Formulation; its intracellular hydrolysis led to AZT being an effective anti-HIV agent in vitro, but required liposomal prodrugs. Further development of this work led us to 3P NMR spectroscopy since it allows both the monitoring of ATP release after internalization of Chol-ATP into liposomes and the distinction between external and internal species in a well-compartmented system. Moreover, 3P NMR resonances are highly sensitive probes of local pH changes. Our approach consists of recording 3P NMR spectra of Chol-ATP in the presence of small unilamellar vesicles (SUV) in a phosphate-buffered system using a pH gradient to distinguish between resonances of ATP molecules located inside and/or outside the liposomes. Essential requirements of such an experiment are that the compound of interest does not act as a detergent damaging the liposomal structure, and that its hydrolysis kinetic is compatible with the incubation time in the presence of the vesicles. In order to observe the resonances of ATP at different pH values, the liposomes must be able to maintain the proton gradient (ΔpH) across the membrane within the required acquisition time of the 3P NMR spectrum (one hour). To monitor Chol-ATP transport through membranes and the subsequent ATP release, the specific pattern of a 3P NMR spectrum of ATP entrapped in liposomes was first characterized. For this purpose, we prepared vesicles containing ATP. Figure 1 shows 3P NMR spectra of ATP (internal concentration 50 mM) entrapped in liposomes prepared in a phosphate-buffered medium recorded at different times. The comparison of these spectra provided three important pieces of information: a) The appearance of an inorganic phosphate peak, at a resonance frequency identical to the initial step before the pH jump, indicates the preservation of the pH 5 in the inner volume of the liposomes during the acquisition time. This, and the fact that no extravesicular ATP resonances occurred, demonstrates the integrity of the vesicles (Figure 1 b). b) After the pH jump the integral value for the two inorganic phosphate resonances gives an accurate determination of the distribution between the inner (6%) and outer volume (94%). As a result, the concentration of internal ATP was equivalent to 3 mM with respect to the total volume. c) A direct comparison of the lineshapes of intra- and extravesicular ATP resonances, while...
both species have been set at equivalent concentrations, revealed that the internal ATP phosphorus resonances were considerably broadened (Figure 1c). Moreover, the broadening was much more marked for $\beta$- and $\gamma$-phosphate resonances than for $\alpha$ ones. It is expected that the observation of free ATP internalized in liposomes will be very difficult.

To study the transmembrane transport of Chol-ATP the compound was incubated for nine days with SUV, which did not contain entrapped Chol-ATP, and monitored by $^{31}$P NMR spectroscopy (Figure 2). The lifetime of the compound was also controlled by $^{31}$P NMR spectroscopy in the same buffered medium (pH 5) in the absence of liposomes ($t_{1/2} = 69$ h). The total disappearance of the $\gamma$-phosphate signal of Chol-ATP after 96 h can be explained by an association of the molecule in the water–membrane interface. Moreover the absence of any sharp resonance of phospholipids provided evidence that there was no destruction of the liposomal structure due to Chol-ATP. When aqueous NaOH was added in the NMR tube the resonances of ATP, as well as of inorganic phosphate (both extravesicular), were shifted (Figure 2d). During the required acquisition time of the $^{31}$P NMR spectrum the adjustment of the pH between the different compartments started. This induced a pH-dependent shift of the resonances of ATP and consequently they were broadened. Considering the typical large pattern of entrapped ATP, one can assume that under these conditions the corresponding signals could be broadened beyond detection. Despite these shortcomings, a weak signal centered at $\delta = -8.2$ was observed (Figure 2d). This resonance was in agreement with the predicted chemical shift of the $\gamma$-phosphorus of entrapped ATP, calculated through the pH value which was indicated by the resonance of the internal inorganic phosphate peak. Thus, the release of ATP from Chol-ATP, inside the liposomes, can be assumed. To confirm this result, we added the detergent Triton X100 to the solution to break the liposomal structure. Consequently, the signals of the phospholipids appeared, indicating their micellization. Hence, the $\Delta$pH was neutralized and the resonances of extra- and intravesicular phosphorus coalesced. The areas of the signals of the $\beta$- and $\gamma$-phosphate groups of the total free ATP (Figure 2e) increased by 10% after the detergent addition, when compared to the area of the signal for free external ATP (Figure 2d); this increase can be considered as the amount of entrapped ATP. Concomitantly, the signal of the $\gamma$-phosphate of residual intact Chol-ATP (Figure 2e) reappeared.

As a further proof, we checked the evolution of the $\Delta$pH between the compartments during Chol-ATP internalization. It is indeed expected that the hydrolysis of Chol-ATP leads to the release of protons (Scheme 1). In a control experiment, a pH jump in the same phosphate-buffered system containing only liposomes was applied and the development of the proton gradient was monitored by $^{31}$P NMR spectroscopy over a long period (Figure 3A). In contrast to the decreasing $\Delta$pH value in the control experiment, an initial increase of the
proton gradient in the presence of Chol-ATP was observed, reflecting the hydrolysis of Chol-ATP and thus the release of the intravesicular ATP (Figure 3B).

In summary, we have demonstrated that ATP bearing a cholesteryl moiety at the γ-phosphate group can be transported across the membrane bilayer. It is now of interest to evaluate these model drug carriers of adenosine and various therapeutic nucleosides in integrated systems or living cells.

Experimental Section

Cholesteroloxycarbonyl-adenosine 5′-triphosphate: White powder. The 1H NMR spectrum (D2O) showed the characteristic signals of adenosine; protons of the cholesteryl moiety were observed between δ = 2.05 and 0.57. 31P NMR (D2O, proton-decoupled, pH 7): δ = –10.35 (d, J P,P = 19.8 Hz), –18.82 (br, δH = 21.51 (br, δP). Mass spectrum (electrospray); calcd for CaH107N6O8P(NBu3)2: 919.35; found 919.4. HPLC (gradient; 5–70% acetonitrile in 0.01M triethylammonium acetate, pH 7): t1/2 = 1704 min. Chol-ATP (HNBu3 or Na salt) is freely soluble in water and organic solvents (dichloromethane, acetonitrile, alcohols).

Vesicles preparation: SUV of defined size were prepared by reverse-phase evaporation using a mixture of egg phosphatidylcholine and phosphatidic acid (mole ratio 9:1) according to Rigaud and Pitard.[10] The phosphate-buffered solution referred to in the text contained KH2PO4 (20 mM), K2SO4 (15 mM), and Na2SO4 (15 mM) at pH 5.0 (90% H2O/10% D2O). After extrusion through 200-nm nucleopore membranes, the final lipid concentration was about 30 mg mL−1. ATP was finally thoroughly removed by filtration of the SUV through pD 10 columns (Pharmacia).

31P NMR experiments: 31P NMR spectra were recorded on a Bruker DMAX-300 operating at 121.49 MHz, and referenced relative to external H3PO4 85%. Two levels of broad band proton decoupling were applied for all the experiments. Samples were prepared immediately prior to use. In a glass vial, Chol-ATP (9 mg) was directly dissolved in the SUV preparation (550 μL, lipid concentration 30 mg mL−1). phosphate-buffered solution pH 5.0, 90% H2O/10% D2O). After quick introduction into the spectrometer, an initial 31P NMR spectrum (t = 0) was recorded (90’ pulse 6 μs, relaxation delay 5 s, 16 scans). In general, successive short spectra (16 to 64 scans) were acquired at the beginning of the incubation to monitor the pH gradient development. Better signal-to-noise spectra (512 scans) were then recorded to monitor the slow kinetics of internalization and hydrolysis of Chol-ATP. The pH jump was performed by injection of a few microliters of NaOH 1N into the NMR tube.

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[9] Hydrolysis of Chol-ATP in aqueous solution showed a selective cleavage at the mixed anhydride bond with a half-life time (63 h) large enough to allow diffusion of Chol-ATP molecules into the liposomes before their hydrolysis.

Ca2AuN: A Nitride Containing Infinite Zigzag Gold Chains**

Paul F. Henry and Mark T. Weller*

Previous investigation of the calcium –gold –nitrogen ternary phase field[1] resulted in the characterization of Ca2AuN,[2] which crystallizes with the cubic perovskite structure. This structural type is well represented in nitride chemistry, for example Ca3XN (X = P, As, Sb, Bi, Ge, Sn, and Pb).[3] The nitrogen atom in these compounds is surrounded by a Ca4Au3N3 core.

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