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Cellular distribution of nicotinamide adenine dinucleotide glycohydrolase in the central nervous system

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NICOTINAMIDE adenine dinucleotidase (NAD-glycohydrolase, EC 3.2.2.6) was first discovered by Mann and Quastel[1] and later described by Handler and Klein.[2] Its cellular distribution was originally studied by Alivisatos and Denstedt[3,4] in rabbit erythrocytes more than two decades ago. In those cells, the enzyme is clearly and uniquely attached to the plasmalemmal membranes and its orientation is such that it catalyzes the hydrolysis of pyridine dinucleotides present in the surrounding plasma; intracellular NAD^+ or $NADP^+$ is not affected. A somewhat similar distribution of this enzyme was later described in Ehrlich ascites tumor cells,[5,6] while in human[7] and bull[8] semen it is found soluble and extracellular in the seminal plasma. The localization and turnover of NADase in rat liver plasmalemmal and microsomal membranes was also reported.[9]

Considering the importance of pyridine nucleotides in processes involved in brain metabolism[10,11] and its abundant content of NADase,[12] we investigated the subcellular distribution of this enzyme. Experiments were performed on both viable cells isolated from rat cerebral cortex and subcellular fractions obtained from differential centrifugation of calf brain.

In whole cell preparations; aggregates of broken fragments (mostly granules), counted in three different preparations, amounted to no more than 15 per cent of all visible objects in our preparations.

NADase activity of various preparations was measured as described previously.[8]

As shown in Table 1, sonication of the "intact" cell preparation did not result in a profound increase in NADase activity, indicating an overwhelmingly "pericellular" (located at the cell surface) distribution of this enzyme. To further demonstrate the presence and the polarity of NADase on the plasma membrane, we demonstrated that ^{14}C-labeled NAD^+ present in the milieu did not enter the intact nerve cells. Brain neurons (1·3 mg protein) prepared as described in the legend of Fig. 1 were incubated in Earle's balanced salt solution (1 ml total volume) containing 20 mM nicotinamide and uniformly ^{14}C-labeled NAD^+ (sp. act. 8·3 μCi/μmole, New England Nuclear) at five different concentrations between 6×10^{-5} M and $1{\cdot}8 \times 10^{-4}$ M. After incubation for 10 min at 37°, the cells were washed four times with Earle's solution. The cells were collected by centrifugation and the remaining (intracellular) radioactivity was determined, after digestion in hydroxide of Hyamine, by liquid scintillation counting. No more than 0·2 per cent of the NAD^+ present in the medium was found to enter the cells.

TABLE 1. LOCALIZATION OF NADASE IN RAT BRAIN PREPARATIONS*

Preparation	Experiment 1	2	3	4
	($NAD^+ \times 10^{-6}$ moles split in 60 min/mg protein)			
Sonicated rat brain homogenate	1·356	1·489	1·080	0·586
Intact rat brain cells†	1·117	1·285	0·799	0·539
Per cent pericellular activity‡	82·4	86·3	74·0	92·0

* The assay system consisted of 0·35 mM NAD^+, either in the presence or absence of sonicated rat brain homogenate, in Earle's balanced salt solution at pH 7·4 (0·5 mg to 1 mg protein) or in the presence of intact rat brain cells (obtained by trypsin treatment as in the legend of Fig. 1) suspended in the Earle's medium, corresponding to 0·5 mg to 1 mg protein/1 ml. Each sample was made up to a final volume of 1 ml with Earle's solution. Incubation was performed at 37° for 10 min with adequate, but not excessive shaking. The reaction was stopped by the addition of 2·5 ml of 2 M NaCN in ice and the intact NAD^+, cyanide complex determined as described by Kaplan *et al.*[13] Supernatants of the centrifuged reaction mixtures were read at 327 nm in a Zeiss spectrophotometer. Values are averages of triplicate determinations.

† Obtained through trypsin treatment (see legend of Fig. 1).

‡ The average value of pericellular NADase is approximately 83·7 per cent (see also Ref. 9).

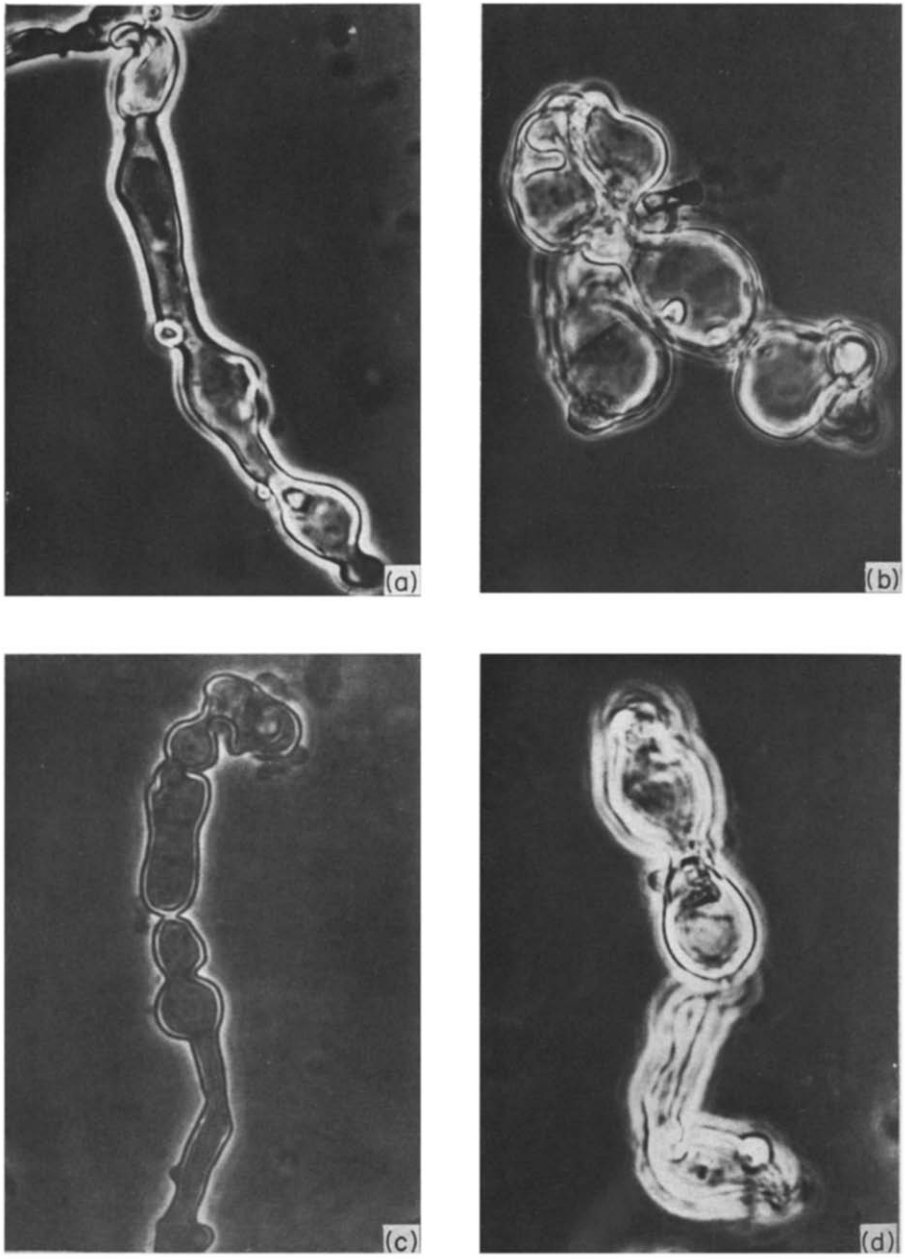

FIG. 1. Isolated cells derived from mouse brain. Cells (A–D) were isolated from brain by treatmen with trypsin. Mice brains were excised immediately after sacrificing the animals and cut into two halves anteroposteriorly with a sharp razor blade. One half was sterilely minced and washed in Earle's balanced salt solution. The small fragments were then incubated in a 0·25% trypsin-containing solution in buffered phosphate and saline pH 8·0 and at 4° on a magnetic stirrer. Every 20 min, for a period of 1·5–2 hr, depending on the yield of viable cells, the supernatant was collected (without centrifugation) and replaced by fresh trypsin solution. The pooled collected cell suspensions were washed three times in Earle's balanced salt solution, and finally were suspended in that solution according to the weight per volume (10 per cent) dictated by the homogenate prepared from the other half brain. The viability of the suspended cells was estimated by the method of trypan blue exclusion (see text). Microscopic magnification was in all instances 300×. Over-all magnifications, including photographic magnifications, were approximately 2200×. Thus, the longest diameter of individual cell structures in Fig. 1, B would correspond to approximately 27 μm (middle structure of Fig. 1, B).

Bock *et al.*,[9] using the well established marker enzyme, glucose 6-phosphatase,[14] found large amounts of NADase activity (approximately 37 per cent) in the microsomal fraction of rat liver and concluded that the enzyme is associated *in situ* with the endoplasmic reticulum membrane, especially that of rough microsomes.

The molecular identity of plasmalemmal and of smooth vesicular or microsomal rat liver NADases was indicated in their experiments by the similar behavior of these fractions during solubilization and purification, and by the reaction of the two fractions with the acetylpyridine analogue of NAD. It was found that the ratio of splitting for NAD and of its acetylpyridine analogue was the same for the plasmalemmal and microsomal fractions of NADase. Finally, no gross differences were found between the half-life of microsomal and plasmalemmal NADase.

When we subfractionated brain tissue by a modification[15] of the methods described by Whittaker *et al.*[16] and De Robertis and Azcurra,[17,18] approximately 30 per cent of the NADase activity found in the homogenate was recovered with the "crude" mitochondrial fraction, while up to 35 per cent of the activity was found in the microsomal fraction. However, when the "crude" mitochondrial fraction was subfractionated, the major portion (65 per cent) of the NADase activity appeared with the synaptosomes,

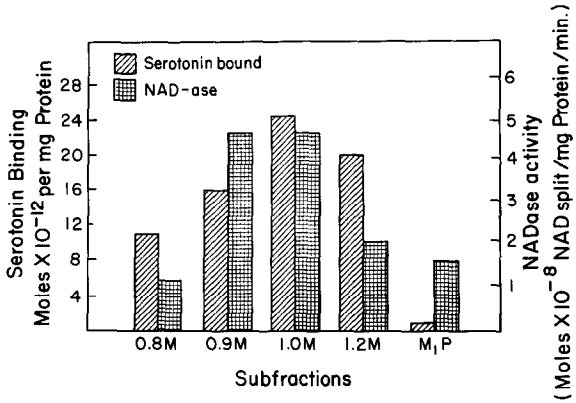

FIG. 2. Distribution of NADase activity and serotonin binding sites in subsynaptosomal fractions. Fractions were prepared by a modification[15] of the method of De Robertis and Azcurra.[17,18] Myelin was recovered in the 0·8 M fraction; 0·9 M, 1·0 M and 1·2 M fractions contained synaptic membrane fragments and M_1P contained mostly mitochondria and membrane fragments. NADase activity was assayed as described in Table 1. Serotonin binding in the various fractions was determined after stabilization with sodium borohydride as described by Alivisatos *et al.*[19]

and most of this activity was present in material designated by means of marker enzymes (i.e. Na^+K^+-activated ATPase) as synaptic membranes[15] after the disruption of the synaptosomes. In the experiments of Bock *et al.*,[9] the highest specific activity of NADase was found in the light of plasmalemmal subfraction consisting of smooth vesicles. Thus, the activity residing in the microsomal fraction of brain tissue may be a result of contamination by vesicles arising from plasma membranes upon cellular disruption. This contention is supported by the fact that sonic disruption of cells resulted in only an 8–26 per cent increase in NADase activity compared to intact cells (see Table 1).

Since we have previously described[19] the distribution of borohydride stabilizing binding of serotonin in subsynaptosomal fractions, we compared this distribution to that of NADase. It was demonstrated (Fig. 2) that NADase and the borohydride stabilizable binding of serotonin show a rather similar distribution.

It may be concluded that pericellular NADase may be used as a fairly reliable and stable marker, of known polarity, for serotonin binding sites of end synaptosomal membranes. As a matter of fact, our studies of the cellular distribution of NADase are relatively free of ambiguities involved in indirect assignments obtained through certain marker-enzymes (e.g. 5′-nucleotidase[20]). At any rate, they are roughly in agreement with others' results of the localization of NADase in various cells (e.g. in liver[9]) and Ehrlich ascites cells.[5,6]

The possibility that NAD^+ may diffuse out of the cell is now being probed with the aid of labeled histamine, included in the incubation medium of intact nerve cells. In view of the pericellular distribution of NAD^+ase, if leakage of NAD^+ (or $NADP^+$) occurs, stable labeled histamine dinucleotide[21] could be isolated from the medium.

Since most kinds of cells appear to possess pericellular enzymes which would destroy NAD^+, no NAD^+ can exist in the interstitial space or in incubation media.[3,4] Thus, the reverberating question of the role of an extremely active enzyme (NAD^+ase) must again be considered.

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REFERENCES

1. P. J. G. MANN and J. H. QUASTEL, *Biochem. J.* **35,** 502 (1941).
2. P. HANDLER and J. R. KLEIN, *J. biol. Chem.* **143,** 49 (1942).
3. S. G. A. ALIVISATOS, Ph.D. Thesis, McGill University, Montreal, Canada (1951).
4. S. G. A. ALIVISATOS and O. F. DENSTEDT, *Science, N.Y.* **114,** 281 (1951).
5. A. BEKIERKUNST, *Archs. Biochem. Biophys.* **118,** 549 (1967).
6. K. W. BOCK, V. GANG, H. P. BEER, R. KRONAU and H. GRUNICKE, *Eur. J. Biochem.* **4,** 357 (1968).
7. E. LEONE and L. BONADUCE, *Biochim. biophys. Acta* **31,** 292 (1959).
8. A. A. ABDEL-LATIF and S. G. A. ALIVISATOS, *J. biol. Chem.* **237,** 500 (1962).
9. K. W. BOCK, P. SIEKEVITZ and G. E. PALADE, *J. biol. Chem.* **246,** 188 (1971).
10. S. H. BARONDES, *J. biol. Chem.* **237,** 204 (1962).
11. S. G. A. ALIVISATOS and B. TABAKOFF, in *Chemical Modulation of Effective Behavior* (Ed. H. C. SABELLI), p. 41–66. Raven Press, New York (1973).
12. H. MCILWAIN and R. RODNIGHT, *Biochem. J.* **44,** 470 (1949).
13. N. O. KAPLAN, S. P. COLOWICK and A. NASON, *J. biol. Chem.* **191,** 461 (1951).
14. L. E. TICE and R. J. BARNETT, *J. Histochem. Cytochem.* **10,** 754 (1963).
15. B. TABAKOFF, L. MEYERSON and S. G. A. ALIVISATOS, *Brain Res.* **66,** 491 (1974).
16. V. P. WHITTAKER, I. A. MICHAELSON and R. J. A. KIRKLAND, *Biochem. J.* **90,** 293 (1964).
17. E. DE ROBERTIS, *Science, N.Y.* **156,** 907 (1967).
18. J. M. AZCURRA and E. DE ROBERTIS, *Int. J. Neuropharmac.* **6,** 15 (1967).
19. S. G. A. ALIVISATOS, F. UNGAR, P. K. SETH, L. P. LEVITT, A. J. GEROULIS and T. S. MEYER, *Science, N.Y.* **171,** 809 (1971).
20. P. EMMELOT, C. J. BOS, E. L. BENEDETTI and P. RUMKE, *Biochim. biophys. Acta* **90,** 126 (1964).
21. G. A. ALIVISATOS, *Nature, Lond.* **181,** 271 (1958).

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