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Phase separation in solutions of noradrenaline and adenosine triphosphate: influence of bivalent cations and drugs

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Summary

1. From aqueous solutions of biogenic amines, such as noradrenaline plus adenosine triphosphate (ATP), a second liquid phase spontaneously separates in the presence of small amounts of bivalent cations such as calcium. This separation is reversible and temperature-dependent ; the concentration of amine and ATP in the bottom phase is several times higher than in the supernatant.

2. Analytical ultracentrifugation provides evidence that the second phase consists of high molecular weight aggregates of the amine and ATP.

3. The separated second phase of the noradrenaline-ATP system dissolves isothermally on addition of tyramine and amphetamine which *in vivo* are known to liberate biogenic monoamines and which have a low tendency to aggregate with ATP. The apparent molecular weights of noradrenaline-ATP aggregates are decreased by tyramine and amphetamine. Dopamine does not diminish the second phase and it can also form aggregates of high molecular weight with ATP.

4. Bivalent cations in high concentrations diminish or abolish the separation of a second phase.

5. Small amounts of reserpine affect phase separation.

6. It is concluded that the physico-chemical properties of aggregates of biogenic amines with ATP may be of importance for understanding the storage and release of the amines *in vivo*.

Introduction

Biogenic monoamines, for example 5-hydroxytryptamine or (−)-noradrenaline (NA), and adenosine triphosphate (ATP) form aggregates of high molecular weight *in vitro* and probably also in the subcellular organelles storing the amines *in vivo*. The aggregation increases with rising concentration of the solutes and with decreasing temperature ; it also depends on the molar ratio between amines and ATP as well as on the nature of the amine. Thus, 5-hydroxytryptamine shows marked aggregation *in vitro* and NA somewhat less, whereas other amines such as histamine, tyramine and tryptamine aggregate very little (Berneis, Da Prada & Pletscher, 1969a, 1969b ; Berneis, Pletscher & Da Prada, 1969 ; Pletscher, Berneis & Da Prada, 1969). Furthermore, small concentrations of bivalent cations (Ca, Mg) enhance the aggregation, but large concentrations cause dispersion (Berneis *et al.*, 1969).

This paper describes the formation of biphasic liquid systems which results from aggregation of NA and ATP. Evidence is presented that phase separation can be influenced by bivalent cations and drugs known to liberate biogenic monoamines *in vitro* and *in vivo*. For preliminary findings see Berneis *et al.* (1969) and Pletscher *et al.* (1969).

Methods

Apparent average molecular weights were determined by equilibrium centrifugation according to the method of Van Holde & Baldwin (1958) and Yphantis (1960) in a Spinco analytical ultracentrifuge equipped with Schlieren optics. The experiments were carried out with 17% w/w aqueous solutions of mixtures of NA (98% pure ; Fluka) plus adenosine triphosphoric acid (75% pure ; Fluka) (ratio by weight 0·9 ; molar ratios about 3·5 : 1) containing $CaCl_2$ and in one experiment also $MgCl_2$. The solutions had a pH of about 6·5. In other experiments, one of the following amines was added: dopamine hydrochloride (Hoffmann-La Roche) ; *p*-tyramine hydrochloride (Fluka) ; (±)-amphetamine hydrochloride (Sigma). In order to compensate for the high concentrations, a 3 mm centrepiece in place of the standard 12 mm centrepiece was used. To obtain a rapid equilibrium between sedimentation and diffusion, a short column of the solution was used. This was realized by first filling the cell almost completely with silicone oil (FC 43, Beckman) and finally introducing 10 µl of the solution to be investigated. Equilibrium was established after 1–3 h, depending on the viscosity of the solution. Apparent average molecular weights were calculated according to Yphantis (1960) from the concentration gradient in the middle of the column as obtained directly with the Schlieren optics, the partial specific volume (0·60 ml/g), the speed of rotation, and the concentration of the solutes. Calculations were based on the total concentration of amines plus ATP, but electrolytes were not included. In the experiments where the molar ratio $CaCl_2$/NA was 0·24, the molar ratio between NA and ATP had to be reduced to about 3 to avoid precipitation.

The effect of Ca and drugs on phase separation was generally studied in solutions of NA plus ATP (total concentration 17% w/w, ratio by weight 0·91, molar ratio about 3·5 : 1, pH about 6·5) which had been stored under nitrogen at 4° C for 12 h. In the experiments with reserpine, ATP was first mixed with half the final volume of water ; reserpine (Siegfried) and thereafter NA were dissolved in that mixture, and finally a solution of $CaCl_2$ in the other half of the volume of water was added. The final solution was kept at room temperature 6–12 h before cooling. As the commercially available NA batches differ slightly in solubility, the amount of NA had occasionally to be somewhat reduced.

Results

Effect of bivalent cations on aggregation

Small concentrations of Ca markedly enhance the aggregation of mixtures of NA plus ATP (Fig. 1). Without Ca, the average apparent molecular weights increase moderately with decreasing temperature. A molar ratio of $CaCl_2$/NA equal to 0·08, which is near to the alkaline-earth ion content of the chromaffin granules of bovine adrenal medulla (Smith, 1968), drastically increases the apparent molecular weight. At 6·5° C, aggregation approaches infinity, and consequently a second liquid phase

separates (Fig. 2, tube 1). This bottom phase is transparent and highly viscous, and its concentration in amine and ATP may be 60% or more and thus several times higher than in the supernatant. When the molar ratio of $CaCl_2$/NA is 0·24, the effect on apparent molecular weights is still more pronounced. Consequently, the temperature at which separation of a second phase occurs is shifted to about 21° C. Mg ions have a similar effect to Ca ions, whereas monovalent metal ions (Na and K) do not enhance the aggregation (Berneis *et al.*, 1969). However, if the concentration of bivalent alkaline ions is further increased—for example by an addition of $MgCl_2$ (molar ratio $MgCl_2$/NA equals 2), the second phase disappears again (Fig. 1, curve 4). This effect is probably due to a reduction of apparent molecular weights (Fig. 1) (Berneis, Pletscher & Da Prada, 1969). Large amounts of Ca ions also cause dispersion, but in contrast to Mg a precipitate is formed.

Similar effects of bivalent cations have been obtained with mixtures of 5-hydroxytryptamine and ATP (Pletscher, 1969).

Effect of various aromatic amines on NA aggregation

The effect of various amines on apparent molecular weights of a standard solution of NA plus ATP containing small amounts of Ca is shown in Fig. 3. Amphetamine and tyramine, in contrast to dopamine, markedly reduce the apparent molecular weights. The degrading action of amphetamine and tyramine increases with increasing concentration of the drugs, amphetamine being somewhat more effective than tyramine (Fig. 4).

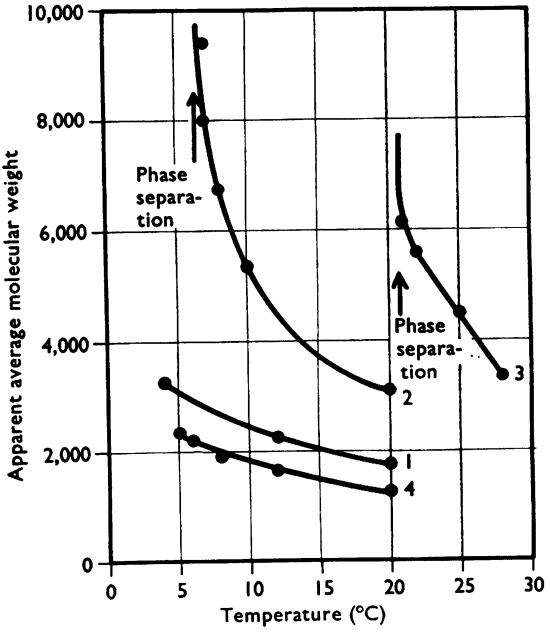

FIG. 1. Temperature dependence of apparent average molecular weights of mixtures of noradrenaline (NA) and ATP, molar ratio 3·5:1, total concentration 17% w/w. 1, No further addition; 2, addition of $CaCl_2$ (molar ratio $CaCl_2$/NA=0·08); 3, molar ratio $CaCl_2$/NA=0·24 (molar ratio NA/ATP reduced to 3); 4, molar ratio $CaCl_2$/NA=0·08; molar ratio $MgCl_2$/NA=2.

Figure 2 shows the effect of the aromatic amines on phase separation. The bottom phase which has separated from a solution of NA plus ATP containing small amounts of Ca does not decrease, but even increases in volume if an amount of dopamine is added so that the molar ratio dopamine/NA is close to 1·0. In contrast, no bottom phase is separated after the addition of corresponding amounts of *p*-tyramine (tube 3) or amphetamine (tube 4).

Effect of reserpine on phase separation

0·0003 mmol reserpine, when present in a solution of 0·27 mmol NA, 0·074 mmol ATP and 0·023 mmol $CaCl_2$ in 0·5 ml of H_2O, induces at room temperature the formation of a voluminous precipitate containing reserpine. Precipitation is optimal in solutions saturated with respect to NA and proceeds for several hours. On

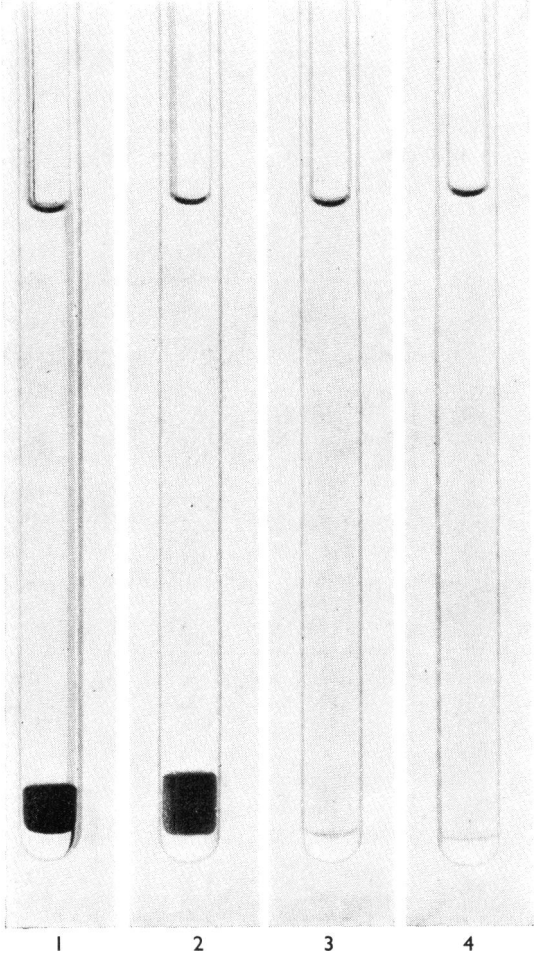

FIG. 2. Effect of different amines on phase separation of a 17% w/w aqueous solution of noradrenaline (NA) and ATP. Molar ratio NA/ATP=3·5, $CaCl_2$ added (molar ratio $CaCl_2$/NA=0·08). Tube 1, No further addition ; tube 2, addition of dopamine ; molar ratio dopamine/NA=0·9 ; tube 3, addition of *p*-tyramine ; molar ratio *p*-tyramine/NA=0·9 ; tube 4, addition of amphetamine ; molar ratio amphetamine/NA=0·9.

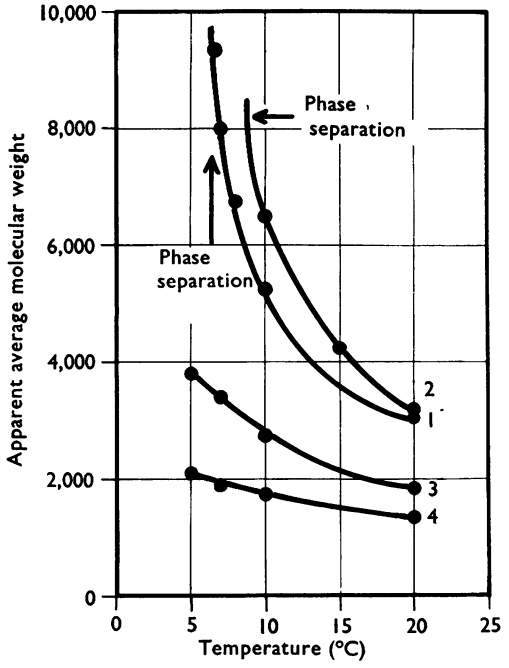

FIG. 3. Effect of added amines on temperature dependence of apparent average molecular weight of a 17% w/w solution of noradrenaline (NA) plus ATP, molar ratio 3·5:1, containing $CaCl_2$ (molar ratio $CaCl_2/NA = 0{\cdot}08$); additional amines equimolar with NA. 1, No additional amine; 2, dopamine; 3, *p*-tyramine; 4, amphetamine.

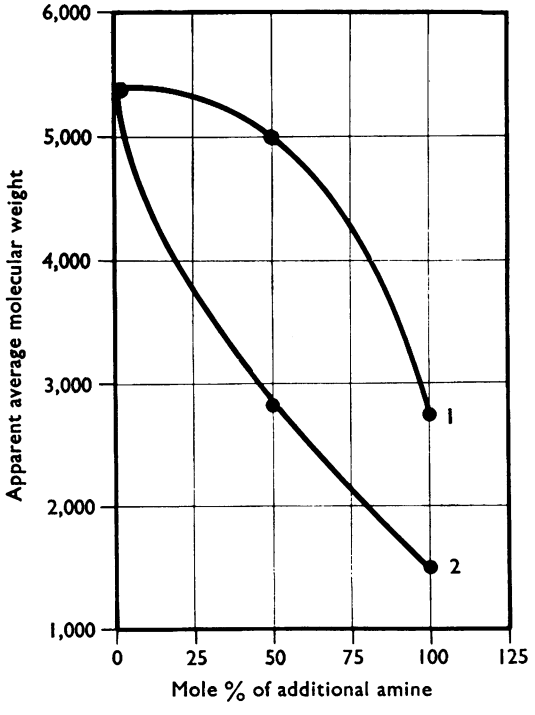

FIG. 4. Effect of various concentrations of added amines on apparent average molecular weights at 10° C of a 17% w/w solution of noradrenaline (NA) plus ATP, molar ratio 3·5:1, containing $CaCl_2$ (molar ratio $CaCl_2/NA = 0{\cdot}08$). Added amines: 1, *p*-tyramine; 2, amphetamine.

cooling, a liquid bottom phase separates which has a smaller volume ($\frac{1}{2}$–$\frac{2}{3}$) than that obtained without reserpine. The composition of the reserpine-containing precipitate is under investigation.

Discussion

The bottom phase which separates from solutions of amines plus ATP in the presence of small amounts of alkaline earth metal ions shows remarkable similarities to the intracellular organelles storing monoamines *in vivo* in the following respects:

(*a*) In both systems the amines are contained in a highly concentrated form. The upper phase *in vitro* and the cytoplasm surrounding the storage organelles *in vivo* contain a smaller concentration of the amines.

(*b*) The amines probably form aggregates of high molecular weights with ATP in both the bottom phase *in vitro* (Fig. 1) and the storage organelles *in vivo* (Berneis *et al.*, 1969a). A molar ratio NA-ATP of about 3·5 was chosen in the present experiments, as this mixture has a pH value of about 6·5 without further additive. *In vivo*, similar (D'Iorio & Eade, 1956) and somewhat higher (Smith, 1968) ratios have been determined.

(*c*) At low temperatures, aggregation between amines and ATP *in vitro* is enhanced, and the exchangeability of the amines in isolated storage organelles is decreased (Berneis *et al.*, 1969 ; Smith, 1968).

(*d*) Bivalent cations in small concentrations enhance the aggregation of NA and ATP and thus the formation of a bottom phase *in vitro*, whereas high concentrations cause dispersion. Bivalent cations also affect the amine storage of the organelles. Thus, Ca and Mg are known to enhance the uptake of catecholamines by isolated storage organelles—for example of the adrenal medulla and the hypothalamus—and Ca is also involved in the release of catecholamines (Iversen, 1967 ; Philippu, Burkat & Becke, 1968).

(*e*) Aromatic amines (for example, tyramine and amphetamine) decrease the apparent molecular weights of aggregates formed by NA and ATP and diminish or eliminate the bottom phase. The drugs exert their maximum action at concentrations about equimolar to those of NA. In organelles storing monoamines, tyramine and amphetamine cause liberation of endogenous amines, whereby one molecule of tyramine seems to displace one or a few molecules of the endogenous monoamines (Schümann & Philippu, 1961 ; Da Prada & Pletscher, 1969a).

It is therefore suggested that the physico-chemical properties of aggregates between amines and ATP are of importance for the storage and release of biogenic monoamines *in vivo*. This does not, of course, exclude other mechanisms such as an active transport of the amines, for example at the level of the cytoplasmic membrane of the amine-containing cells. Furthermore, the *in vitro* two-phase system can represent only a very crude model of the storage organelles as these contain constituents, such as proteins, not present *in vitro*. In this connection, it is of interest that the content of 5-hydroxytryptamine organelles of blood platelets, in contrast to that of the chromaffin granules of the adrenal medulla (Smith, 1968), has only a low protein content (1–3% w/v compared with the 5-hydroxytryptamine content). Further work will show whether proteins and/or other substances are

also involved in the storage of the amines within the organelles and whether their presence *in vivo* may explain some differences found with regard to the *in vitro* model.

The structure of the amine-ATP aggregates as well as the mechanism of action of the bivalent cations and drugs in interfering with the aggregation is not yet known. In analogy to other systems—for example aqueous solutions of purine (Van Holde & Rosetti, 1967)—it may be suggested that amines and ATP form subunits (consisting of several molecules of NA per molecule of ATP) which aggregate by vertical stacking due to Van der Waal's forces (Berneis *et al.*, 1969a ; Pletscher *et al.*, 1969). Other amines, such as dopamine, *p*-tyramine and amphetamine, may be competitively bound to ATP when added to the NA-ATP system and thus replace NA in the subunits. Dopamine would not diminish the bottom phase as the subunits of ATP and dopamine also form aggregates of high molecular weights (Berneis *et al.*, 1969). The subunits containing *p*-tyramine (Berneis *et al.*, 1969) or amphetamine (unpublished results), however, seem to have only a low tendency for aggregation which may explain the disappearance of the bottom phase if either of the two drugs is added to the *in vitro* NA-ATP system.

Reserpine seems to affect phase separation by a yet unknown mechanism. Whether this observation has a bearing on the *in vivo* effect of the drug remains to be elucidated. It is, however, of interest that in the *in vitro* NA-ATP system reserpine acts rather slowly and in low molar concentrations (close to 1/1,000 of those of NA). In biological substrates, too, reserpine has a relatively slow action and is effective in small amounts. Thus, one molecule of the drug has been shown to correspond to several hundreds or thousands of molecules of the "liberated" endogenous amine (Alpers & Shore, 1969 ; Da Prada & Pletscher, 1969b).

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