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DETERMINATION OF THE ISOELECTRIC POINT OF RAT LIVER MITOCHONDRIA BY CROSS-PARTITION

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SUMMARY

The isoelectric points of rat liver mitochondria and mitochondrial membrane fractions determined by two independent methods, cross-partition in sodium salts and isoelectric focusing in Ampholine gradients, are in good agreement The determined isoelectric points are 5 2–5 4 for whole mitochondria, 5 3 for the inner membrane fraction and 4.8–4 9 for the outer membrane fraction This work shows that cross-partition which has previously only been applied to proteins can also be used to determine the isoelectric points of cell organelles and membranes It is suggested that cross-partition in phase systems can be used to study charge and conformational changes in membranes

INTRODUCTION

When aqueous solutions of dextran and polyethyleneglycol are mixed above certain concentrations, liquid two-phase systems are obtained [1, 2] Macromolecules, cells and cell organelles will partition between the two phases and the interface The partition in a two-phase system with a given polymer composition can be changed by introducing different kinds of salts and by changing the pH [1, 2] Albertsson [1] has been able to arrange salts in a series which increases (or decreases) the partition coefficients of all negatively charged materials in the same sequence (The partition coefficient K is defined according to $K = C_T/C_B$ where C_T and C_B are the concentrations of the material in the top and bottom phases, respectively) Walter et al [3] showed the reverse effect of the salt series on the partition of positively charged materials This phenomenon is due to unequal distribution of the salt ions [4] between the two liquid phases A potential difference between the phases is thus created and the distribution of a substance will be charge-dependent [1] If the pH-dependent partition of an amphoteric material is studied in two series of phase systems containing two different salts, a defined cross-point will be obtained [2] This cross-point or iso-partition point occurs at the pH at which the partition coefficient of the material is equal in the two series of salts.

It has been shown for proteins that these cross-points have values close to the isoelectric points [5, 6]

The work described in this communication shows that cross-points can be obtained for cell organelles, such as mitochondria, and for mitochondrial membrane.

EXPERIMENTAL

Materials

Dextran 500, Batch 5996, molecular weight (M_w) = 500 000, was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden and polyethyleneglycol (PEG), grade Carbowax 4000, molecular weight (M_n) = 3000–3700, was supplied by Union Carbide Chemicals, New York, U S.A.

Ampholine pH 3–6 was from LKB-Produkter AB, Bromma, Sweden

All other chemicals used were of analytical grade.

The water was double distilled in quartz.

Partition in the phase systems

The phase systems used were prepared by weighing out:

(A), 1 50 g of 20 % (w/w) Dextran, 0.75 g of 40 % (w/w) polyethyleneglycol 4000, 0.50 g of 0.05 M citrate–phosphate buffer, 0.44 g of sucrose 0.81 g of water and 0.50 g of 0 5 M Na_2SO_4 (Test Series A1) or 0 50 g of 1.0 M NaCl (Test Series A2)

(B), 2.0 g of 20 % (w/w) Dextran, 1.0 g of 40 % (w/w) polyethyleneglycol 4000 0.10 g of citrate–phosphate buffer, 0.44 g of sucrose, 0.81 g of water and 0 50 g of 0.5 M K_2SO_4 (Test Series B1) or 0.50 g of 1 0 M KCl (Test Series B2). The pH of the buffer varied between 3.0 and 7.0 in all test series.

After temperature equilibration at +2 °C, 0.50 ml of mitochondria in 0.32 M sucrose was added to each tube in the test series except the blanks, to which 0 50 ml of 0 32 M sucrose was added. One blank was prepared for each polymer composition. The final phase systems had the following compositions in addition to mitochondria. Concentrations are expressed as mmoles per kg phase system

Systems to Test Series A1. 6 % (w/w) Dextran, 6 % (w/w) polyethyleneglycol 4000, 320 mmoles sucrose/kg, 5 mmoles citrate–phosphate buffer /kg and 50 mmoles Na_2SO_4/kg.

Systems to Test Series A2 The same composition as in Test Series A1 but 100 mmoles NaCl/kg instead of 50 mmoles Na_2SO_4/kg.

Systems to Test Series B1. 8 % (w/w) Dextran, 8 % (w/w) polyethyleneglycol 4000, 320 mmoles sucrose/kg, 1 mmole citrate–phosphate buffer/kg and 50 mmoles K_2SO_4/kg

System to Test Series B2. The same composition as in Test Series B1 but 100 mmoles KCl/kg instead of 50 mmoles K_2SO_4/kg

The phase systems were thoroughly mixed by inverting the test tubes. After phase separation, 1 0 ml of top phase and 1.0 ml of bottom phase were carefully pipetted from the system in each tube, and each was diluted with 2.0 ml of 0.32 M sucrose The absorbance at 520 nm was measured against a top or bottom phase blank The remaining phase systems were broken by the addition of 2.0 ml of water to each tube and the pH was measured.

The rat liver mitochondria used were prepared according to the method of Schneider and Hogeboom [7] and the inner and outer membrane fractions were prepared according to Sottocasa et al [8].

The microsomal contamination of the mitochondrial preparation was 10–15 % based on measurements of the glucose-6-phosphatase activity The preparations of inner and outer membrane fractions were examined in the electron microscope

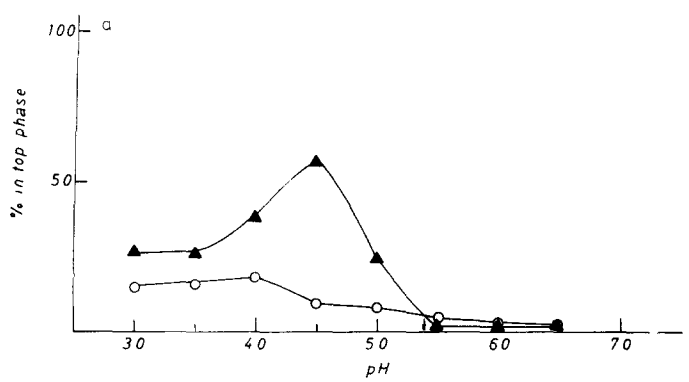
Isoelectric focusing

Isoelectric focusing was done in a glass U tube (inner diameter 0.7 mm, length of arms 12 cm) with an electrode vessel on top of each arm The bottom of the tube was filled with 2.0 ml of 40 % (w/v) sucrose in 0 01 M NaOH and a linear gradient (4 0 ml) from 5 to 40 % (w/v) sucrose with 1 % (w/v) Ampholine was added to the left arm of the U-tube 10 ml of 22 5 % (w/v) sucrose in 0 01 M NaOH was added to the right arm and electrode vessel (cathode) of the tube and 1 % (w/v) H_3PO_4 was layered above the sucrose gradient and in the left electrode vessel (anode) until hydrostatic equilibrium was reached After a pH gradient had been obtained by electrophoresis for 3–5 h at 200 V, the sample in 0 7 M sucrose was carefully added to the gradient and electrophoresis was run at 400 V for 3–16 h Migration was followed by a graduated scale The electrophoresis was stopped when migration had ceased In that way aggregation and concomitant sedimentation of particles during concentration into narrow bands was minimized Fractions of 10 droplets each were collected by means of an outlet under the gradient, and the absorbance at 520 nm or 280 nm and the pH were measured. The construction of the U tube and the pH gradient were checked by running ovalbumin and pig hemoglobin All runs were performed in a waterbath at +2 °C

RESULTS

Partition in two-phase systems

The partition of a substance in a two-phase system can either be expressed as the partition coefficient, as stated in the introduction or as the quantity of substance distributed to the top phase or bottom phase, expressed as percentage of the total quantity of substance in the system. Although there generally is adsorption of particles at the interface, essentially the same cross-point values will be obtained regardless of whether the partition coefficient or the percentage in top phase is used, as illustrated in Figs 1a and 1b



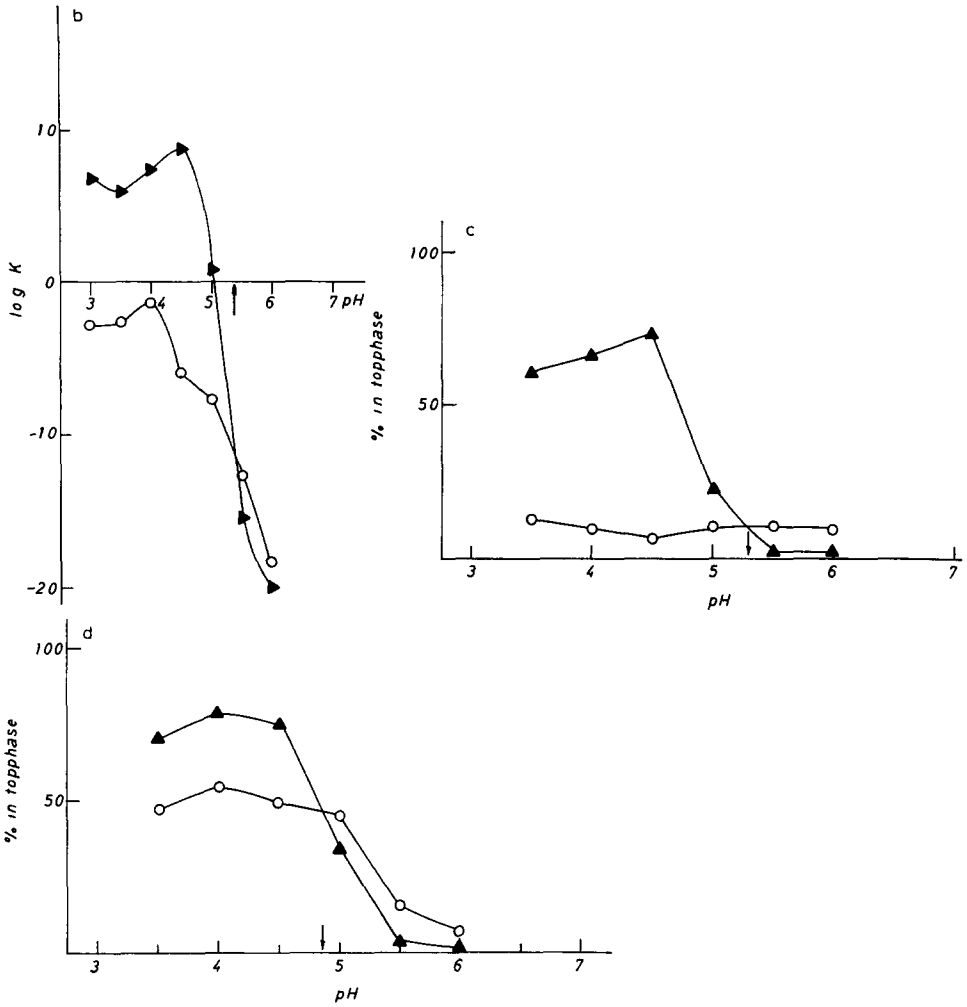


Fig 1 Distribution of rat liver mitochondria and mitochondrial membranes as a function of pH in the systems 6 % (w/w) Dextran 500, 6 % (w/w) polyethyleneglycol 4000, 5 mmoles citrate–phosphate buffer/kg, 320 mmoles sucrose/kg and 50 mmoles Na_2SO_4/kg (○) or 100 mmoles NaCl/kg (▲) The quantity of mitochondria or mitochondrial membranes in the top phase is expressed in a, c and d as percentage of the total quantity in the system The distribution in b is expressed as the partition coefficient K ($K = C_T/C_B$ where C_T and C_B are the concentrations of mitochondria in the top phase and bottom phase respectively). (a) and (b) Whole mitochondria (c) Inner membrane fraction (d) Outer membrane fraction

Fig. 1 shows that defined cross-points could be obtained for the mitochondrial material studied. The inner membrane fraction had the same cross-point as the whole mitochondria, while the outer membrane fraction had a lower value If potassium salts are used instead of sodium salts, lower cross-point values will be obtained (Table I). The difference between inner and outer membrane fractions however, remains Although the levels of particles in the top phases are low for pH $>$ 5 5 the obtained values on the cross-points were reproducible within the range of ± 0.1 pH units.

TABLE I

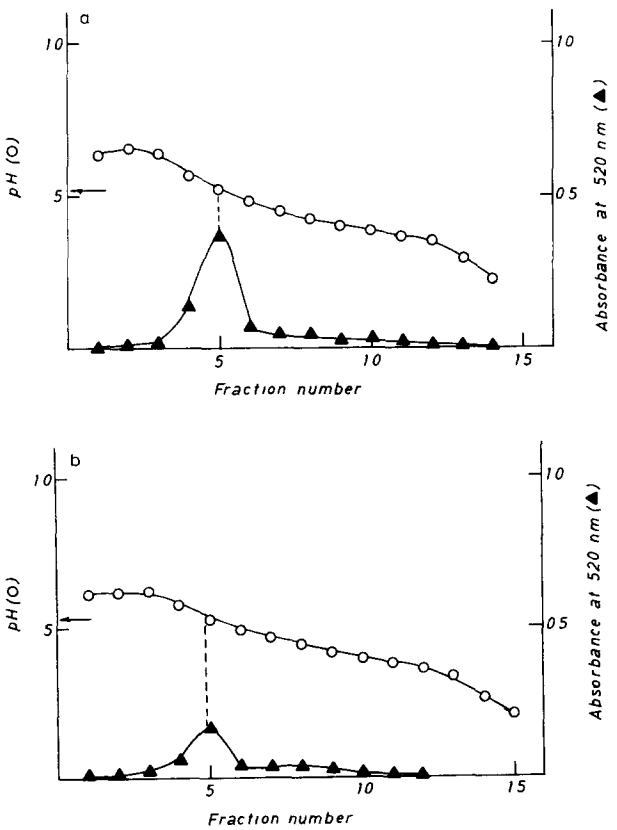
CROSS-POINTS AND ISOELECTRIC POINTS FOR RAT LIVER MITOCHONDRIA AND MITOCHONDRIAL MEMBRANES

For composition of System A and B see Experimental, The isoelectric points were obtained by isoelectric focusing

	Cross-point (pH)		Isoelectric point (pH)
	System A	System B	
Rat liver mitochondria	5 4	4 8	5 2
Inner membrane fraction	5 3	4 9	5 3
Outer membrane fraction	4 9	4 6	4 8

Isoelectric focusing

Electrophoresis of the mitochondrial materials in Ampholine gradients gave reproducible isoelectric points (Fig. 2) which agreed well with the cross-point obtained in systems containing sodium salts (Table I) These isoelectric points were essentially the same whether the electrophoresis was run for 6 or 16 h or whether the pH gradient was between pH 3 and 6 or between pH 3 and 10



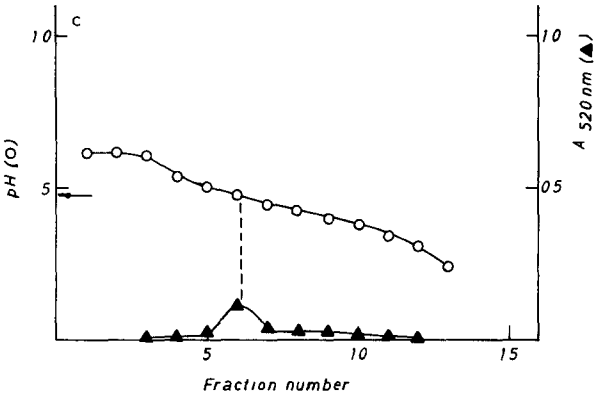


Fig 2 Isoelectric focusing of rat liver mitochondria and mitochondrial membranes in an Ampholine gradient pH 3–6 The volume of each fraction was ten droplets, corresponding to 0 4 ml. (a) Whole mitochondria, electrophoresis was run for 16 h (b) Inner membrane fraction, electrophoresis was run for 14 h (c) Outer membrane fraction, electrophoresis was run for 16 h

DISCUSSION

It has previously been shown that if the partition of a protein in two series of two-phase systems with a given polymer composition but containing two different alkali salts (such as NaCl and Na_2SO_4) is plotted against the pH, the two lines will intersect at a defined point, the cross-point. This point has been shown to be close to the isoelectric point of the protein [5, 6].

In this work it has been possible to obtain defined values on the pH scale at which a membrane material will show cross-partition The cross-points observed in the presence of sodium salts agree well with isoelectric points obtained by electrophoresis in Ampholine gradients (Table I). There is however, a discrepancy in regard to the type of cation used in the phase systems. As seen in Table I, the systems containing K^+ gave consistently lower cross-point values. (The differences in the polymer concentrations are of relatively little importance in comparison with the difference in cation) Cation effect was also found for proteins [6]. The lower cross-point values obtained in the presence of potassium salt may reflect differences in the interaction between the cations and the membranes These interactions may cause conformational changes which alter the charge distributions on the surfaces exposed to the polymeric phases. Lower values on the isoelectric point of rat liver mitochondria were obtained by Plummer [9] and Katyare et al. [10] With a microscope they observed the electrophoretic mobility of mitochondria at different pH and obtained isoelectric points at pH 4 4 and 4 8, respectively The higher values obtained in this work may, however, be explained by experimental differences, such as different ionic media

The cross-point values obtained suggest that the surfaces of the membranes exposed to the phases are essentially covered with only slightly acidic molecules, probably mainly proteins. Pure rat liver mitochondria contain three major phospholipids: phosphatidylcholine, phosphatidylethanolamine and cardiolipin Cardiolipin occurs mainly in the inner mitochondrial membrane. It is not likely that cardiolipin is much exposed to the phases because a lower value on the cross-point

would then be obtained for the inner membrane To what extent neutral lipids, phosphatidylcholine and phosphatidyletanolamine are exposed at the surfaces are difficult to judge from the obtained cross-points. This is true of both the inner and the outer membrane On the other hand, it can be seen in Fig 1 that the outer and inner membranes have different partitions as a function of pH, which suggests that there are surface differences It is also well known that there are large differences between the inner and outer membranes with respect to lipid composition and lipid–protein ratios [11] It is interesting that the cross-point for mitochondria is the same as that for the inner membrane fraction but different from that of the outer membrane fraction It has, however, been shown by counter-current distribution technique that both the whole mitochondria preparation and the inner membrane fraction are heterogenous Whether the cross-points obtained represent mean values of different populations is unknown At present it is not clear which surface or surfaces determine the partition of the membranes The polyethyleneglycol molecules are likely to interact with the outer and inner surfaces of the outer membrane and the outer surface of the inner membrane of the mitochondria, while the large dextran molecules probably interact mainly with the outer surface of the outer membrane It is also not clear which surfaces are exposed to the medium when inner and outer membranes are prepared

Investigations are in progress to discover how structure and metabolic activities of the mitochondria are altered in the vicinity of the cross-point An advantage of cross-partition over isoelectric focusing is that cross-partition studies require less than 15 min to perform and the actual equilibrium requires only a few seconds Negative effects of the low and unphysiological pH will thus be minimized Another advantage is the greater ease of adjusting the phase medium with respect to salt composition, metabolically active substances and osmotic pressure

Since different preparative methods will yield mitochondria that differ with respect to adsorbed and released molecules on the membranes, it is likely that the cross-points will be found to vary It would be of interest to see whether changes in the charge and conformation of the membranes induced by metabolically active substances can be described by changes in the cross-points It has been shown (to be published) that the presence of substrates and inhibitors in the phase systems change the partition of mitochondria

Studies with proteins in these phase systems [12] have demonstrated that the logarithm of the partition coefficient is a linear function of net charge of the protein If the same holds for macromolecular complexes it may be possible to determine net charge of membrane particles It would also be of interest to compare the cross-points of various membranes with their chemical compositions

REFERENCES

1 Albertsson, P -Å (1971) Partition of Cell Particles and Macromolecules, 2nd Edn, Wiley-Interscience, New York
2 Albertsson, P -Å (1970) Adv Protein Chem 24, 309–341
3 Walter, H , Garza, R and Coyle, R P (1968) Biochim Biophys Acta 156, 409–411
4 Johansson, G. (1970) Biochim Biophys Acta 221, 387–390
5 Albertsson, P -Å , Sasakawa, S and Walter, H (1970) Nature 228, 1329–1330
6 Walter, H , Sasakawa, S and Albertsson, P -Å (1972) Biochemistry, 11, 3880–3883

7 Schneider, W C and Hogeboom, G. H (1950) J Biol Chem , 183, 123–128
8 Sottocasa, G L., Kuylenstierna, B., Ernster, L. and Bergstrand, A (1967) J Cell Biol 32, 415–418
9 Plummer, D. T. (1965) Biochem J. 96, 729–732
10 Katyare, S S , Fatterpacker, P. and Sreenivasan, A. (1970) Biochem. J. 118, 111–121
11 Ernster, L and Kuylenstierna, B (1970) in Membranes of Mitochondria and Chloroplasts (Racker, E , ed) pp 191–192, Van Nostrand Reinhold Co, New York
12 Johansson, G (1973) Thesis, Umeå University