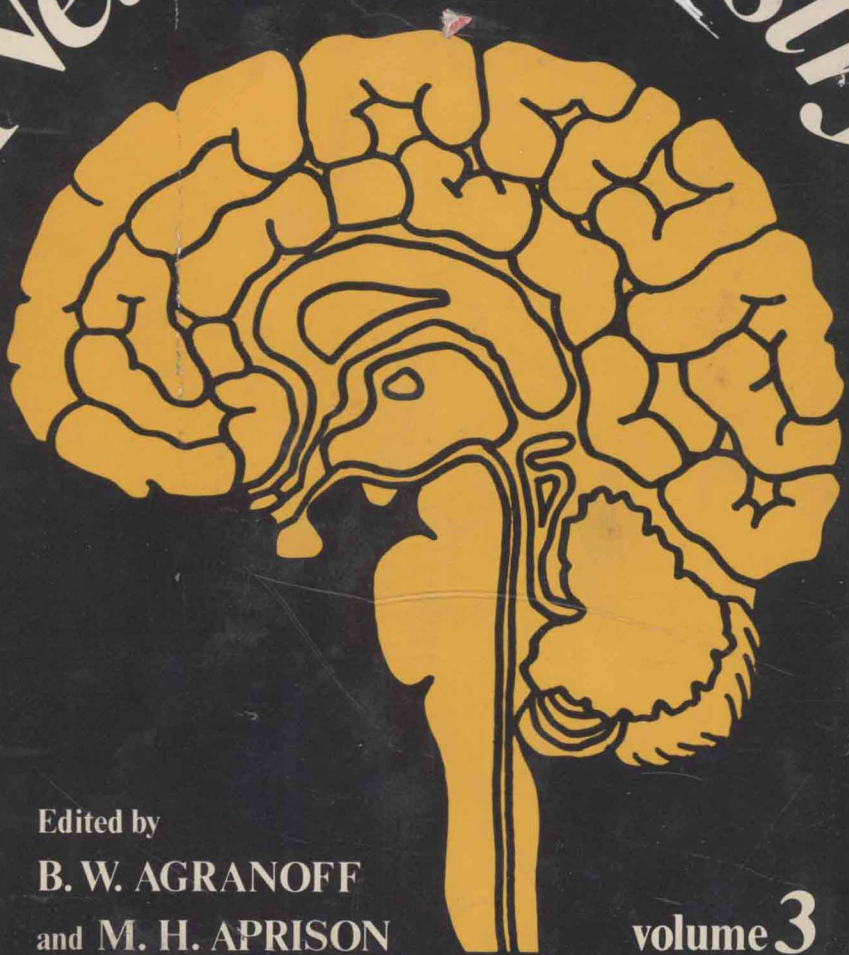
Advances in Neurochemistry



Edited by
B. W. AGRANOFF
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volume 3

Advances in
Neurochemistry

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PREFACE

The original premise of the Editors in initiating this series was that there existed a readership of neurochemists with considerable biochemical background who would make use of a series dedicated to both new developments and specialized reviews in neurochemistry. Having selected our authors, we have offered them virtually complete freedom to reflect and speculate in a field in which they have achieved prominence. The response to the first two volumes has been rewarding. The present one continues in this tradition. While we have not attempted to publish specialized volumes, the present volume contains two somewhat related chapters (Chapters 4 and 5, on the role of amino acid neurotransmitters). The first three chapters examine three diverse approaches, each of current interest, in neurochemical approaches to the molecular bases of neuronal and glial structure.

B. W. Agranoff
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CONTENTS

CHAPTER 3

MOLECULAR COMPOSITION AND FUNCTIONAL ORGANIZATION OF SYNAPTIC STRUCTURES

SAHEBARAO P. MAHADIK, HADASSAH TAMIR, and MAURICE RAPPORT

CHAPTER 4

THE MULTIPLE ROLES OF GLUTAMATE AND ASPARTATE IN NEURAL TISSUES

RICHARD P. SHANK and LEWIS T. GRAHAM, JR.

Erratum: In Volume 2 of this series, Chapter 2, Table 5 (page 147), the units of V_{max} for brain slices taken from the work of Kiely and Sourkes (1972) and Denizeau and Sourkes (1977) should all be mmol liter^{-1} min^{-1}.

CHAPTER 1

2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHODIESTERASE

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1. INTRODUCTION

2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNPase, EC 3.1.4.37*) has been widely used for several years as a marker for the presence of myelin in the central nervous system, but no review is available on its application and limitation as a marker enzyme and there is no information on its role in myelin. This was the first enzyme to be unequivocally characterized as a myelin component; previously it had been thought that myelin was enzymatically inert (Adams *et al.*, 1963). Several other enzymes have now been

*2',3'-Cyclic nucleotide 3'-phosphodiesterase was recently assigned the enzyme commission number 3.1.4.37. (IUPAC-IUB Enzyme Commission, 1976). Several workers had erroneously assigned the number 3.1.4.16 (2',3'-cyclic nucleotide 2'-phosphodiesterase) because of an error in the previous reference list for this entry. We have chosen the abbreviation CNPase for this enzyme following the style used for a number of enzymes (e.g., ATPase, RNase). Several other abbreviations have been used including CNP and CNPH.

proposed as being myelin-associated (for references see Carnegie and Sims, 1977; Norton, 1977), but evidence of myelin association is incomplete for some of these, such as protein kinase (Carnegie *et al.*, 1974; Miyamoto and Kakiuchi, 1974; Steck and Appel, 1974; Miyamoto, 1976) and phosphoprotein phosphatase (Miyamoto and Kakiuchi, 1975). In the case of nonspecific esterase (Keoppen *et al.*, 1969; Frey *et al.*, 1971; Rumsby *et al.*, 1973; Mitzen *et al.*, 1974) and arylamidase (Banik and Davison, 1969; Riekkinen and Clausen, 1970; Riekkinen and Rumsby, 1972; Mezei and Palmer, 1974) there is some controversy as to whether the enzymes are truly myelin-associated or are bound as an artifact of the preparation of myelin. There is strong evidence that cholesterol ester hydrolase is a myelin enzyme (Eto and Suzuki, 1973). The evidence that CNPase is a true myelin component is presented herein.

The physiological function of the enzyme is unknown but activity is measured by determining conversion of 2′,3′-cyclic nucleotides to 2′-nucleotides. CNPase-like activity has been found in other tissues and other nervous system subfractions but specific activities are generally much lower. This widespread distribution of activity could be an indication of a role of some general importance for the enzyme. The relationship of the enzyme to myelin and the properties of isolated and membrane-bound forms of CNPase are examined.

Since much of this chapter is concerned with CNPase in central nervous system (CNS) myelin a brief comment on the structure of this type of myelin is included. CNS myelin coats nerve axons and provides an insulating material which allows for more rapid transmission of electrical signals within the axon than would otherwise be possible. The myelin sheath is composed of a number of layers of membranous material which produces a characteristic multilamellar pattern in electron micrographs. Myelin has a high lipid-to-protein ratio (4:1 wt/wt) and the protein content would appear from gel electrophoresis to be simplified when compared to other membranes. Two major proteins, basic protein (18,000 daltons) and proteolipid protein (25,000 daltons), account for approximately 80% of the total myelin protein, the remainder being high-molecular-weight components. Among the lipid fraction, the cerebrosides are the most characteristic constituent, being present in much higher levels in myelin than in any other nervous system fraction or subfraction. The oligodendroglial cell is responsible for production of myelin. Since the composition of myelin from developing animals is different from that of the adult, it is possible that some of the components may be incorporated into myelin after it has been formed and deposited by the oligodendroglia. (For a more detailed review on myelin structure see Norton, 1972.)

2. ASSAY OF CNPase

2.1. Need for Activation

Early work on CNPase demonstrated that pretreatment of material using sonication or detergents resulted in an increase in the measured specific activity of the enzyme and allowed more reproducible results to be obtained. The significance of this activation is discussed fully in Section 5.1. It must be stressed that the assay values obtained are dependent on the method used for activating the enzyme. Treatment with the detergents Triton X-100 and sodium deoxycholate has been the most widely used activation procedures. However, sodium deoxycholate is not readily soluble at pH values below neutrality and activation at pH 7.5 followed by dilution and assay at pH 6.2 is necessary, whereas activation with Triton X-100 is unaffected by a change from pH 7.5 to 6.2 and both activation and assay can be performed at the lower pH.

2.2. Comparison of Assays

CNPase activity is assayed by measuring the conversion of 2',3'-cyclic nucleotides to 2'-nucleotides by estimation of either the product formed or the substrate remaining. Both fixed-time assays and continuous monitoring systems have been used. Although continuous monitoring assays are essential for accurate kinetic studies these have not been favored where the enzyme is used as a marker because they usually require more sophisticated equipment and in some cases are not as sensitive as fixed-time assays.

The major features of the assay methods available for CNPase are summarized in Table 1. None of these methods is applicable to all situations and the choice of a suitable assay is dependent on the nature of the samples being examined, the equipment and materials available, the number of assays required, and the sensitivity desired. Early methods (for references see Table 1) can be separated into two groups. The first involved determination of either substrate or product following separation by techniques including paper electrophoresis, paper chromatography, and thin-layer chromatography. These suffer from the disadvantage that spotting and elution are fairly tedious and time-consuming and limit the reproducibility. Furthermore the number of assays that can be handled at one time is limited by facilities for chromatography. The second group of assays involved determination of product formed by selectively removing the phosphate from the 2'-nucleotides with alkaline phosphatase followed by

TABLE 1. Comparison of CNPase Assays

Assay method	Substrate ([m*M*] in assay)	Total hours[a] (20 samples)	Actual man-hours[a] (20 samples)	Sensitivity (cf. I_{a1})	Separation of 2′- and 3′-nucleotides	Reference[c]
I. Fixed-time assays						
a. Spectrophotometric determination after:						
Paper chromatography	2′,3′-Cyclic AMP (7.5)	≈20	2.5–3.5		Yes	Kurihara and Tsukada, 1967
Thin-layer chromatography	2′,3′-Cyclic AMP (7.5)	4–6	2–3	Equal	Yes	Zanetta *et al.*, 1972; Glastris and Pfeiffer, 1974
Column chromatography	2′,3′-Cyclic CMP (na)	na	na		No	Lundblad and Moore, 1969
Alkaline phosphatase treatment for selective release of phosphate from product	2′,3′-Cyclic AMP (7.5)	1.25–2.5[b]	1–2[b]	Equal	No	Olafson *et al.*, 1969; Prohaska *et al.*, 1973; Kurihara and Takahashi, 1973
Precipitation	2′,3′-Cyclic AMP (7.5)	1.25–1.5	1–1.25	Equal	No	Sims and Carnegie, 1976
b. Fluorometric determination after:						
Precipitation	1,N^6-Ethenoadenosine-2′,3′-cyclic monophosphate (5)	1–1.25	0.75–1	Greater	No	Trams, 1973
Alkaline phosphatase treatment and separation of substrate and dephosphorylated product on column chromatography	1,N^6-Etheno-2-azaadenosine-2′,3′-cyclic monophosphate (4.95)	2–2.5	1.5–2	Greater	No	Lo *et al.*, 1975
II. Continuous monitoring assays						
a. ΔOD_{286}	2′,3′-Cyclic CMP (1)	2–3	≈2	Less	No	Hugli *et al.*, 1973
b. pH stat titration of release of second phosphoryl group	2′,3′-Cyclic AMP (7.5)	≥2	≈2	Less	No	Kurihara and Takahashi, 1973
c. ΔOD_{340} after coupled reaction with glucose-6-phosphate dehydrogenase	2′,3′-Cyclic NADP (1)	1–1.5	≈1.25	Equal	Yes	Sogin, 1976

[a] Approximate values determined from information in literature or from authors' own experience (na, not available).
[b] Time required dependent on variation of method used.
Source: Modified from Sims and Carnegie (1976).

determination of the phosphate by colorimetric analysis. This was originally proposed as a one-step reaction (CNPase and alkaline phosphatase acting simultaneously), but was later modified to two separate steps because the enzymes have different pH optima and K_m values. Although these assays do not suffer the drawbacks of chromatographic separation, their applicability is limited because a number of additives in the assay (e.g., some detergents) interfere with the action of alkaline phosphatase or the phosphate analysis.

CNPase acts on all naturally occurring 2',3'-cyclic nucleotides with optimal activity toward 2',3'-cyclic adenosine monophosphate (2',3'-cyclic AMP—Section 6.1). Generally this is the substrate of choice and was the substrate used in the assays just described. Several fixed-time assays have been proposed which utilize other substrates. One such method, based on a ribonuclease assay, used 2',3'-cyclic cytidine monophosphate and separation of the reaction mixture on an ion exchange column (Lundblad and Moore, 1969). Two fluorometric methods have also been proposed using derivatives of 2',3'-cyclic AMP, 1,N_6-ethenoadenosine-2',3'-cyclic monophosphate (Trams, 1973) and 1,N_6-etheno-2-azaadenosine-2',3'-cyclic monophosphate (Lo *et al.*, 1975). Separation of the substrate and product was achieved by selective precipitation in the first case and by an involved procedure requiring alkaline phosphatase treatment and chromatographic separation in the other. Although these substrates improved sensitivity they have not been widely used, probably because of the need to prepare these unusual derivatives. Furthermore, the K_m values for these substrates are very high (about 10 mM) compared to that of 2',3'-cyclic AMP (0.2–1.2 mM). Since the substrate is used at around 5 mM for reasons of cost and solubility this will affect the linearity of response. The greater sensitivity of these assays is of little consequence when dealing with myelin or whole brain homogenates but may be useful in examining the much smaller levels of activity in nonmyelin subfractions and tissues from outside the nervous system.

Continuous monitoring assays have been difficult to devise because of the similarities between the substrate and product in properties which can be readily measured. The use of pH stat titration to determine hydrolysis of the phosphoryl ester bond has been described but is insensitive and uses comparatively large amounts of substrate (Kurihara and Takahashi, 1973). A second method used 2',3'-cyclic cytidine monophosphate as substrate and involved measurement of small changes in the absorption spectrum at 286 nm as the product was formed (Hugli *et al.*, 1973).

Two other assays have recently been published and would seem to offer advantages for the assay of CNPase under most conditions. An assay that has been successfully used under widely ranging conditions in this

laboratory (Sims and Carnegie, 1976) for several years is based on the early fixed-time methods of Drummond *et al.* (1962) and Kurihara and Tsukada (1967). However, chromatographic separation of the reaction mixture was replaced by selective coprecipitation of 2′-AMP with cadmium carbonate (Figure 1). Because the absorbance of substrate remaining is measured directly, substances which absorb at 260 nm may interfere with the assay. However, the large dilutions involved in the procedure mean that only substances with high absorption maximum in this region show any effect. In most cases, these effects are small enough to be easily controlled, e.g.,

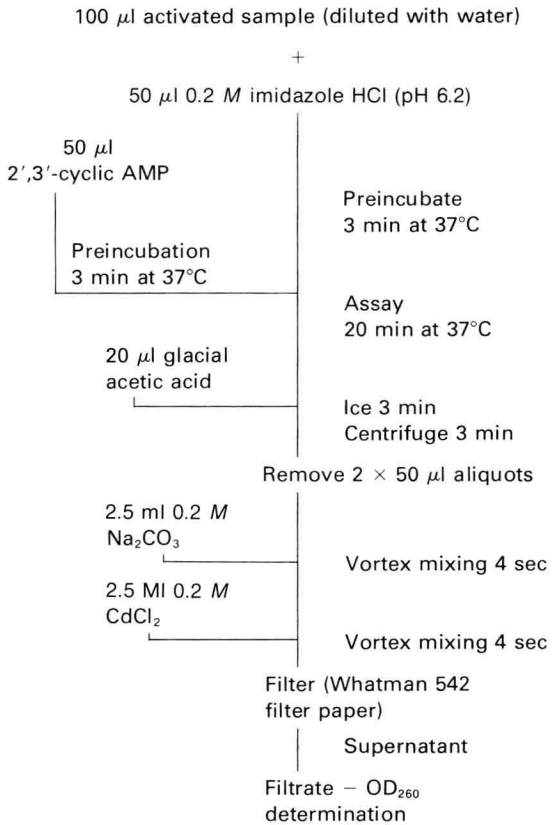

FIGURE 1. Precipitation assay for CNPase (Sims and Carnegie, 1976). Assay at 37°C has been used as a routine in this laboratory but assay at 30°C may be substituted to obtain slightly higher results.