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INFLUENCE OF THE CONFORMATIONAL STATE ON THE ISOELECTRIC POINTS OF RAT BRAIN SYNAPTOSOMES, MITOCHONDRIA AND MITOPLASTS

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The isoelectric points of rat brain synaptosomes, mitochondria and mitoplasts have been determined by using different charged two-phase systems containing dextran and poly(ethylene glycol). The cross-partition diagrams of these organelles show isoelectric points at pH 4.1, 4.5 and 4.7, respectively. The influence of the conformational state of mitochondrial membranes upon their partition in two-phase systems has been studied. Shrunk mitoplasts showed a large change in their partition behavior as reflected by an increased affinity for the lower dextran phase, while shrinkage of mitochondria did not affect their partition. Shrunk mitoplasts showed the same isoelectric point of pH 4.7 as swollen mitoplasts, which indicates that no charge changes occurred on the outer side of the inner mitochondrial membrane during shrinkage of mitoplasts.

Introduction

It is well stablished that the salt composition determines the electrostatic potential between the phases of aqueous dextran-poly(ethylene glycol) two-phase systems [1,2]. Therefore, the partition of particles in appropriate salt-containing phase systems is dependent on their surface charge. The isoelectric point of particles can thus be determined if the partition on dextran-poly(ethylene glycol) two-phase systems is studied as a function of pH with two different salt media. If the partition percent of the particles in the upper phase is plotted against the pH, two curves can be obtained that cross each other in the isopartition point. This cross (isopartition) point occurs at the pH at which the partition is independent of the electrostetic potential difference between the phases. It has been shown that there is significant agreement between the isopartition point determined in aqueous dextran-poly(ethylene glycol) two-phase systems and the isoelectric point of particles obtained by other methods [3–5].

Reversible structural modifications accompany change in the metabolic steady-state of mitochondria, and such modifications have been interpreted as small changes in total mitochondrial size resulting from energy-linked swelling-contraction phenomena [6–10]. Since the partition of a particle in a two-phase system is based on its surface properties, it was of interest to study the effect of the conformational state of the mitochondrial membrane on the partition of these organelles in dextran-poly(ethylene glycol) two-phase systems.

Materials and Methods

Dextran T_{40}(lot No. 4987) and T_{500}(lot No. GI21917) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Poly(ethylene glycol) 4000 was obtained from Sigma Chemical Co, St Louis.

Preparation of synaptosomes

12 forebrains from male rats weighing 150–200 g were finely chopped with scissors and washed

with ice-cold isolation medium to remove as much blood as possible. The chopped brain tissue was resuspended in 10 vol. of 0.32 M sucrose/1 mM EDTA/1 mM Tris-HCl (pH 7.4) (medium A) and homogenized with a Dounce homogenizer fitted with a Teflon pestle at 400 rev./min with five up-down strokes. The homogenate was centrifuged at $1100 \times g$ for 10 min. The supernatant was further centrifuged at $19000 \times g$ for 20 min. The pellet was resuspended in 5 ml of medium A and layered on top of 20 ml of 6% Ficoll (w/w) in medium A and centrifuged at $19000 \times g$ for 30 min. The supernatant solution was removed and discarded. The fluffy layer was carefully collected and designated 'crude synaptosomes'. This fluffy layer was diluted with 30 ml of medium B, consisting of 0.32 M sorbitol/0.1 mM EDTA/5 mM potassium phosphate (pH 7.8), and re-centrifuged at $19000 \times g$ for 30 min. The pellet was resuspended in 2 ml of medium B. Purification of synaptosomes by a batch procedure of phase partition was carried out with the same composition of phase as previously described [11]. After partition synaptosomes were obtained from the upper phase. Further washing of the upper phase with freshly prepared lower phase was carried out. The upper phase was diluted with medium B, centrifuged and the pellet resuspended in medium B as described before [11].

Preparation of mitochondria and mitoplasts

Mitochondria from rat brain were obtained as previously described [11], except for the salt composition of medium B and the phase system, which contained 5 mM ammonium hydroxide/phosphoric acid buffer, pH 7.8, instead of 5 mM potassium phosphate buffer, pH 7.8. 5 mM ammonium chloride was added to the phase system to adjust the partition of mitochondria to the lower phase.

Mitoplasts were obtained by treatment of pure mitochondria with digitonin (0.4 mg/mg protein) at 0 °C for 15 min with gentle shaking every 3 min. The mixture was diluted with 10 ml of medium B and mitoplasts were collected by centrifugation at $19000 \times g$ for 10 min.

Determination of isoelectric points

Two series of phase systems with different salt composition were prepared. The final concentrations in both series of 2 g systems after addition of 0.25 ml samples were: 6.4% (w/w) dextran T_{500}, 6.4% (w/v) poly(ethylene glycol) 4000, 0.1 mM EDTA, 0.32 M sorbitol with either 15 mM Tris-phosphate buffer (initial pH 7.8) or 5 mM Tris-phosphate buffer (initial pH 7.8) plus 15 mM KCl. The pH values of the phase systems were adjusted to 3.5, 4.0, 4.5, 5.0 and 5.5, respectively, with 0.1 M citric acid before addition of sample. Other polymer concentrations were used as indicated.

Samples of mitochondria, synaptosomes and mitoplasts were prepared by dilution of the pure organelles with 20 ml of medium B containing the same salt composition as the corresponding phase system and adjusted to the corresponding pH value with 0.1 M citric acid. The suspensions were centrifuged at $19000 \times g$ for 20 min, and the pellet was resuspended in medium B.

After the addition of samples, the phase systems were mixed by 20 inversions of the tubes and allowed to separate for 20 min. All partition work was performed at 4 °C. The upper phase was carefully removed with a Pasteur pipette and the two phases were diluted with an equal volume of water. The amount of material in the top phase was calculated from the absorbance at 280 nm or from enzyme activities in diluted samples.

Enzyme assays

Acetylcholinesterase was assayed according to Ellman et al. [12], cytochrome *c* oxidase according to Turner [13], and fumarase according to Racker [14]. Mitochondrial protein was determined by the biuret method using bovine serum albumin as standard [15].

Electron microscopy

The suspended mitochondria, synaptosomes and mitoplasts were fixed with 1% glutaraldehyde. After 2 h, the samples were centrifuged at $9000 \times g$ for 5 min, and the pellet was washed with Millonig medium [16], postfixed with 2% OsO_4 in Millonig medium, and then dehybrated stepwise with ethanol and propylene oxide and embedded in Epon. Sections were cut on an LKB ultramicrotome, and stained with uranyl acetate and lead citrate.

Results and Discussion

Synaptosomes purified with a potassium-containing two-phase system showed a high degree of purity (Fig. 1A). Fig. 1B shows an electron micrograph of mitochondria isolated with an ammonium-containing two-phase system. The preparation contains pure mitochondria without any synaptosomal contamination. Further confirmation of the purity of the mitochondria was obtained from their low enzymatic activities of lactate dehydrogenase (7 ± 1 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) and acetylcholinesterase (4 ± 1 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}), markers of synaptosomal contamination. The enzymatic activities of cytochrome *c* oxidase (501 ± 70 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) and fumarase (454 ± 100 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) of this mitochondrial preparation are also in agreement with the results already found in mitochondria prepared with potassium-containing two-phase systems [11]. The respiratory activities of mitochondria obtained with ammonium-containing phase systems are summarized in Table I. This mitochondrial preparation showed a significant stimulation of the state-3 rates by high potassium concentration in the medium. This effect was previously described in metabolically active mitochondria, purified by

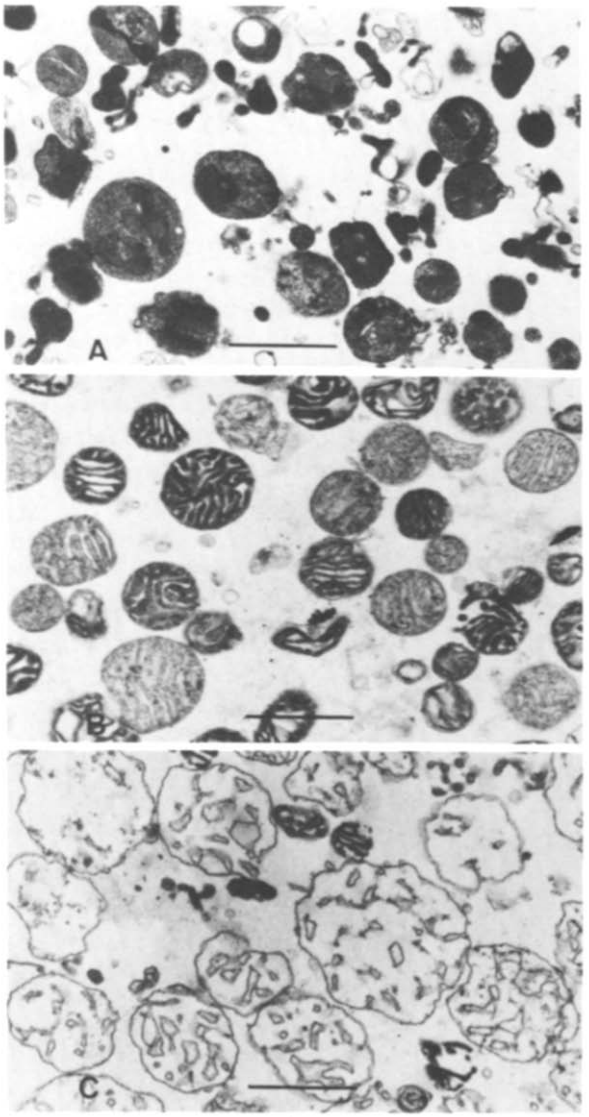


Fig. 1. Electron micrographs of rat brain synaptosomes (A), mitochondria (B) and mitoplasts (C). The bars represent 1.0 μm.

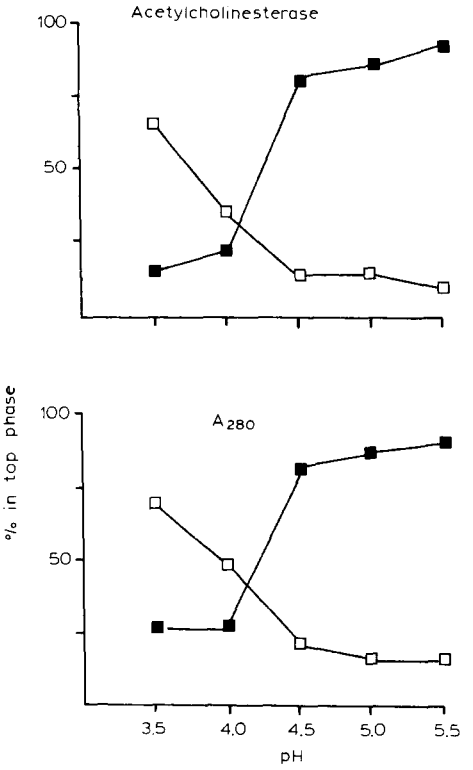


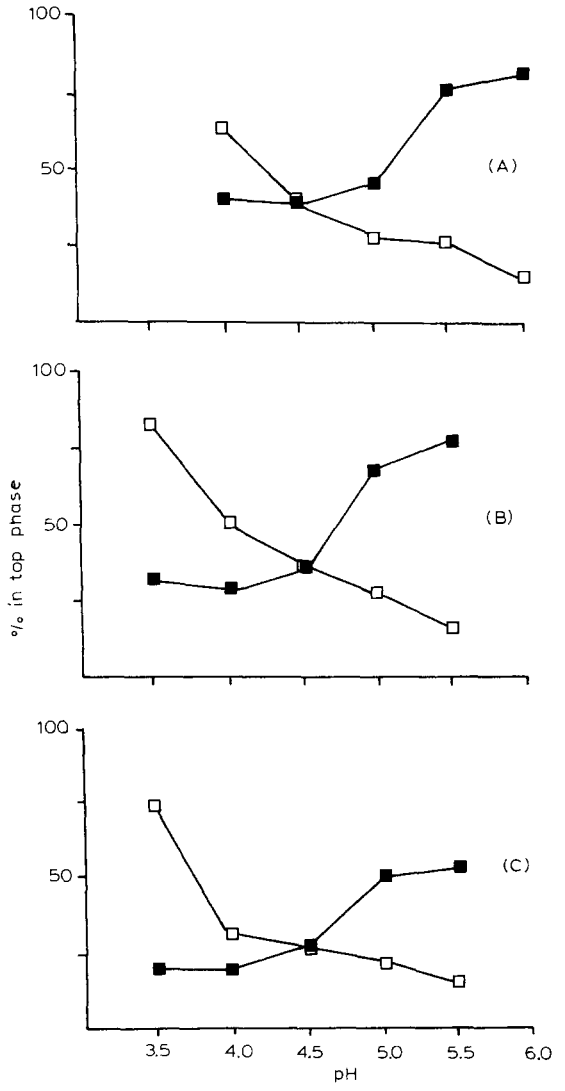
Fig. 2. Isoelectric point of rat brain synaptosomes determined by cross-partition in 6.4–6.4% (w/w) dextran-poly(ethylene glycol) two-phase systems. ■———■, phase systems containing 15 mM Tris-phosphoric acid buffer; □———□, phase systems containing 5 mM Tris-phosphoric acid buffer plus 15 mM KCl.

TABLE I

RESPIRATORY ACTIVITIES OF MITOCHONDRIA OBTAINED WITH AMMONIUM-CONTAINING TWO-PHASE SYSTEMS

Respiration was measured polarographically at 25 °C. Incubation media containing 5 mM or 100 mM KCl were used according to the method of Lai and Clark [17]. State 3 was induced by addition of ADP and calculations were carried out according to Chance and Williams [18]. State 3 and state 4 rates are expressed as natoms O/min per mg protein. Except in the case of succinate the experiments were carried out in the presence of 2.5 mM malate. ADP/O ratios and respiratory control ratios (RCR) are the average of four determinations on sucessive additions of ADP.

	KCl	ADP/O	State 4	State 3	RCR
10 mM succinate	5	1.9	27± 6	112±19	4.1
	100	1.8	59±10	149±10	2.5
5 mM glutamate	5	2.8	12± 3	63±20	5.2
	100	2.7	32± 3	87±10	2.7
5 mM pyruvate	5	2.9	17± 3	74±12	4.3
	100	2.9	63± 6	134±18	2.1



Ficoll gradients [17], but was not observed in highly purified mitochondria prepared by potassium-containing phase-systems [11]. Since the target of this work required good metabolic integrity of the organelles we have used in all the experiments mitochondria purified by phase systems containing ammonium instead of potassium salts. Mitoplasts were essentially free of outer mitochondrial membrane (Fig. 1C).

Fig. 2 shows the cross-partition of total protein and acetylcholinesterase activity of synaptosomes. Both cross-partition diagrams show an isoelectric point at pH 4.1, and agree well also in other respects, indicating that the preparation of synaptosomes was pure. The isoelectric pH of mitochondria was obtained using three different phase systems, all with the same salt composition as described above, but with different polymer concentrations; Fig. 3. In all these cases, the isoelectric point was at pH 4.5 showing that the cross-point was independent of the polymer concentration. The isoelectric point of mitoplasts was found to be at pH 4.7 (Fig. 4), i.e., slightly higher than for intact mitochondria.

In previous works, Ericson [3] and Lundberg and Ericson [19] using two-phase systems, re-

Fig. 3. Isoelectric point of rat brain mitochondria as determined by cross-partition using phase systems of different polymer concentrations. ■———■, phase systems containing 15 mM Tris-phosphoric acid buffer; □———□, phase systems containing 5 mM Tris-phosphoric acid buffer plus 15 mM KCl. A, 6.2–6.2%; B, 6.4–6.4%; C, 6.8–6.8%.

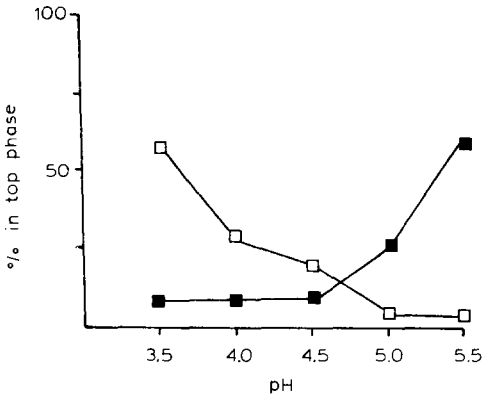


Fig. 4. Isoelectric point of rat brain mitoplasts determined by cross-partition in 6.2–6.2% (w/w) dextran-poly(ethylene glycol) two-phase system. ■———■, phase systems containing 15 mM Tris-phosphoric acid buffer; □———□, phase systems containing 5 mM Tris-phosphoric acid buffer plus 15 mM KCl.

ported isoelectric points of 5.3 and 5.6, respectively, for liver mitochondria. The isoelectric point of brain mitochondria is markedly different to that obtained with liver mitochondria by these authors. These different results could be explained by differences in purity and origin of the mitochondrial preparations.

Fig. 5 shows the distribution of mitochondria and mitoplasts in a two-phase system as a function of polymer concentration. Since the isoelectric points of these particles are quite similar, the differences observed in the partition may be explained by the different lipidic content and composition of the inner and the outer mitochondrial membranes [20–22].

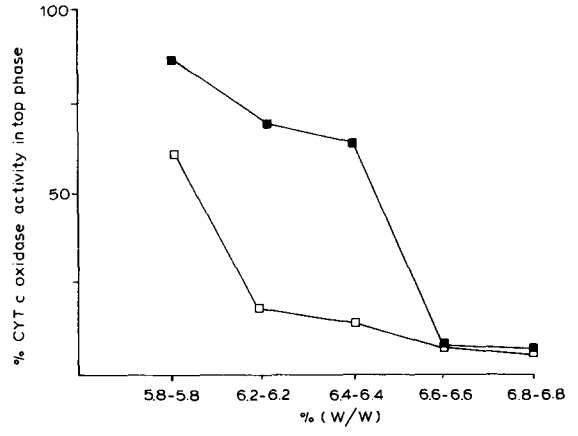


Fig. 5. Partition of rat brain mitochondria and mitoplasts in two-phase systems as a function of polymer concentration. ■———■, mitochondria; □———□, mitoplasts.

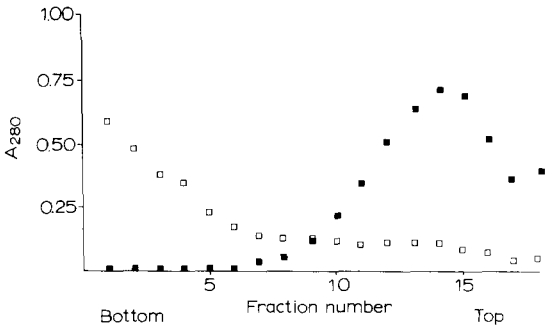


Fig. 6. Sedimentation of rat brain mitochondria treated with sodium dithionite in iso-osmotic gradients of dextran T_{40}. ■———■, untreated mitochondria; □———□, mitochondria treated with 5 mM sodium dithionite to induce irreversible shrinkage. Sodium dithionite was removed by adding 20 ml of medium B and centrifugation at 19000 × *g* for 10 min. Treated mitochondria were layered on a continuous (0–20% w/w) dextran T_{40} gradient prepared in a medium containing 0.45 M sucrose, 0.1 mM EDTA and 5 mM ammonium hydroxyide/phosphoric acid buffer, pH 7.8. Centrifugation was carried out at 23000 × *g* for 10 min and 1-ml fractions were collected with a peristaltic pump.

TABLE II

EFFECTS OF IRREVERSIBLE SHRINKAGE OF RAT BRAIN MITOPLASTS AND MITOCHONDRIA ON THEIR PARTITION IN DEXTRAN-POLY(ETHYLENE GLYCOL) TWO-PHASE SYSTEMS

Mitoplasts and mitochondria were treated with 5 mM sodium dithionite. After shrinkage (monitored by the absorbance change at 600 nm; data not shown) sodium dithionite was removed as described in the legend for Fig. 6. The particles were resuspended in medium B and the partition of mitoplasts and mitochondria was carried out, as previously described in Materials and Methods, in phase systems containing 5.8–5.8 and 6.2–6.2% (w/w) dextran-poly(ethylene glycol), respectively.

	% in top phase		
	A_{280}	Cyt. *c* oxidase	Fumarase
Mitoplasts			
Control	81	80	79
Shrunk	26	29	28
Mitochondria			
Control	54	50	55
Shrunk	57	49	54

In order to establish the influence of the conformational state of mitochondrial membrane upon their partition in two-phase systems we studied the partition of shrunk mitoplasts and mitochondria. Shrinkage of mitoplasts and mitochondria was produced by addition of sodium dithionite, as deduced by the absorbance changes which occurred (data not shown). However, after induction of the shrinkage, dithionite had to be removed to avoid interference of this salt in the partition of the organelles. To prove that the induced shrinkage of the organelles was irreversible and remained after the elimination of the dithionite, we determined their conformational state by testing the sedimentation patterns of control and dithionite-treated mitochondria in iso-osmotic gradients of dextran T_{40}. As shown in Fig. 6, mitochondria treated with sodium dithionite appeared in the bottom of the gradient, while untreated mitochondria remained at the top.

Shrunk mitoplasts showed a large change in their partition by increasing the affinity for the lower dextran phase. On the other hand, shrinkage of mitochondria did not affect their their partition (Table II). In order to establish whether the charge changes of the inner membrane of mitochondria during shrinkage were responsible for the change in the partition of the mitoplasts, we determined the isoelectric points of shrunk mitoplasts and found the same isoelectric point of pH 4.7 (Fig. 7)

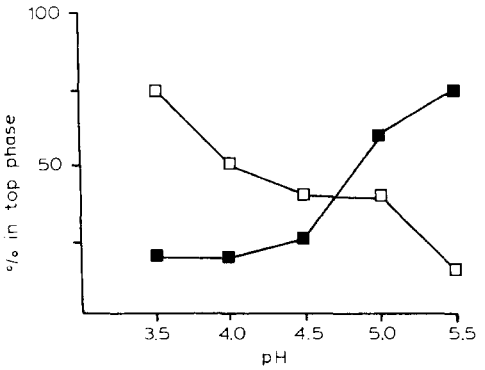


Fig. 7. Isoelectric point of shrunk rat brain mitoplasts as determined by cross-partition in 6.2–6.2% (w/w) dextran-poly(ethylene glycol) two-phase systems. ■———■, phase systems containing 15 mM Tris-phosphoric acid buffer; □———□, phase systems containing 5 mM Tris-phosphoric acid buffer plus 15 mM KCl. Mitoplasts were shrunk with 5 mM sodium dithionite and the salt was removed as described in the legend for Fig. 6.

as found earlier for untreated mitoplasts. Lundberg and Ericson [19] found that the isoloelectric point of inside-out submitochondrial particles decreased after addition of ATP, suggesting a charge change at the inner side of the inner mitochondrial membrane during shrinkage. The results presented here indicate that no such charge change occurred on the outer side of the inner mitochondrial membrane.

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References

1 Albertsson, P.A. (1971) Partition of Cell Particles and Macromolecules, 2nd Edn., Almgrist and Wiksell, Stockholm, and Wiley New York
2 Larsson, C. and Andersson, D. (1979) in Plant Organelles, Methodological Surveys (B), Biochemistry, Vol. 9 (Reid, E., ed.) pp. 35–46, Ellis Horwood, Chichester
3 Ericson, I. (1974) Biochim. Biophys. Acta 356; 100–107
4 Blomquist, G. (1976) Biochim. Biophys. Acta 420, 81–86
5 Akerlund, H.E., Andersson, B. Persson, A. and Albertson, P.A. (1979) Biochim. Biophys. Acta 552, 2238–2246
6 Chance, B. and Packer, L. (1958) Biochem. J. 68, 295–297
7 Packer, L. (1960) J. Biol. Chem. 235, 1, 242–249
8 Packer, L. (1961) J. Biol. Chem. 236, 1, 214–220
9 Packer, L. (1962) J. Biol. Chem. 237, 4, 1327–1331
10 Azzi, A. and Azzone, G.B. (1965) Biochim. Biophys. Acta 105, 265
11 López-Pérez, M.J., París, G. and Larsson, C. (1981) Biochim. Biophys. Acta 635, 359–368
12 Ellmann, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
13 Turner, G. (1973) Eur. J. Biochem. 40, 201–206
14 Racker, E. (1950) Biochim. Biophys. Acta 4, 211–214
15 Gornall, A.G., Bardewill, C.S. and Davis, M.M. (1949) J. Biol. Chem. 177, 751–766
16 Milloning, G. (1961) J. Biophys. Biochem. Cytol. 11, 736
17 Lai, J.C.K. and Clark, J.B. (1976) Biochem. J. 154, 423–432
18 Chance, B. and Williams, G.K. (1972) Adv. Enzymol. 17, 65–134
19 Lundberg, P. and Ericson, I. (1975) Biochim. Biophys. Res. Commun. 65 (2), 530–536
20 Parsons, D.F. and Yano, Y. (1967) Biochim. Biophys. Acta 135, 362–364
21 Stoffel, W. and Schiefer, H.G. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1017–1026
22 Parkes, J.G. and Thompson, W. (1970) Biochim. Biophys. Acta 196, 162–169