777.
Wade, H. E., and Morgan, D. M. (1953), *Nature (London) 171*, 529.

Inactivation of Nicotinamide–Adenine Dinucleotide Glycohydrolase from Livers of Different Mammalian Species by Nicotinamide–Adenine Dinucleotide*

Saul Green† and Areta Dobrjansky

ABSTRACT: Nicotinamide–adenine dinucleotide glycohydrolase (EC 3.2.2.5) from microsomes and nuclei of mouse Ehrlich ascites cells is rapidly inactivated by its substrate, NAD^+, at pH 8.0. The possibility that NADase from tissues of other mammalian species may be similarly inactivated by NAD^+ has been investigated. NADases from mouse, rat, and rabbit livers were all significantly inactivated by NAD^+ at pH 8.0, whereas the NADases from dog, bovine, and pig livers were insensitive to the inactivation. Mouse, rat, and rabbit liver NADase had pH optima of 6.3–6.8 and molecular weights of 69,000 or higher while dog, bovine, and pig liver NADases had pH optima of around 9.0 and had molecular weights of 40,000 or less. Again, the mouse, rat, and rabbit liver enzymes were reversibly inactivated by 6 M urea. In contrast, the dog, bovine, and pig liver enzymes were irreversibly inactivated by this reagent. Purified rat liver microsomal NADase could be dissociated into two subunits of about 38,000 each and reassociated into an active form of the enzyme with a molecular weight of 70,000 by decreasing the concentration of urea to 1.5 M urea or lower. The results of this present work indicate that mammalian liver microsomal NADases may be divided into two classes on the basis of the following criteria: (a) inactivation by NAD^+ at pH 8.0, but not at pH 6.0; (b) pH–activity relationship; (c) molecular weight; (d) effect of urea.

Tissue levels of cellular nicotinamide–adenine dinucleotide glycohydrolase (EC 3.2.2.5) (NADase) have been reported to change markedly in certain diseases (Goldman *et al.*, 1970) and after the use of certain agents (Green, 1966; Tsukagoshi *et al.*, 1968). Although Swislocki *et al.* (1967) reported variations in the properties of this enzyme from different tissues, no information is available concerning a property recently reported by us (Green and Dobrjansky, 1971) on the interaction of NAD glycohydrolase with the substrate, NAD^+. We found that the enzyme NADase from mouse Ehrlich ascites cell nuclei or microsomes was inactivated by its substrate, NAD^+, and that this inactivation was very rapid at pH 8.0. The present paper is concerned with determining the generality of this observation. The effect of NAD^+ at pH 8.0 on microsomal NADase from mouse liver as well as from the livers of other mammalian species has been investigated in order to examine the relationship between this NAD^+ effect and the properties of the individual NADases.

* From the Division of Biochemistry, Sloan-Kettering Institute for Cancer Research, New York, New York 10021. *Received July 15, 1971.* This work was supported in part by Grant CA-08748 from the National Cancer Institute.

† To whom to address correspondence.

TABLE I: Effect of NAD^+ on Activity of Microsomal NADase[a] from Different Mammalian Livers.

Source	Sp Act. (Units/mg of Protein)	NADase Act. after Incubn with NAD^+ (%)	
		pH 6.0	pH 8.0
Mouse	1.16	97	49
Rat	1.42	93	50
Rabbit	1.20	104	43
Dog	0.58	105	106
Bovine	1.00	104	96
Pig	0.39	96	103

[a] Enzyme source was a sonicated suspension of washed liver microsomes (see Methods). Experimental conditions for incubation with NAD^+ have been described in a previous publication (Green and Dobrjansky, 1971). The pH of the reaction mixture in the determination of all NADase activity was 6.5. All values are the mean of duplicate experiments.

Materials

Enzymes, substrates, and reagents were obtained from the following sources: twice-crystallized alcohol dehydrogenase (yeast) (EC 1.1.1.1), once-crystallized, salt-free, soybean trypsin inhibitor, Worthington Biochemical Corp., Freehold, N. J.; β-NAD, nicotinamide, cytochrome *c* (horse heart) type III, Sigma Chemical Co., St. Louis, Mo.; lipase (pancreatic lipase steapsin 2000) (EC 3.1.1.3), Wilson Laboratories, Chicago, Ill.; twice-crystallized bovine serum albumin, Pentex Biochemicals, Kankakee, Ill.; Sephadex G-100–120, medium grade, Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Bull semen was obtained through the courtesy of N. Y. Artificial Breeders Cooperative, Inc., Ithaca, N. Y.

Methods

Preparation of Microsomal NADase from Rat, Mouse, Dog, Bovine, Pig, and Rabbit Liver. All procedures were carried out at 4°. Liver (60 g), in 150 ml of 0.05 M potassium phosphate buffer (pH 7.0), containing 0.25 M sucrose, was homogenized in a Potter-Elvejem glass homogenizer, equipped with a Teflon pestle, and filtered through a double thickness of cheesecloth. The filtrate was made to 300 ml with the buffered sucrose and centrifuged for 15 min at 30,000*g* in a Model L-2 Spinco ultracentrifuge to remove intact cells, connective tissue, nuclei, mitochondria, and debris. The supernatant solution was centrifuged at 105,000*g* for 1 hr, and the precipitated microsomes were suspended by homogenization in 150 ml of 0.154 M KCl and sonicated for 1.0 min in a 100-W, 20-kc MSE ultrasonic disintegrator. The sonicate, representing 75–85% of the original tissue NADase activity, was stable for up to 1 year when stored at −20°.

Other Methods. When purified NADase was required, it was solubilized with bovine pancreatic lipase and processed according to the method of Green *et al.* (1969). NADase activity of all preparations was determined with alcohol dehydrogenase as described in a previous paper (Green and Bodansky, 1965). A unit of NADase activity is defined as the amount of enzyme needed to hydrolyze 1 μmole of NAD^+/hr under the specified conditions of the assay. Total protein was determined by the method of Folin as modified by Lowry *et al.* (1951).

Results

Inactivation of Mammalian Liver NADases by NAD^+. The sensitivity of NADase from several mammalian species to inactivation by NAD^+ at pH 8.0 was investigated. All procedures were carried out at 4°. The assay of NADase activity was at 37°. An aliquot of a suspension of microsome-bound NADase in 0.154 M KCl from mouse, rat, rabbit, dog, bovine, or pig liver, adjusted to contain 4.0 units of enzyme/ml, was mixed with an equal volume of 1.0 mM NAD^+ in either 8.0 mM glycylglycine buffer (pH 8.0) or 8.0 mM potassium phosphate buffer (pH 6.0). The NADase activity was recovered from the mixture by centrifugation at 105,000*g*. The total contact time between the NAD^+ and NADase was 25 min. This procedure has been described in detail in a previous publication (Green and Dobrjansky, 1971). There was no inactivation of NADase in any of the enzyme preparations incubated with NAD^+ at pH 6.0 (Table I). In marked contrast, mouse, rat, and rabbit liver NADases were substantially inactivated at pH 8.0. There was no inactivation of the NADase from dog, bovine, or pig liver at this pH. Apitz *et al.* (1971) have recently reported that the activity of a preparation of pig brain NADase which was not inactivated by NAD^+ at pH 7.8, or by 0.13–0.50 mM dithiothreitol (Cleland, 1964) was rapidly and irreversibly inactivated in the simultaneous presence of both. This did not occur with dog, bovine, and pig liver microsomal NADase under the conditions of our experiments.

pH Optimum of Microsomal NADase from Mammalian Livers. The finding that NADases from different species varied in their sensitivities to inactivation by NAD^+ at pH 8.0 suggested that other properties of this enzyme might vary as well. Accordingly, the pH optimum of microsomal liver NADase from several species of mammals was determined. Each sample was standardized to 2.0 units/ml after assay at 37° at pH 6.5, the established pH optimum for NADase from rat and mouse tissue (Green and Bodansky, 1964). The pH of each of the 1.0-ml reaction mixtures, which contained 0.25 mM NAD^+ and 1.0 unit of NADase, ranged from 5.2 to 10.9 in either 0.10 M potassium phosphate or glycylglycine buffer. The reaction was started by addition of the enzyme, warmed to 37°, to the mixture. The reaction was stopped after incubation at 37° for exactly 2.0 min by the addition of 0.5 ml of ice-cold 0.4 M nicotinamide. The unhydrolyzed NAD^+ in the mixture was determined with alcohol dehydrogenase (Green and Bodansky, 1965). A control containing no NADase was included at each pH to account for any nonenzymatic breakdown of NAD^+ during incubation at 37°. The NADases from bovine, pig, and dog liver, each of which was insensitive to inactivation by NAD^+ at pH 8.0, had broad pH optima at around pH 9.0 and had 60, 81, and 80% of the activity at pH 7.4, respectively. In contrast, the NADases from rat, mouse, and rabbit liver, enzymes which were sensitive to inactivation by NAD^+ at pH 8.0, had sharp pH optima at 6.8, 6.3, and 6.5, respectively.

Solubilization and Determinations of the Molecular Weights of Microsomal NADases. Properties of the membrane-bound NADases of various mammalian tissues have been reported not to be altered after solubilization by treatment with lipase (Swislocki *et al.*, 1967). In a preliminary experiment, we determined the optimal conditions for lipase solubilization of NADase from the various mammalian livers. An aliquot of

TABLE II: Solubilization of NADase from Mammalian Liver Microsomes by Lipase.

Source	Incubn Time (hr)	NADase Act. in 105,000g × 1 hr Supernatant Soln (Units/ml)	NADase Act. Solubilized (%)
Mouse	1[a]	22	50
Rat	1	16	48
Calf	1	3	10
Rabbit	3	3	14
Dog	1	12	70
Pig	1	1[b]	9

[a] Incubation with lipase at 37° for more than the time shown resulted in an increased loss of active enzyme in the 105,000g supernatant solution. [b] Original liver NADase activity was very low (0.39 unit/mg of protein). The solubilized enzyme lost 50% of its activity after 24 hr at 4° and was completely inactivated by freezing and thawing.

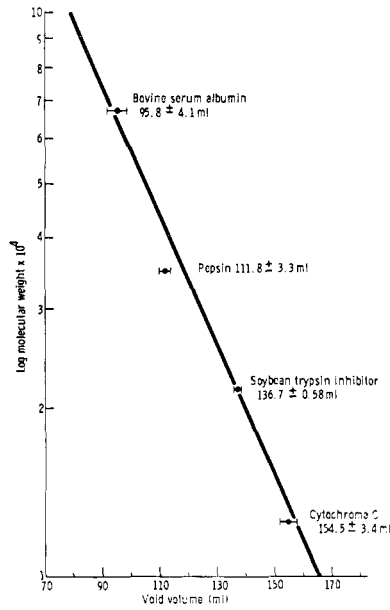

FIGURE 1: Calibration of Sephadex G-100 column. The column was equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) and was calibrated with 10 mg of each of the following proteins in 2.0 ml of the same buffer: bovine serum albumin (67,500), pepsin (35,000), soybean trypsin inhibitor (21,500), and cytochrome c (12,500). Peak elution volumes were determined by continuous scanning at 280 mμ.

a sonicated suspension of liver microsomes in 0.154 M KCl from each species source was centrifuged for 1 hr at 105,000g. Each pellet was taken up in three volumes of 0.1 M potassium phosphate buffer (pH 7.0) and the total NADase activity and protein determined. Each suspension was then diluted to a final protein concentration of 20 mg/ml with the same buffer, one volume of 2.5% bovine pancreatic lipase in 0.1 M potassium phosphate buffer (pH 7.0) was added to nine volumes of microsomal suspension and the mixture was placed in a 37° water bath. At 1-hr intervals, aliquots were centrifuged at 105,000g and the NADase activity in the supernatant solution and in the pellet were determined. The extent of lipase solubilization of NADase from the liver microsomes is shown in Table II. The sum of the NADase activity present in the 105,000g supernatant solution and in the precipitated material from each sample accounted for more than 90% of the original total enzyme activity and indicated that the low content of soluble NADase in the supernatant solution from preparations of bovine, rabbit, and pig livers was not due to inactivation by the lipase treatment, but rather was due to the failure of the lipase to release bound enzyme.

Determination of the Molecular Weight of NADases. The molecular weights of the lipase-solubilized NADases were determined at 4° on a 2.5 × 50 cm Sephadex G-100 column, according to the method of Andrews (1964). The gel column was equilibrated for 72 hr with 0.1 M phosphate buffer (pH 7.0) until the flow rate was constant at 10–15 ml/hr. The column was calibrated by passage of 10 mg of each of the following purified proteins in 2 ml of buffer: bovine serum albumin (mol wt 67,500), pepsin (mol wt 35,000), soybean trypsin inhibitor (mol wt 21,500), and cytochrome c (mol wt 12,600).

The peak elution volume for each standard protein was determined by continuously scanning the filtrate at 280 nm. Each standard protein was run through the column three to five times. The mean elution volume and the average deviation of this volume for each standard protein was calculated. The equation for the best straight-line relationship between each of these points and the log of the molecular weight of the corresponding standard protein was determined by the method of least squares. The resulting standard curve for determination of molecular weight from this Sephadex G-100 column is shown in Figure 1. At weekly intervals during the course of the study each standard protein was reapplied to the column to verify its calibration.

The concentration of NADase in each preparation (Table II, column 4) was adjusted to 15 ± 5 units/ml either by dilution with 0.1 M phosphate buffer (pH 7.0) or by concentration *via* lyophilization followed by dialysis; a 2.0-ml sample of each NADase was applied to the standardized Sephadex G-100 column. The peak elution volume of each NADase was found by determination of the NADase activity with alcohol dehydrogenase in the 5-ml fractions which were collected. When the NADase activity was located, its identity was verified by assay with cyanide (Kaplan, 1955). Table III shows that NADases from the rat, mouse, and rabbit liver, enzymes which were inactivated by NAD^+ at pH 8.0 (see Table I), had molecular weights of approximately 70,000 or more. In contrast, the NADase from dog, bovine, and pig livers, enzymes which were not inactivated by NAD^+ at pH 8.0, had molecular weights of 40,000 or less. Of interest was the finding that the rabbit liver NADase consisted of a mixture of an 89,000 species (40% of the total activity) and a 160,000 species (60% of the total activity). Samples of 500- and 1000-fold purified rat or mouse liver NADases, respectively, were also run through the column and were found to have the same molecular weights as the relatively crude lipase-solubilized enzymes from the corresponding species. These values are in agreement with those of Swislocki *et al.* (1967).

Effect of 6 M urea on the Activity of Liver NADase. Since rabbit NADase existed in two forms (89,000 and 160,000) and since the molecular weights of the other NADases appeared to be multiples of 20,000 subunits, the possibility existed that the larger forms of the enzymes might be dis-

TABLE III: Molecular Weights of Mammalian Liver Microsomal NADases on Sephadex G-100.[a]

Source	Peak Elution Vol. (ml) Mean	Rel Av Dev (%)	Mol Wt Mean	Rel Av Dev (%)
Mouse (3)[b]	92.6	0.5	69,000	2.4
Rat (5)	91.8	2.8	70,800	7.0
Rabbit (3)	83.0	0.8	89,200	3.9
	60.3	1.8	163,300	4.5
Dog (4)	110.0	0.5	42,700	5.1
Calf (3)	135.0	2.6	22,700	9.2
Pig (2)	137.0	1.1	22,200	7.7

[a] See text for details. [b] Numbers in parentheses are the number of separate determinations carried out.

TABLE IV: Effect of 6 M Urea on Mammalian Liver NADase Activity.

Source	Rel NADase Act.[a]
Rat	105
Mouse	86
Rabbit	113
Calf	15
Dog	0
Pig	0

[a] NADase activity was determined after 2 hr in 6 M urea at 4° in a dilution of the enzyme–urea mixture where the final concentration of urea was 0.6 M.

sociable into smaller subunits. Accordingly, solid urea was added to soluble rat, mouse, rabbit, dog, pig, and bovine liver NADase in 0.1 M potassium phosphate buffer (pH 7.0) at 4° to a final concentration of 6.0 M. After standing for 2 hr, the activity of the NADase was determined by warming a 0.5-ml aliquot to 37° and adding 0.5 ml of 0.1 M potassium phosphate buffer (pH 6.0), containing 0.5 mM NAD^+ and 6.0 M urea. The mixture at pH 6.5 to 6.6 was incubated at 37° for 2 min and the reaction was stopped by addition of 0.5 ml of ice-cold 0.4 M nicotinamide. The NAD^+ content of the reaction mixture was then assayed with alcohol dehydrogenase and ethanol in glycylglycine buffer as has been previously described (Green and Bodansky, 1965). The final concentration of urea in the NAD^+ assay mixture was 1.5 M. Concentrations of urea up to 3.0 M had previously been found not to interfere with the reduction of NAD^+ by alcohol dehydrogenase in the presence of ethanol at pH 9.0. The results of these assays showed that rat, mouse, rabbit, bovine, pig, and dog liver NADase had not hydrolyzed any NAD^+ in the presence of 6 M urea. Each preparation of NADase in phosphate-buffered 6.0 M urea was then diluted tenfold with 0.1 M potassium phosphate buffer (pH 7.0). An aliquot of 0.5 mM NAD^+ in pH 6.0 phosphate buffer alone was added and the NADase activity was again determined.

Table IV shows that dilution of the 6 M urea–enzyme incubation mixture resulted in complete restoration of the rat mouse, and rabbit NADase activity. These findings suggested two possibilities, namely, the inhibition of NADase action on NAD^+ by 6 M urea, or the dissociation of NADase by 6 M urea into inactive units. In either case, dilution would cause reversal of inhibition or reassociation. In contrast, the bovine enzyme had only 15% of its original activity, and the dog and pig enzymes were completely inactive and were not reactivated to any extent by dilution.

The possibility that a reversible dissociation of the high molecular weight species of NADase could take place was investigated with a preparation of concentrated, highly purified rat liver NADase (Green *et al.*, 1969) to which urea was added to a final concentration of 6 M. A Sephadex G-100 gel was prepared and equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 6 M urea. The column was equilibrated for 72 hr with the same buffered urea solution and was calibrated for use in the determination of molecular weights with bovine serum albumin, soybean trypsin inhibitor, and cytochrome *c*. The details of the calibration procedure have been described earlier in this paper. A plot of the elution volumes of each standard protein *vs.* the corresponding log of the molecular weight yielded a straight-line standard curve (Figure 2) with a different slope from that shown in Figure 1. A 2-ml sample of the rat liver NADase in 0.1 M potassium phosphate buffer (pH 7.0) containing 6 M urea was placed on the column, and eluted with the same buffered urea. Fractions of 5 ml were collected, aliquots were diluted tenfold and assayed for NADase activity. This experiment was carried out in duplicate. In each experiment, NADase activity was found after dilution, in fractions corresponding to an elution volume of 71 ± 0.5 ml which, according to the standard curve (Figure 2), represented a protein of around 38,000.

Since, as we stated earlier, the presence of the active enzyme in the fractions collected was detected after a tenfold dilution of the urea concentration, the question arose whether the active rat liver NADase had reassociated or was active as a 40,000 species. Fractions eluted from the phosphate-buffered urea Sephadex G-100 column, which contained the NADase activity, were pooled and dialyzed against 0.1 M potassium phosphate buffer (pH 7.0), until free of urea. A 2-ml aliquot containing 20 units of NADase activity was placed on the urea-free Sephadex G-100 column (Figure 1). The elution volume of this NADase was 89 ml, or a volume equivalent to a protein having a molecular weight of about 71,000.

In additional studies with this preparation of rat liver NADase, we found that the dissociation by 6 M urea and its reassociation by dilution or dialysis had not affected its sensitivity to inactivation by NAD^+ at pH 8.0 or its pH optimum.

Studies with Purified Bull Seminal NADase. Zervos *et al.* (1970) have recently reported that purified bull seminal NADase was inactivated in the presence of its substrate, NAD^+, at pH 7.0. In view of the fact that this was a soluble form of NADase and of our finding that bovine liver NADase was not inactivated by NAD^+, it was of interest to determine whether our findings with the microsomal enzyme applied in this case. Bull seminal plasma NADase was purified 80-fold by the method of Abdel-Latif and Alivisatos (1962) through the stage of the $(NH_4)_2SO_4$ fractionation. The final dialyzed preparation had no measurable 5′-nucleotidase, pyrophos-

phatase, or phosphatase activity and the hydrolysis of NAD^+ at pH 6.5, like the 1000-fold preparation of Abdel-Latif, was completely inhibited by 0.10 M nicotinamide. This preparation was used in each of the following studies.

The activity–pH relationship of the NADase was measured in phosphate buffers between pH 5.5 and 8.0 and in glycylglycine buffers between pH 8.0 and 10.0. These studies showed no sharply defined pH optimum for this enzyme. The activity increased only 50% from pH 5.5 to 8.5 and remained at the elevated level to pH 10.1.

The time course of the hydrolysis of NAD^+ at 37° by bull seminal NADase was determined in the presence of 0.5 mM NAD^+, at both pH 6.0 and 8.0. We found that the action of NADase ceased after 8 min at which time only 10–15% of the total NAD^+ was hydrolyzed. This is in agreement with the observations of Zervos *et al.* (1970) at pH 7.1. In the absence of NAD^+, this NADase was stable up to 30 min at both pH 6.0 and 8.0 after which time a slow deterioration in activity took place.

The bull seminal NADase was diluted with 0.15 M KCl to a final concentration of 20 units/ml and mixed with an equal volume of 1.0 mM NAD^+ in either 0.015 M potassium phosphate buffer (pH 6.0) or 0.015 M glycylglycine buffer (pH 8.0). After 25 min at 4° the pH of each mixture was adjusted to 7.0 and each was dialyzed at 4° against 0.1 M potassium phosphate buffer (pH 7.0) until free of measurable NAD^+. Similar preparations were incubated in the absence of NAD^+ and served as controls. We found that the NADase was inactivated by NAD^+ to the extent of 62% at pH 6.0 and to the extent of 65% at pH 8.0.

Solid urea was added to bull seminal NADase in 0.1 M potassium phosphate buffer (pH 7.0) to a final concentration of 6 M. The mixture was allowed to stand for 2 hr at 4°. No hydrolysis of NAD^+ took place in the presence of 6 M urea. In contrast, decreasing the urea concentration in the enzyme solution by diluting 1:10 with buffer resulted in the recovery of 82% of the original NADase activity.

A 2-ml sample of bull seminal NADase was placed on the standardized Sephadex G-100 column described in Figure 1, and was eluted from the column with 0.10 M potassium phosphate buffer (pH 7.0). The peak void volume of the enzyme was found to be 100 ml and corresponded to a molecular weight of about 58,000.

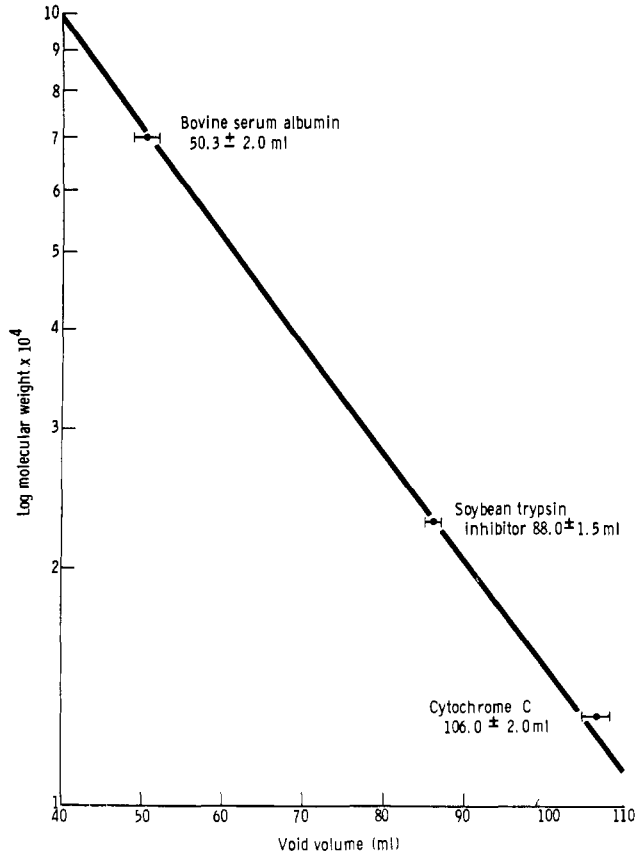

FIGURE 2: Calibration of Sephadex G-100 column containing 6.0 M urea. The column was equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 6.0 M urea. The column was calibrated with 2.0-ml aliquots of the 6 M urea phosphate buffer containing the same standard proteins used in the standardization of the Sephadex column described in Figure 1.

Discussion

We have recently reported that NADase from nuclei and microsomes of mouse Ehrlich ascites cells was inactivated by its substrate NAD^+ at pH 8.0, but not at pH 6.0 (Green and Dobrjansky, 1971). The present paper deals with these and other characteristics of NADases from microsomes of mouse, rat, rabbit, dog, bovine, and pig livers, as well as the soluble NADase of bull seminal plasma.

The results of our work indicate that the particulate-bound mammalian liver microsomal NADases may be divided into two classes on the basis of the following criteria: (a) inactivation by NAD^+ at pH 8.0, but not at pH 6.0; (b) pH–activity relationship; (c) molecular weight; (d) effect of 6.0 M urea. NADases from mouse, rat, and rabbit livers are all significantly inactivated by NAD^+ at pH 8.0, whereas the NADases from dog, bovine, and pig livers were insensitive to this inactivation. Again, the NADases in the former group all showed a marked optimum at pH values between 6.3 and 6.8, had molecular weights of about 69,000 or higher and were reversibly inactivated by 6 M urea. In contrast, the second group of microsomal NADases (from dog, bovine, and pig liver) showed an optimum activity at about pH 9.0, had molecular weights of about 40,000 or less, and were irreversibly inactivated by 6 M urea.

The soluble NADase from bull seminal plasma could not be clearly included in either of these two groups. Like the microsomal NADases of the first group, it had a high molecular weight (58,000), was inactivated by NAD^+ at pH 8.0, and was reversibly inactivated by urea. It resembled the enzymes of the second group in that it had a broad pH optimum near 9.0. Unlike either group, it was also inactivated by NAD^+ at pH 6.0.

The finding that rat liver NADase could be dissociated into stable subunits by 6 M urea and then reassociated into an active form of the enzyme by removal of the urea is of interest. Since the subunits could be demonstrated only in the presence of 6 M urea and, since NADase did not hydrolyze NAD^+ in the presence of 6 M urea, no statement can be made at this time concerning the enzymatic activity of the subunits. That mouse and rabbit NADases were reversibly inactivated by 6 M urea suggests the possibility that these NADases could also be dissociated into stable subunits. In contrast, the bovine, pig, and dog NADases were probably irreversibly denatured.

Studies are currently in progress to determine whether a relationship exists between the molecular weight of NADase, its reversible dissociation by 6 M urea and its inactivation by NAD^+.

Acknowledgment

We are indebted to Dr. Oscar Bodansky for his continued support and valuable advice and discussion during the course of these studies.

References

Abdel-Latif, A. A., and Alivisatos, S. G. A. (1962), *J. Biol. Chem. 237*, 500.
Andrews, P. (1964), *Biochem. J. 91*, 222.
Apitz, R., Mickelson, K., Shriver, K., and Cordes, E. H. (1971), *Arch. Biochem. Biophys. 143*, 359.
Cleland, W. W. (1964), *Biochemistry 3*, 480.
Goldman, D. S., Rouach, T. M., and Bekierkunst, A. (1970), *Amer. Rev. Resp. Dis. 102*, 556.
Green, S. (1966), *Cancer Res. 26*, 2481.
Green, S., and Bodansky, O. (1964), *J. Biol. Chem. 239*, 2613.
Green, S., and Bodansky, O. (1965), *J. Biol. Chem. 240*, 2574.
Green, S., and Dobrjansky, A. (1971), *Biochemistry 10*, 2496.
Green, S., Dobrjansky, A., and Bodansky, O. (1969), *Cancer Res. 59*, 1568.
Kaplan, N. O. (1955), *Methods Enzymol. II*, 660.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Swislocki, N. I., Kalish, M., Chasalow, F., and Kaplan, N. O. (1967), *J. Biol. Chem. 242*, 1089.
Tsukagoshi, S., Kao, M. H., and Goldin, A. (1968), *Cancer Chemother. Rep. 52*, 569.
Zervos, C., Apitz, R., Stafford, A., and Cordes, E. H. (1970), *Biochim. Biophys. Acta 220*, 636.

Bovine Liver Glutamate Dehydrogenase. Equilibria and Kinetics of Inactivation by Pyridoxal*

Dennis Piszkiewicz and Emil L. Smith†

ABSTRACT: Pyridoxal inactivates glutamate dehydrogenase presumably by forming an imine with the ϵ-amino group of lysine-97, as in the case of pyridoxal 5′-phosphate. The equilibrium constants for imine formation at varying pH values (K_{pH}) have been calculated from the initial concentrations of enzyme and pyridoxal and the final degree of inactivation. The variation of K_{pH} with pH has been related to the dissociation constants of the reactive ϵ-amino group, pyridoxal, and the product imine, and a single equilibrium constant for imine formation. When this treatment was applied to the inactivation of glutamate dehydrogenase by pyridoxal with the known K_a values of pyridoxal and reasonable, assumed values for the K_a values of the ionizating groups of the imine product, the reactive ϵ-amino group was found to have $pK_a = 7.9 \pm 0.2$. Inactivation of glutamate dehydrogenase by pyridoxal was found to be a kinetically second-order process for which the rate constant of inactivation was dependent upon the mole fraction of a conjugate base having $pK_{app} = 8.1 \pm 0.2$; this group was concluded to be the ϵ-amino group of lysine-97. The enzyme was protected from pyridoxal inactivation by DPNH and TPNH.

Glutamate dehydrogenase (L-glutamate:DPN(TPN) oxidoreductase (deaminating), EC 1.4.1.3) occupies an important position in the nitrogen metabolism of mammals since it catalyzes a reaction which is the major pathway for the interconversion of α-amino group nitrogen and ammonia (Meister, 1965; Frieden, 1963a). Although other important enzymes of amino acid metabolism generally employ pyridoxal 5′-phosphate as a cofactor in transamination or decarboxylation reactions, glutamate dehydrogenase is inhibited by this compound through the formation of an imine with the ϵ-amino group of a lysyl residue (Anderson *et al.*, 1966). In addition, this enzyme is subject to allosteric regulation by a variety of nucleoside polyphosphates (Frieden, 1963a,b); for example, GTP acts as an inhibitor and ADP as an activator. In its active form the dehydrogenase is composed of six-subunit polypeptide chains (Eisenberg and Tomkins, 1968).

A recent report from this laboratory has presented a tentative but almost complete amino acid sequence of the subunit polypeptide chain of the enzyme from bovine liver which indicated that the six subunits are identical, each with a molecular weight of 56,000 (Smith *et al.*, 1970). We have also described the specific nitration of a tyrosyl residue which is accompanied by a loss of allosteric inhibition by GTP (Piszkiewicz *et al.*, 1971), and we have identified lysine-97 as the residue which is labeled during the reversible inhibition of the enzyme by pyridoxal 5′-phosphate (Piszkiewicz *et al.*, 1970).

Pyridoxal has also been shown to inactivate glutamate dehydrogenase (Anderson *et al.*, 1966), and it is likely that this is also the result of imine formation with the ϵ-amino group of lysine-97. The present study describes the equilibria and kinetics of inactivation of the dehydrogenase by pyridoxal, and the effects of substrate, cofactors, and allosteric modifiers on the rates of enzyme inactivation. Its purpose was to determine the mechanism of inactivation and to probe the

* From the Department of Biological Chemistry, UCLA School of Medicine, and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received June 7, 1971.* This investigation was supported by Grant GM 11061 from the National Institute of General Medical Sciences, U. S. Public Health Service.

† To whom inquiries and reprint requests should be sent.