High-molecular-weight catecholamine–ATP aggregates are absent from the chromaffin-granule aqueous phase

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Direct n.m.r. studies show that the viscous liquid phase which separates from solutions containing physiological concentrations of noradrenaline, ATP and Ca^{2+} is not present in the chromaffin-granule interior. This finding is verified for both adrenaline and noradrenaline by using ^{13}C n.m.r. spectra.

Several investigations have shown that catecholamines are stored in chromaffin granules in a highly mobile aqueous environment (Sharp & Richards, 1977a,b; Sharp & Sen, 1978; Sen *et al.*, 1979; Daniels *et al.*, 1976, 1978; Steffen *et al.*, 1974). High reorientational mobility of the low-molecular-weight components is difficult to reconcile with the known low internal osmolality of the granules, which are in osmotic equilibrium with the cytosol, and has led to the suggestion that catecholamines may be present in an ordered condensed phase (Morris *et al.*, 1977). An alternative possibility is that a liquid phase containing the low-molecular-weight solutes in highly aggregated form may be present. Interesting examples of both solid and liquid phases have been demonstrated (Maynert *et al.*, 1971; Berneis *et al.*, 1969, 1970a,b). Of particular relevance is the observation of a separation of two liquid phases from solutions containing physiological concentrations of noradrenaline, ATP and Ca^{2+} (Berneis *et al.*, 1970a,b). The lower phase is highly viscous and contains high-molecular-weight aggregates that could readily account for the osmotic properties of the chromaffin-granule interior. Rather surprisingly, the phase separation has not been observed in comparable systems containing adrenaline.

In a previous study we demonstrated that most of the adrenaline and ATP in chromaffin granules is present in an n.m.r.-visible aqueous phase, and that little, if any, of the low-molecular-weight components are present as solid (Sen *et al.*, 1979). The present paper examines the possible involvement of the highly aggregated noradrenaline–ATP– Ca^{2+} liquid phase in catecholamine storage. N.m.r. spectra of this phase provide conclusive evidence against its occurrence inside chromaffin granules.

Experimental

Preparation of the biphasic noradrenaline–ATP– Ca^{2+} system

A 17% solution of noradrenaline (Aldrich) and monosodium ATP (Sigma) containing CaCl_2 in a

molar ratio of 3:1:0.24 (noradrenaline/ATP/ CaCl_2) was prepared (Berneis *et al.*, 1970a,b). A small amount of undissolved solid was observed and was removed by centrifugation. The supernatant was stored in an n.m.r. tube at 4°C overnight. The top layer was removed before recording n.m.r. spectra of the viscous bottom layer.

N.m.r. measurements

Spectra were obtained by using a Fourier-transform JEOL JNM/PFT-100 n.m.r. spectrometer (100 MHz ^1H , 25.3 MHz ^{13}C) or a JEOL FX-90Q spectrometer (22.5 MHz ^{13}C). Medullary-tissue slices were removed from bovine adrenal glands within 45 min of slaughter and placed in a 10 mm n.m.r. tube with 0.2 ml of 0.145 M- K_2SO_4 (in $^2\text{H}_2\text{O}$). Chromaffin granules were prepared for n.m.r. measurements as described previously (Sen *et al.*, 1979).

Results

^1H n.m.r. spectra of the viscous dense layer of the noradrenaline–ATP– Ca^{2+} system

Fig. 1 shows 100 MHz ^1H n.m.r. spectra of the viscous phase recorded at several temperatures. The numbering and assignment of resonances are shown in Fig. 2. The breadth of the peaks, especially at 4 and 10°C, is in marked contrast with the much narrower lines observed in spectra of isolated chromaffin granules (Sharp & Richards, 1977a,b; Sen *et al.*, 1979; Daniels *et al.*, 1978) or tissue slices (Daniels *et al.*, 1976). The linewidths increase dramatically with decreasing temperature, reflecting the known formation of high-molecular-weight aggregates. Peak integrals in the spectrum at 35°C are consistent with a 3:1 molar ratio of noradrenaline to ATP.

Quantitatively, the ratio of linewidths in the viscous phase compared with those of chromaffin-granule suspensions is about 3 for the noradrenaline catecholamine peak and about 4 for the side-chain methylene resonance. This difference clearly indicates that high-molecular-weight aggregates of the

Fig. 1. Proton n.m.r. spectra (100 MHz) of the viscous bottom phase after separation from a biphasic noradrenaline-ATP- Ca^{2+} solution

The frequency scale is relative to 2,2-dimethyl-2-silapentane-5-sulphonate at 1000 Hz. Abbreviations used: NAdr, noradrenaline; arom, aromatic; H_1 , see Fig. 2.

Fig. 2. Numbering system used in the assignments of ATP and catecholamine resonances

(a) ATP; (b) noradrenaline ($\text{R} = \text{H}$) and adrenaline ($\text{R} = \text{CH}_3$).

type known to be present in the viscous phase do not characterize the chromaffin-granule interior. Moreover, the difference in motional correlation times is considerably greater than is indicated by the difference in linewidths, since the proton linewidth in chromaffin granules is dominated by static interactions associated with magnetic-susceptibility changes at phase boundaries (Sharp & Richards, 1977a,b). The specifically motional part of the linewidth is at least an order of magnitude smaller than that seen in Fig. 1. The temperature coefficient of the linewidth is also markedly different in the two systems.

^{13}C n.m.r. spectra of adrenal-medulla tissue

Noradrenaline appears to be present in dense-cored vesicles largely segregated from adrenaline (Coupland & Hopwood, 1966), and the degree to which the intragranular matrices in the two types of vesicles correspond is not known. In particular, it is possible that only the dense-cored vesicles contain the aggregated phase, since phase separation has not been observed in the adrenaline-ATP- Ca^{2+} system. In ^{13}C n.m.r. spectra, unlike ^1H n.m.r. spectra, side-chain noradrenaline peaks are resolved from those of adrenaline and provide motional probes of the intragranular medium in both populations of granules. For this reason, ^{13}C spectra of medullary-tissue slices were recorded (Fig. 3). The glands were dissected and measurements begun within approx. 45 min of slaughter. The main peaks were seen with a signal-to-noise ratio of about 5 after 20 min.

The spectral profile is predominantly that of chromaffin granules; the spectrum of a chromaffin-granule preparation is included in Fig. 3 for comparison. In Fig. 3(a), noradrenaline-side-chain resonances are resolved at 70.4 p.p.m. (CHOH) and 46.5 p.p.m. (CH_2) and show an intensity ratio of approx. 3:1 relative to the corresponding adrenaline peaks, which is near the expected ratio in medullary tissue. Three sharp peaks, labelled A, B and C in Fig. 3, are present in spectra of adrenal-medulla-tissue slices and in the HClO_4 extract of medullary tissue but not in chromaffin-granule spectra. These peaks are very closely matched by reference spectra of *myo*-inositol (R. Sen & R. R. Sharp, unpublished work), which appears to be present in high concentration in the cytosol.

The linewidths of adrenaline and noradrenaline peaks are similar, and there is no evidence of differential mobility of the two catecholamines. It appears that the internal matrices of the two populations of granules are very similar, at least with respect to fluidity. A comparison of ^{13}C spin-lattice relaxation-time (T_1) measurements on isolated chromaffin-granule preparations and on tissue slices further indicate that the isolation procedure itself

Fig. 3. ^1H -decoupled natural-abundance ^{13}C spectra (a) Bovine adrenal-medulla tissue, 63 800 scans, 10°C ; (b) chromaffin granules suspended in a $0.145\text{ M-K}_2\text{SO}_4$ medium, 70 020 scans, 10°C . The chemical-shift scale is in p.p.m. relative to internal 2,2-dimethyl-2-silapentane-5-sulphonate, assuming the adrenaline (Adr) methyl peak is at 34.2 p.p.m. Peak assignments of catecholamines and ATP are indicated. Noradrenaline (NAAdr) is shown by dotted lines. Peaks A, B and C are discussed in the text.

Fig. 4. ^1H -decoupled ^{13}C spectra of the dense noradrenaline-ATP- Ca^{2+} phase

The reference of chemical shifts is internal 2,2-dimethyl-2-silapentane-5-sulphonate at 0 p.p.m. Each spectrum represents 5000 transients and was obtained by using a 50° pulse-width and a 0.3 s delay. The resonance frequency was 22.5 MHz. Peak assignments are given in Fig. 3.

causes little alteration of the internal phase (Sen, 1980).

^{13}C n.m.r. spectra of the dense phase of the noradrenaline-ATP- Ca^{2+} system

^{13}C spectra of the viscous noradrenaline-ATP- Ca^{2+} phase is shown in Fig. 4. As is apparent in the proton spectra (Fig. 1), the linewidths are systematically much broader in the viscous phase than in the chromaffin-granule interior, especially in spectra at 4 – 10°C . Resonances of protonated carbon atoms, which are relaxed relatively efficiently by large dipolar couplings, show particularly dramatic differences. The three protonated aromatic

resonances of noradrenaline (C-2, C-5, C-6) are not resolved in the dense phase at 10°C , and all the protonated resonances of ATP are broadened almost beyond the limit of detectability. In contrast, in chromaffin-granule and tissue spectra, all of the unobscured protonated ^{13}C resonances are well resolved at 10°C , except for C-8 on ATP, which is selectively broadened, owing to interactions with dissolved paramagnetic metal ions (Sen & Sharp, 1980).

Linewidths of protonated ^{13}C resonances in homogeneous solution can be used to measure rotational correlation times of the ^{13}C - ^1H bond vectors (Sharp & Richards, 1977b). At 25°C , the methine ^{13}C resonances of noradrenaline are about 50 Hz broad, and those of ATP are 75–100 Hz broad. By using the theoretical plots published previously (Sharp & Richards, 1977b; Fig. 3), we estimate rotational correlation times near 50 ns and 100 ns for noradrenaline and ATP respectively. At 10°C the noradrenaline linewidths imply a reorientational correlation time near 100 ns; the ATP

linewidths can only be estimated very roughly at 10°C, but the breadth indicates $\tau_R \sim 300$ ns. These values are two orders of magnitude greater than those inferred from T_1 data in chromaffin-granule spectra at 10°C (Sharp & Richards, 1977b); Sen, 1980), where values near 0.5 and 1 ns were found for catecholamines and ATP respectively. This large difference in reorientational correlation times provides quantitative confirmation that the chromaffin-granule interior is much more mobile than the dense noradrenaline-ATP- Ca^{2+} liquid phase.

Discussion

Proton and ^{13}C n.m.r. spectroscopy have shown that the viscous highly aggregated phase that forms spontaneously from noradrenaline-ATP- Ca^{2+} solutions is not present in chromaffin granules. Although our previous ^{13}C relaxation data in isolated chromaffin granules demonstrated a viscosity increase relative to simple aqueous adrenaline/ATP solutions, this increase is comparatively small (a factor of about 3) and cannot be interpreted in terms of the highly aggregated noradrenaline-ATP- Ca^{2+} phase. This conclusion has been verified both in isolated chromaffin-granule preparations and in intact tissue slices examined within 1 h of slaughter. Thus the possibility that the phase is relevant *in vivo* but is altered during sample procedure seems remote. Furthermore, the presumably distinguishable populations of noradrenaline and adrenaline vesicles do not appear to differ significantly in solute mobility in the aqueous phase. Certainly the resolved noradrenaline peaks, which arise from methylene and methine carbon atoms on the aliphatic side chain, indicate that the molecular reorientational mobility is essentially identical with that of adrenaline. Thus we conclude that the internal aqueous phases of both populations of vesicles have similar viscosity, and in particular that

neither population of vesicles contains the dense noradrenaline-ATP- Ca^{2+} phase.

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