# ON THE ROLE OF ATP AND DIVALENT METAL IONS IN THE STORAGE OF CATECHOLAMINES

## $^1$H NMR studies of bovine adrenal chromaffin granules

Joseph GRANOT* and Kurt ROSENHECK

*Department of Structural Chemistry, The Weizmann Institute of Science, Rehovot and Department of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel*

Received 14 August 1978

### 1. Introduction

The storage vesicles of catecholamines in the adrenal medulla, i.e., the chromaffin granules, contain a remarkably high content of catecholamine (> 0.5 M) and nucleotides (mainly ATP, ~0.1 M), as well as substantial amounts of acidic protein (chromogranin A) and divalent metal ions [1,2]. The mechanism by which such high concentrations are stored is unknown. Suggestions have been made that catecholamines participate in non-diffusible storage complexes with ATP [3,4] or with ATP together with divalent metal ions [5], or that catecholamines form aggregates with ATP which may play a role in their storage [6]. The possible formation of either binary catecholamine–ATP complexes or ternary complexes with metal ions, in aqueous solution, has been demonstrated by several spectroscopic studies [7–15]. Recently, on the basis of potentiometric and ultraviolet absorption measurements of ternary catecholamine–metal ion–ATP complexes, a 'metal-coordination hypothesis' [16] was proposed in which the storage of catecholamine is attributed to the formation of multinuclear structures which contain catecholamine, ATP, divalent metal ions, and possibly phospholipids. It suggests that catecholamine chelate divalent metal ions either through ionized ring hydroxyls or through the amine and the β-hydroxyl of the side chain. However, examination of this hypothesis reveals several inconsistencies. Appreciable coordination of catecholamine with $Ca^{2+}$ and $Mg^{2+}$ is found to take place only at pH > 8 [12,15]. With equimolar $Ca^{2+}$ or $Mg^{2+}$ the chelation by catecholamine is negligible at physiological pH [5]; at this pH a 100-fold excess or more over the catecholamine is needed to produce significant chelation. Metal ions such as $Cu^{2+}$ and $Fe^{2+}$ associate somewhat more strongly with catecholamine at neutral pH [13,14], but their concentrations in the storage vesicles are too low to account for the storage of catecholamine. Furthermore, at physiological pH the amine group of catecholamine is protonated and will not bind to positively-charged metal ions. The finding that the pH inside the chromaffin granules is actually acidic, i.e., pH ~ 5.5 [17], substantiates the arguments against the 'metal-coordination hypothesis' as a mechanism for the storage of catecholamine.

In a series of NMR studies of catecholamine in aqueous solutions [18–21] it has been demonstrated that at basic pH, catecholamine being in the zwitterionic or the anionic forms, chelate metal ions either in binary or in ternary complexes with ATP. No chelation occurs between catecholamine and metal ions at neutral or acid pH where the major part of the catecholamine (>97%) is in the cationic state. However in the region of this pH, ternary complexes are formed in which the metal ion is chelated by ATP and the catecholamine molecule also binds to the ATP molecule, without having any direct interaction with the metal ion. The stability and structural properties of these complexes, with regard to the

* Present address: The Institute for Cancer Research, Fox Chase, Philadelphia, PA 19111, USA

catecholamine, are actually independent of the type of metal ion chelated by ATP [21]. Thus, in the ternary complexes formed at basic pH, the metal serves as a bridge between the ATP and the catecholamine molecules (type I complex), whereas in the ternary complexes formed at neutral or acid pH, ATP serves as a bridge between the metal ion and the catecholamine molecule (type II), and the latter is actually 'outer-sphere' coordinated with respect to the metal ion.

It is the purpose of the present study to show that ternary complexes of type II are formed in the storage vesicles of catecholamine. Implications of this result with regard to the role of ATP and divalent metal ions in the storage of catecholamine are discussed.

## 2. Materials and methods

Chromaffin granules were prepared from bovine adrenal medulla by the method in [22]. In order to reduce the amount of water the granules were washed 3 times with isotonic sucrose solution in $D_2O$. $^1H$ NMR spectra of the granules were recorded immediately after their preparation on a FT-Bruker WH-270 spectrometer operating at 270 MHz, equipped with a Nicolet model 1180 32-K computer. Homonuclear decoupling was used to suppress the MDO signal. Absorbance measurements were performed on a Cary Model 15 recording spectrophotometer.

## 3. Results and discussion

The type of complexation that takes place between catecholamine and metal–ATP chelates can be determined by introducing the cobaltous ion to form the ternary complex. $Co^{2+}$ provides an excellent probe since it produces completely different effects in the $^1H$ NMR spectra of catecholamine associated in either type of the ternary complex. Formation of the type I complex results in considerable broadening and large down-field shifts in the catecholamine resonances, whereas formation of the type II complex results in relatively slight broadening and significant up-field shifts [19]. Therefore by monitoring the effect of $Co^{2+}$ on the resonances of catecholamine in the chromaffin granules, the type of complexation can be directly determined. In order to use this method in the present case, two conditions have to be satisfied, namely that the $Co^{2+}$ enter into the vesicles and that the catecholamine molecules (or at least most of them) remain stored inside the vesicles during the experiment.

Figure 1A shows the $^1H$ NMR spectrum of chromaffin granules. The lines labeled $\phi_H$ and $CH_3$ are split. The sharp components at the down-field side of these resonances were formerly assigned [11,23] to the epinephrine molecules which have diffused out of the chromaffin granules, while the corresponding up-field components were assigned to the inner epinephrine. This was confirmed in the present work by comparison of the spectra of intact and lysed vesicles. Upon progressive additions of $Co^{2+}$, the resonances of the extra- and intravesicular epinephrine coalesce and are shifted up-field (fig.1B). As discussed above, this indicates the formation of a ternary complex of type II. The resonances of the intravesicular ATP and protein are considerably broadened, actually beyond the detection limit, as a result of direct association with $Co^{2+}$. This effect clearly provides the evidence that $Co^{2+}$ have entered into the chromaffin granules. The ultraviolet spectrum of the same granule suspension was recorded immediately after the NMR measurements and compared with that of a lysed granule suspension. The optical density at the wavelength of maximum absorption of catecholamine (at $\lambda$ = 197.5 nm) was found to be 0.7 of that of the lysed sample. This indicates that about 70% of the total amount of the catecholamine remained intact inside the chromaffin granules [24].

Recent analysis of NMR spectra of bovine chromaffin granules [25,26] showed that the intravesicular medium consists of a fluid phase that is essentially isotropic and is characterized by relatively low viscosity, similar to that of dilute aqueous solution. Furthermore, the proton resonances of both ATP and epinephrine, in the chromaffin granules, were found to be shifted up-field, relative to their free states, in amounts comparable to the association shifts in aqueous solution [11]. In addition, we have now shown that $Co^{2+}$ shifts the catecholamine resonances up-field, also similarly to aqueous solution. It thus appears that there is a marked similarity between the interactions of catecholamine in aqueous solution and in the storage vesicles. Based on these similarities, the quan-

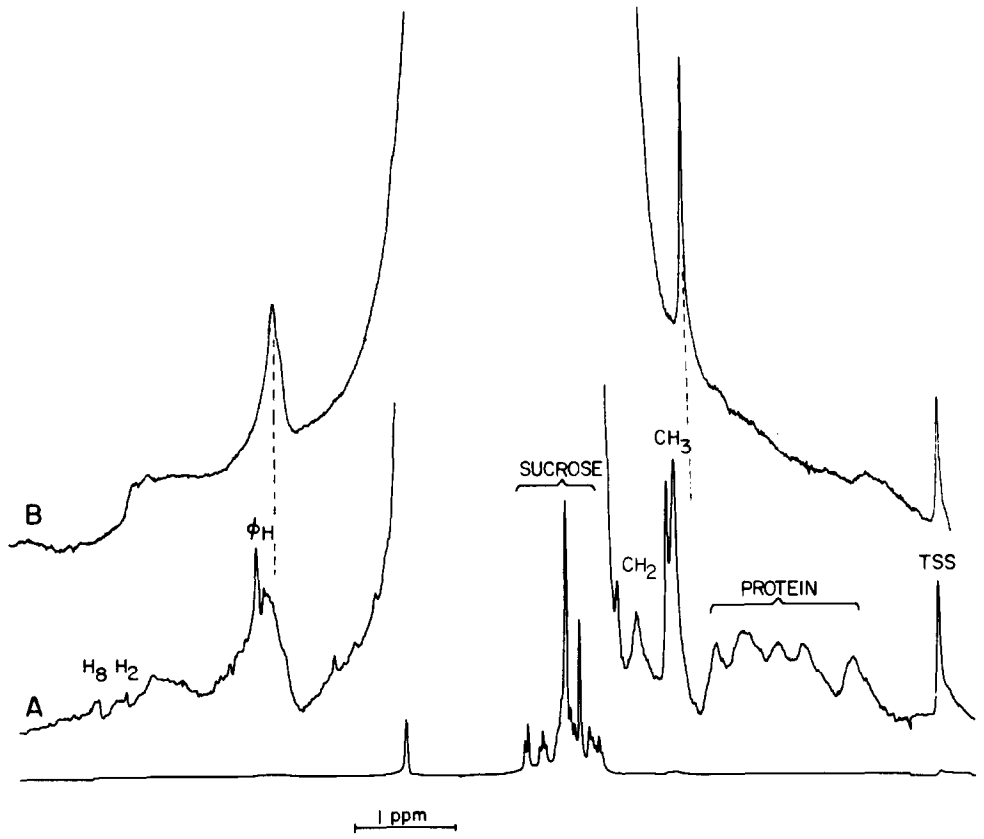


Fig.1. $^1H$ NMR spectra of intact chromaffin granules before (A), and after addition of 40 mM $CoCl_2$ (B) at 7°C. The resonances labeled $\phi_H$, $CH_2$ and $CH_3$ belong to epinephrine, and those labeled $H_8$ and $H_2$ to ATP.

titative results obtained for the aqueous system were used to estimate the amount of catecholamine complexed with ATP, in the absence and presence of divalent metal ions, in their storage vesicles. Fractional concentrations of bound catecholamine calculated with the data reported for the binary [20] and ternary [21] complexes are shown in fig.2. Evidently, when-

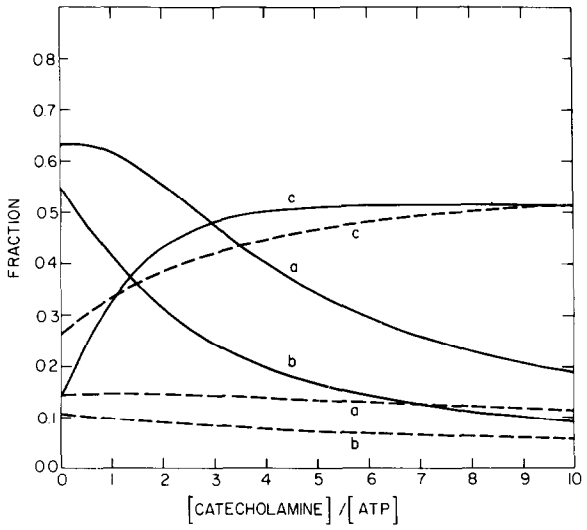


Fig.2. Calculated fractional concentrations of catecholamines bound in binary complexes with ATP (a) or in ternary complexes with 1:1 metal–ATP chelates (b), and of catecholamines liberated from the binary complexes upon the chelation of a divalent metal ion by the ATP molecule (c). The solid and the dashed lines correspond respectively to concentrations of 0.1 M and 0.01 M for ATP or the 1:1 metal–ATP chelate.

ever catecholamines are in excess over ATP, an appreciable fraction is not bound. This is even more marked in the presence of divalent metal ions, since the formation of ternary complexes, from the binary, cause liberation of about half the catecholamine which was found to ATP (cf. curve c of fig.2). As the catecholamine/ATP molar ratio in storage vesicles of catecholamine was found to vary from 4–12 [3,4,27,30], complex formation with ATP or with metal–ATP chelates cannot by itself be responsible for the storage of catecholamine. Recent observations [31] that ATP-free granules can also store catecholamine substantiate this conclusion.

In summary, the present study indicates that in the chromaffin granules there is no significant chelation between catecholamine and divalent metal ions and that the interaction is indirect, and mediated by ATP. Further evidence is provided that although part of the catecholamines are complexed with ATP inside the chromaffin granules, neither the binary nor the ternary complexes in the presence of metal ions can account for the storage of high concentrations of catecholamine. Since the composition of the storage vesicles of catecholamine of sympathetic nerves have been found to be similar to that of chromaffin granules [32], it seems reasonable that this conclusion is relevant in general for the storage vesicles of catecholamine.

## Acknowledgements

We thank Miss A. Zakaria for technical assistance and Professor Z. Luz for helpful comments.

## References

[1] Kirshner, N. and Kirshner, A. G. (1971) Phil. Trans. R. Soc. London B 261, 279–289.
[2] Winkler, H. (1976) Neuroscience 1, 65–80.
[3] Blaschko, M., Born, G. V. R., Di'Orio, A. and Eade, N. A. (1956) J. Physiol. 133, 548–557.
[4] Falk, B., Hillarp, N. A. and Hogberg, B. (1956) Acta Physiol. Scand. 36, 360–376.
[5] Colburn, R. W. and Maas, J. W. (1965) Nature 208, 37–41.
[6] Berneis, K. H., DaPrada, M. and Pletscher, A. (1971) Experientia 27, 917–918.
[7] Weiner, N. and Jardetzky, O. (1964) Naunyn-Schmiedeberg Arch. Exp. Pathol. Pharmakol. 248, 308–318.
[8] Muro, I., Morishima, I. and Yonezawa, T. (1971) Chem. Biol. Interact. 3, 313–324.
[9] Pai, V. S. and Maynert, E. W. (1972) Mol. Pharmacol. 8, 82–87.
[10] Tuck, L. D. and Baker, J. K. (1973) Chem. Biol. Interact. 7, 335–366.
[11] Daniels, A., Korda, A., Tanswell, P., Williams, A. and Williams, R. J. P. (1974) Proc. R. Soc. London B 187, 353–361.
[12] Rajan, K. S., Davis, J. M., Colburn, R. W. and Jarke, F. H. (1972) J. Neurochem. 19, 1099–1116.
[13] Rajan, K. S., Davis, J. M. and Colburn, R. W. (1974) J. Neurochem. 22, 137–147.
[14] Rajan, K. S. and Davis, J. M. (1976) J. Inorg. Nucl. Chem. 38, 897–905.
[15] Rajan, K. S., Skripkus, A., Marks, G. E. and Davis, J. M. (1976) Bioinorg. Chem. 6, 93–117.
[16] Rajan, K. S., Colburn, R. W. and Davis, J. M. (1976) in: Metal Ions in Biological Systems (Sigel, H. ed) pp. 292–321, Marcel Dekker Inc., New York.
[17] Bashford, C. L., Casey, R. P., Radda, G. K. and Ritchie, G. A. (1976) Neuroscience 1, 399–412.
[18] Granot, J. (1976) FEBS Lett. 67, 271–275.
[19] Granot, J. and Fiat, D. (1977) J. Am. Chem. Soc. 99, 4963–4968.
[20] Granot, J. (1978) J. Am. Chem. Soc. 100, 1539–1548.
[21] Granot, J. (1978) J. Am. Chem. Soc. 100, 2886–2891.
[22] Trifaro, J. M. and Dworkind, J. (1970) Anal. Biochem. 34, 403–412.
[23] Daniels, A., Williams, R. J. P. and Wright, P. E. (1976) Nature 261, 321–323.
[24] Neumann, E. and Rosenheck, K. (1972) J. Membr. Biol. 10, 279–290.
[25] Sharp, R. R. and Richards, E. P. (1977) Biochim. Biophys. Acta 497, 14–28.
[26] Sharp, R. R. and Richards, E. P. (1977) Biochim. Biophys. Acta 497, 260–271.
[27] Hillarp, N. A. (1960) Acta Physiol. Scand. 50, 8–22.
[28] DePotter, W. P., Smith, A. D. and DeSchaepdryver, A. E. (1970) Tiss. Cell 2, 529–546.
[29] Lagercrantz, H. and Stajarne, L. (1974) Nature 249, 843–844.
[30] Yen, S. S., Klein, R. L., Chen-Yen, S. H. and Thureson-Klein, A. (1976) J. Neurobiology 7, 11–22.
[31] Uvnas, B. and Aborg, C. H. (1977) Acta Physiol. Scand. 99, 476–483.
[32] Lagercrantz, H. (1976) Neuroscience 1, 81–92.