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OXONOL-V AS A PROBE OF CHROMAFFIN GRANULE MEMBRANE POTENTIALS

D. SCHERMAN and J.P. HENRY

Service de Biochimie-Physique, Institut de Biologie Physico-chimique, 13, rue Pierre et Marie Curie, 75005 Paris (France)

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Summary

The dye, oxonol-V (bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol), can be used to estimate the transmembrane potential of chromaffin granules. The potentials result either from a resting-state Donnan equilibrium (inside negative at pH 6.6) or from an ATP-driven proton pump. The fluorescence and absorption changes generated by ATP addition depended on the pH of the medium and the dye-to-vesicle ratio. Energization resulted in an increase in the number of oxonol-V binding sites, the new binding sites having the same dissociation constant. The rate of dye association was higher with resting than with energized chromaffin granules. The absorption change was associated with a red shift whereas the fluorescence change involved a quenching due to the increase in dye concentration on the membrane. The absorption and fluorescence changes varied linearly with the transmembrane potential difference when the interior potential was positive relative to the medium.

Introduction

The transmembrane potential of organelles which cannot be penetrated with microelectrodes can be estimated using dyes [1—3]. Absorption and fluorescence spectra as well as fluorescence polarization and dichroic effects have been used [2,4]. The molecular mechanisms of the phenomena are not fully

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Abbreviations: TPMP$^+$, triphenylmethylphosphonium cation; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

understood and may vary with the system and with the dye utilized [5—10]. The anionic dye, oxonol-V (bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol), has been used in *Rhodospirillum rubrum* chromatophores and submitochondrial particles and has been shown to undergo a red shift when an inside positive potential was developed by K^+ influx, light or substrate consumption [11]. Pigeon heart mitochondria showed a blue shift on negative potential change following K^+ efflux or substrate consumption. Fluorescence emission changes were dependent upon the membrane system used and the method of potential generation.

Chromaffin granules have been shown to accumulate catecholamines by an ATP-dependent reserpine-sensitive mechanism which involves inward proton translocation [12—14]. The electrogenic ATP-dependent proton pump polarizes positively the granules the membrane of which is impermeant to protons [15, 16]. A probe monitoring instantaneous transmembrane potential changes would be a useful tool in the study of catecholamine transport. In this communication oxonol-V has been added to chromaffin granules. The absorption and fluorescence changes have been correlated with the transmembrane potential and mode of action of the probe investigated.

Materials and Methods

Granule preparation. Bovine chromaffin granules were purified either by differential centrifugation [15] or by isopycnic centrifugation on isotonic metrizamide/sucrose solutions according to the method of Morris and Schovanka [17]. In the latter case, crude granules (2.4 ml) were loaded onto a layer (2.8 ml) of a 58% metrizamide (300 mosM, $\eta_D^{20} = 1.389$)/42% sucrose (300 mosM, $\eta_D^{20} = 1.346$) mixture (v/v) and subjected to centrifugation at 50 000 rev./min for 60 min in an SW65 Beckman rotor. The interface was collected by suction. At the osmolarity used, 85% of the granules (measured by dopamine-β-hydroxylase activity and catecholamine concentration) was found at the interface, while 70% of the mitochondria (tested by monoamine oxidase activity) migrated through the gradient.

Ghost preparation. Ghosts were prepared by osmotic lysis of granules purified by differential centrifugation [14]. The granules were added drop-wise in 20 vols. of a hypotonic salt solution containing 2 mM $MgSO_4$, 10 μM $CaCl_2$, 0.1 mM dithiothreitol and 5 mM Hepes, pH 7.5. After stirring at 0°C for 10 min the suspension was centrifuged (35 000 × *g* for 20 min). The pellet was washed with the same volume of buffer and resuspended in a medium containing 2 mM $MgSO_4$, 0.1 mM dithiothreitol, 0.3 M sucrose and 5 mM Hepes, pH 7.0.

Protein concentrations were estimated according to the method of Lowry et al. [18] with bovine serum albumin as standard, following precipitation in 5% trichloroacetic acid and redissolution in 2% deoxycholate/3% NaOH.

Potential measurements. The procedure of Casey et al. [12] was applied. For each measurement, four 0.25-ml aliquots of chromaffin granules (2 mg of protein in 0.3 M sucrose/10 mM Hepes, pH 7.0) were mixed with the same volume of the different indicated media. After incubation at 20°C, the isotopes (20 μl) were added: two samples were used to estimate the internal exchangeable water space (sorbitol exclusion volume) and two for the transmembrane potential. The first used ^3H_2O and [^{14}C]sorbitol and the latter either $S^{14}CN^-$ and ^3H_2O or [^{3}H]TPMP$^+$ and [^{14}C]sorbitol. Final isotope activities were (in μCi/ml): ^3H_2O, 2; [^{14}C]sorbitol (0.1 mM), 1; $S^{14}CN^-$(25 μM), 1.5; [^{3}H]TPMP$^+$ (40 μM), 4. The samples were equilibrated for 2.5 min at 20°C and centrifuged for 10 min at 27 000 × *g* at the same temperature. Both supernatant and pellet fractions were treated as described in Ref. 12, their radioactivity measured in 10 ml of scintillation mixture (NE 260, Nuclear Enterprise, Edingburgh) or alternatively the pellets dissolved in 0.5 ml of 2% Triton X-100 and the samples counted in a Triton-toluene scintillation fluid. Internal water space, and internal-to-external concentrations ratios of TPMP$^+$ or SCN$^-$ were derived as described in Ref. 12 from the relative activities of the isotopes in pellets and supernatants.

Internal water spaces ranged from 1.8 to 2.5 μl/mg of vesicle protein. Transmembrane potentials (in mV, 20°C) were calculated as follows:

$$\psi_{\text{in}} - \psi_{\text{out}} = 57.6 \log \frac{[\text{SCN}^-]\text{in}}{[\text{SCN}^-]\text{out}} = 57.6 \log \frac{[\text{TPMP}^+]\text{out}}{[\text{TPMP}^+]\text{in}}$$

The results were corrected for isotope binding to the core or membrane of the granules. Fluorescence experiments showed that transport of $S^{14}CN^-$ or [^{3}H]-TPMP$^+$ did not perturb transmembrane potentials of ATP-energized granules. $S^{14}CN^-$ (59 mCi/mmol) and [^{14}C]sorbitol (191 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, U.K.), ^3H_2O was from CEA (France), [^{3}H]TPMP$^+$ was prepared as described in Ref. 19.

Uptake of naradrenaline. The uptake of (—)-[^{3}H]noradrenaline was determined by filtration of the incubation mixture on 0.45 μm Millipore filters [20].

Instrumentation. Fluorescence and absorption changes were measured on a Farrand or on a Jobin-Yvon double monochromator fluorimeter and a DW2 Aminco spectrophotometer. Fluorescence spectra were not corrected either for the lamp output spectrum or for the photomultiplier spectral response. The relative quantum yields were obtained by integration of the uncorrected spectra. For kinetic measurements a stopped-flow apparatus was adapted to the Aminco spectrophotometer.

Results

Oxonol-V fluorescence and absorbance changes induced by chromaffin granules and ATP. When ATP was added to a mixture of oxonol-V and purified chromaffin granules, a large (40%) fluorescence decrease was observed (Fig. 1). ATP also induced a decrease (25%) in the 620—650 nm differential absorbance (Fig. 1). The effects of the concentrations of dye and chromaffin granules on the relative fluorescence and absorption changes are shown in Fig. 2. The ratio, oxonol-V to granule protein, is an important parameter and maximum effects were obtained in the range 30—90 nmol oxonol-V/mg granule protein. The pH of the medium is also important, the optimal optical change being at pH 6.6, with no effect at pH 5.8 and 23% of the maximal value at pH 7.4.

Origin of the ATP-induced change. The observed effect originated in chromaffin granules since other fractions of the density gradient used for their preparation were without activity. Addition of ATP to the mitochondrial frac-

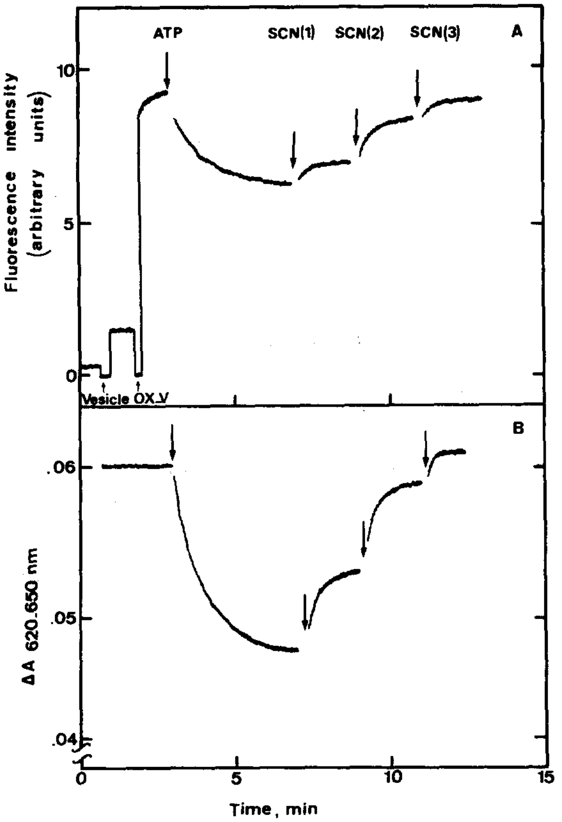

Fig. 1. Oxonol-V spectral changes induced by Mg^{2+}-ATP addition. In 0.45 M sucrose/20 mM Mes (pH 6.6) at 20°C were added: vesicles (0.01—0.2 mg protein/ml), oxonol-V (2 μM), ATP (2.5 mM), MgSO$_4$ (1.25 mM) and KSCN (SCN(1), 2 mM; SCN(2), 10 mM; SCN(3), 30 mM; final concentrations). Final volume 2 ml. (A) Fluorescence decrease. Excitation and emission wavelengths were 580 and 650 nm, respectively. Results were corrected for dilution and light-scattering effects. (B) Absorbance decrease. OX-V, oxonol-V.

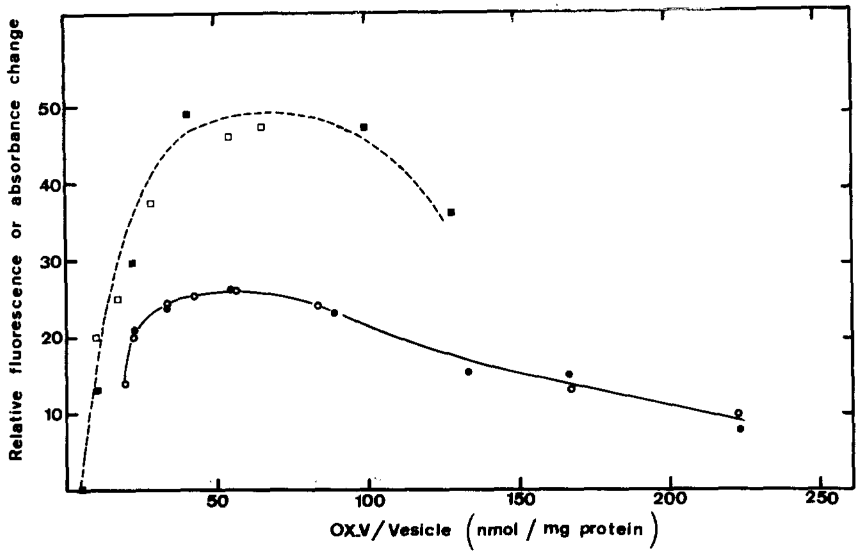

Fig. 2. Optimal dye-to-chromaffin granule ratios for fluorescence (dotted line) and absorbance (solid line) changes induced by ATP. Experimental conditions were as in Fig. 1 with 0.09 (●) and 0.15 mg (■) vesicle protein/ml, with variation of the concentration of oxonol-V: (○,□) 5 μM oxonol-V final concentration, with variation of the granule concentration. OX-V, oxonol-V.

tion did not affect dye fluorescence. Moreover, succinate (20 μM) and NADH (50 μM), which have been shown to induce changes in the spectral properties of oxonol-V bound to mitochondria or submitochondrial particles [11], had no effect on the chromaffin granule preparation. This result also indicates that the chain of electron carriers present in the chromaffin granule membrane and which has been reported to have an NADH-(acceptor) activity [21] is not involved in the observed changes. The effect, nevertheless, originated in the granule membrane since resealed membrane preparations (ghosts) showed the same activity.

In isotonic chloride media (Na^+ or K^+), ATP induced only a limited effect and in sucrose the effect was reversed by SCN^- (20 mM) or by 2.5 μM CCCP, an H^+ ionophore. As Cl^- and SCN^- are permeant anions whilst Na^+ and K^+ are impermeant cations [22,23], the probe appears to be sensitive to the inside positive transmembrane potential which is created by the H^+-translocating ATPase and destroyed by permeant anions [12,13]. Attempts to explore the potential effect further with vesicles suspended in K^+-containing media in the presence of valinomycin were complicated by an interaction between oxonol-V and valinomycin [24]; but in 150 mM potassium methyl sulfate containing valinomycin at 67 nmol/mg protein and oxonol-V at 33 nmol/mg protein we observed a slow but marked decrease in the absorption (21%) and fluorescence (45%) of oxonol-V. A plot of the relative absorption changes versus the log of external K^+ concentration gave a straight line, suggesting a probe matching transmembrane potentials.

Estimation of transmembrane potential from permeant ion distribution. Permeant ions at micromolar concentrations distribute between the inside and the outside of polarized chromaffin granules according to the Nernst equation,

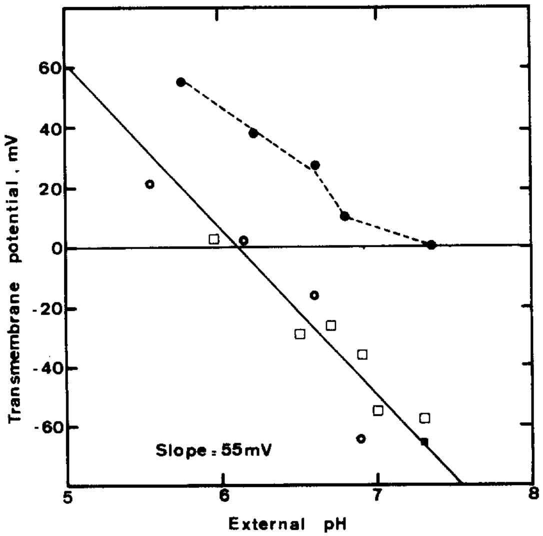

Fig. 3. Resting (solid line) and ATP-induced (dotted line) transmembrane potentials. Measurements were performed either with $S^{14}CN^-$ (○ and ●), or $[^3H]TPMP^+$ (□ and ■) (see Methods). Vesicles were injected into the same volume of 0.6 M sucrose at various pH values: 40 mM Mes (pH < 6.8) or 40 mM Hepes (pH > 6.8). When indicated, 5 mM ATP and 2.5 mM $MgSO_4$ (●) or 2.5 μM CCCP (■) was present. Isotopes were added after a 3 min incubation at 20°C.

without perturbing transmembrane potentials [13,15]. $S^{14}CN^-$ was used to measure the potential as a function of pH, in the absence or presence of ATP (Fig. 3). In their resting state, the vesicles were negatively polarized (—26 ± 10 mV at pH 6.6) as previously noted by Pollard et al. [15]. To confirm the negative potentials at high pH $S^{14}CN^-$ was replaced with the lipophilic cation, $[^3H]$ $TPMP^+$ [19,25,26] (Fig. 3). Since the resting potential decreased linearly with pH with a slope of 55 ± 5 mV it was assumed to be the proton-equilibrium potential. The null point at approx. pH 6 coincided with the internal pH measured by $[^{14}C]$methylamine distribution (data not shown [15,27]). ATP addition induced a positive potential shift of 54 ± 20 mV to a final value of +28 ± 10 mV at pH 6.6. It may be noted that this positive ATP-induced potential shift is independent of the external pH. ATP liberated by partial lysis of the vesicles induced a positive potential shift of the resting potential in several experiments, as indicated by the slight depressing effect of 10 μM CCCP at pH 7.5 (Fig. 3).

Quantitative relationship between the oxonol-V effects and transmembrane potentials. The ATP-induced effect of the transmembrane potential and optical properties of oxonol-V was measured at various SCN^- concentrations. The fluorescence results obtained at optimal fluorochrome-to-vesicle ratios for purified granules are shown in Fig. 4. At pH 6.6 the relative fluorescence change increased linearly with transmembrane potential at approx. 1.6 ± 0.2% per mV in the range 0—40 mV. Different probe-to-vesicle ratios gave converging straight

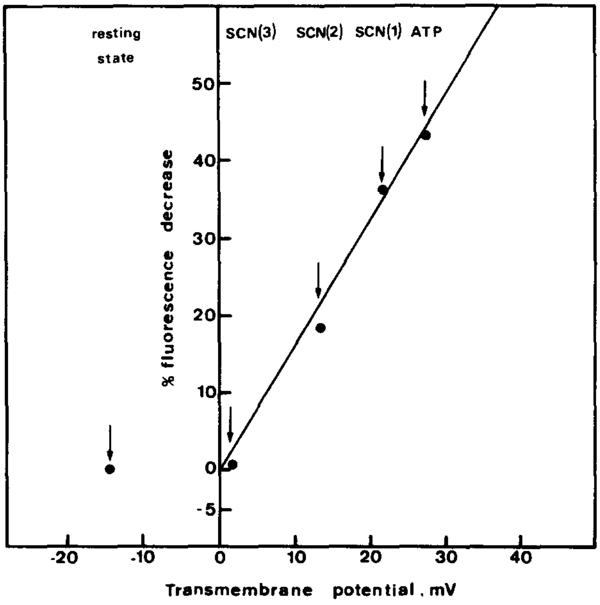

Fig. 4. Relationship between fluorescence changes and ATP-induced and KSCN-reversed transmembrane potentials. Fluorescence changes were recorded as in Fig. 1A (pH 6.6). The same preparation was used to measure transmembrane potentials by $S^{14}CN$ distribution (see Methods); vesicles were preincubated for 3 min with 10 mM ATP and 5 mM $MgSO_4$ at 20°C, then injected in the same volume of 0.6 M sucrose/ 40 mM Mes (pH 6.6) containing the isotopes and, when indicated SCN^- (SCN(1), 2 mM; SCN(2), 10 mM; SCN(3), 30 mM; final concentrations).

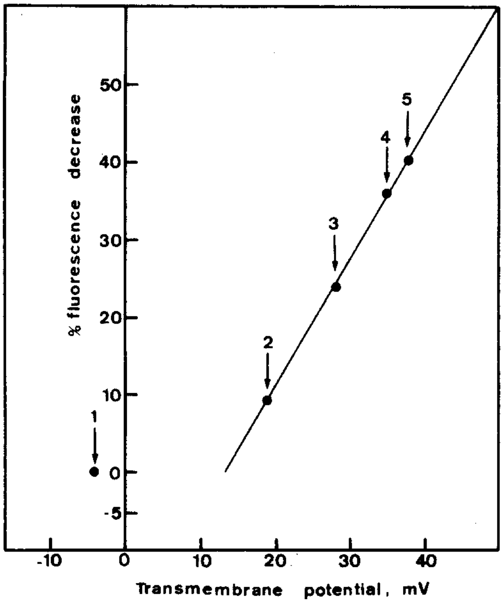

Fig. 5. Relation between fluorescence changes and transmembrane potentials induced by K^+-concentration gradients. Fluorescence changes induced by valinomycin (10 μM) addition were recorded as in Fig. 1A. Incubation media contained Mes (20 mM, pH 6.6), K_2SO_4 at: (1) 5 mM; (2) 25 mM; (3) 50 mM; (4) 75 mM; (5) 100 mM, and sucrose to give 0.475 osM final osmolarity. More than 10 min were required to obtain stable readings. The same preparation was used to measure transmembrane potentials by $S^{14}CN$ distribution (see Methods); the media were as in fluorescence experiments but valinomycin concentration was 2 μM.

lines with lower slopes. The same type of result was obtained with oxonol-V absorption changes (1.0 ± 0.2% absorption change per mV). The experiment performed with ghosts also gave a linear relationship between oxonol-V relative fluorescence changes and transmembrane potentials (1.2 ± 0.2% per mV in the range 0—40 mV).

By addition of valinomycin to granules in K_2SO_4 at various concentrations and measurement of the transmembrane potentials by the thiocyanate technique, an independent calibration curve was obtained (Fig. 5). The relative fluorescence change was a linear function of the positive potential. The slope equalled that previously described (1.6% fluorescence change per mV). The null fluorescence change, however, did not correspond to a null transmembrane potential, perhaps because of the dye-antibiotic interaction. Since a large K^+ permeability can be attributed to valinomycin-treated granules and since low $S^{14}CN^-$ concentrations have been utilized, the observed potentials were assumed to be K^+ equilibrium, thus allowing calculation of the internal K^+ concentrations by the Nernst equation. After 15 min of incubation at 10 mM external K^+, the internal K^+ concentration was found to be 11 mM. Increasing the external salt to 50, 100 and 200 mM increased the internal K^+ concentration to 24, 33 and 44 mM, respectively. During the same time, the internal volume increased from 2.60 ± 0.2 μl/mg protein at 10 mM external K^+ to 3.5 ± 0.2 μl/mg protein at 200 mM K^+. Another characteristic of the potentials generated by incubation of valinomycin-treated granules at constant external K^+ concentration is that they are pH-independent. We took advantage of this property to study the probe sensitivity as a function of pH. Above pH 6.4 the efficiency varied only slowly (1.6% per mV from pH 6.2 to 7.0, and 1.3% from pH 7.4 to 8.5). At acidic values it was much depressed, eg., 0.05% at pH 5.6.

Lack of response of oxonol-V to negative potentials. It has been noted that only positive potentials affect the optical properties of the probe (Figs. 4 and 5). To further substantiate the asymmetry of the optical effects, ghosts resealed in buffers at various pH values were energized at the same external pH (Table I). Such preparations had different resting potentials: rough estimates of their sign and magnitude were obtained by assimilating the resting potential to an H^+-equilibrium potential, assuming a constant internal pH and a permeability to protons large with respect to that of zwitterionic buffer anions. The ATP-induced potential shift (approx. +54 mV) is independent of the pH gradient across the membrane (Fig. 3). It can be seen from Table I that the ATP-induced fluorescence change was constant when the resting potential was estimated to be positive and was decreased as soon as the resting potential became negative. As the oxonol-V efficiency was constant at a given external pH, the experiment clearly showed that the probe only monitored the positive part of the potential shift.

Binding parameters of the probe-granule interaction. Whilst addition of oxonol-V to resting granules enhanced the dye fluorescence, energization of the preparation by ATP induced, in a certain range of the probe-to-granule ratio, a fluorescence decrease (Fig. 6). A considerable fraction of the dye was bound to the granules since significant differences in the dye emission spectra were observed after addition of either resting or energized granules to dye solutions. By performing a titration in which granule concentration was varied at a fixed value of total probe and extrapolating the double-reciprocal plot to infinite concentration [28], it was possible to estimate the limiting fluorescence which corresponded to complete binding of the dye (Fig. 7). It has to be noted that experiments with energized granules were conducted at low granule concentrations (less than 0.1 mg protein/ml) and at 630 nm under conditions where the

TABLE I

ATP-INDUCED FLUORESCENCE CHANGE OF GHOSTS PREPARED AT VARIOUS INTERNAL pH

Fluorescence measurements were as in Fig. 1. Ghosts were prepared as described in Materials and Methods but Hepes was replaced by Tris-succinate at the indicated pH in the lysis and resuspension buffer. Ghosts prepared at pH 8.0 and 8.5, neither transported noradrenaline nor showed any fluorescence change.

Internal pH	Noradrenaline transport (nmol/mg protein per min)	External pH = 6.5		External pH = 7.5	
		Calculated resting potential (mV)	Fluorescence change (%)	Calculated resting potential (mV)	Fluorescence change (%)
5.9	1.60	−34.5	45	−92	26
6.5	1.70	0	57	−57	33
7	1.84	+29	57	−29	42
7.5	0.57	+57	58	0	48

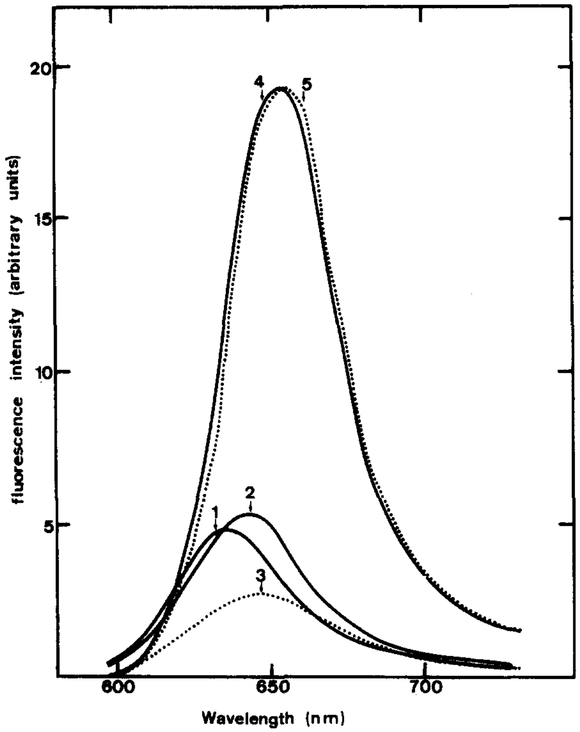

Fig. 6. Emission spectra of oxonol-V (2 μM) in 0.45 M sucrose/20 mM Mes, pH 6.6, at 20°C (experiment 1). Granules were added at concentrations of 0.05 mg protein/ml in experiments 2 and 3, and 1 mg protein/ml in experiments 4 and 5. ATP (2.5 mM) and $MgSO_4$ (1.25 mM) were present in experiments 3 and 5. Excitation wavelength was 580 nm. Spectra were corrected for light scattering.

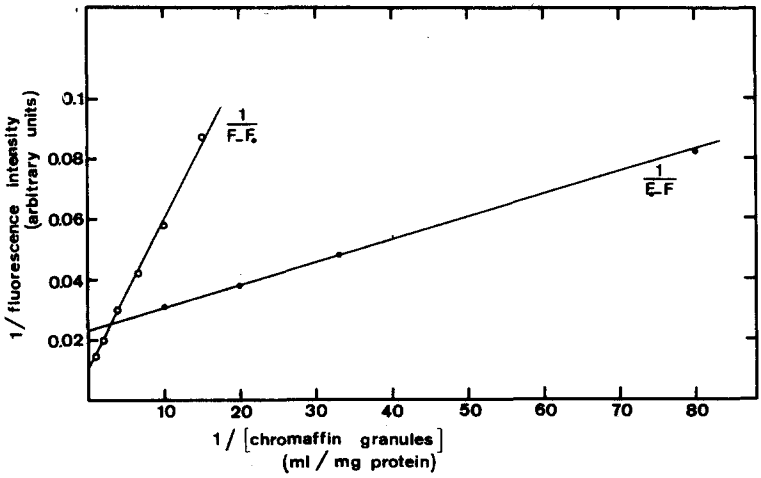

Fig. 7. Double-reciprocal plots of oxonol-V binding to resting and energized chromaffin granule membranes at 25°C. F_0 and F are, respectively, the fluorescence intensities of oxonol-V (1 μM) before and after addition of different amounts of granules. Incubation medium was as in Fig. 1. Excitation wavelength was 580 nm. (○) Resting granules (emission at 650 nm). F_0 was 26.5 (arbitrary units) and the limiting fluorescence was 4.5 F_0. (●) Energized granules (emission at 630 nm). F_0 was 61 and the limiting fluorescence 0.3 F_0.

fluorescence enhancement due to resting granules can be neglected (Figs. 6 and 7). The 'limiting enhancement' or 'limiting quenching' was then used to calculate the fraction of bound oxonol-V [28]. For instance, at 1 μM oxonol-V and 0.1 mg granule protein/ml, 20 and 70% of the probe were bound to resting and energized granules, respectively. The energization thus appears to increase the binding of the dye to the granules.

The presence of binding sites on the granules has been investigated by titration of free and bound dye at constant granule concentration, according to the method of Scatchard. The experiment was performed at 25 and 35°C on resting and energized granules. Straight lines were obtained (Fig. 8), thus showing that in the range of concentration studied, both resting and energized granules each have a major class of binding site, the parameters of which are given in the

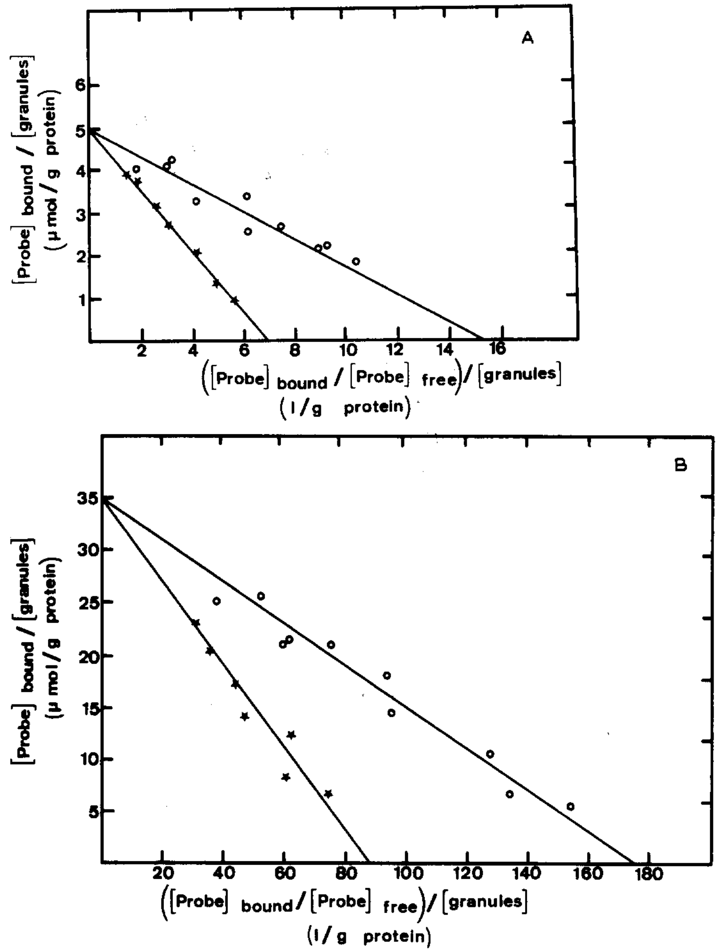

Fig. 8. Scatchard plot of oxonol-V binding to chromaffin granules. Incubation medium was as in Fig. 1, with a 0.2—2 μM probe concentration range. Excitation, 580 nm. (○) T = 25°C, (X) T = 35°C. (A) Resting granules (0.20 mg protein/ml, emission at 650 nm). K_D (25°C) = 0.33 μM, K_D (35°C) = 0.70 μM: number of sites, 5 μmol/g protein at 25 and 35°C. (B) Energized granules (0.05 mg protein/ml, emission at 630 nm). K_D (25°C) = 0.20 μM, K_D (35°C) = 0.40 μM; number of sites, 35 μmol/g protein at both temperatures.

legend of Fig. 8. Whilst energization does not alter the dissociation equilibrium constant by more than a factor of two, a 7-fold increase in the number of sites available to oxonol-V is observed under the same conditions. The same behaviour was also noted at 35°C.

Spectral changes induced by oxonol-V binding to granules. Binding of oxonol-V to resting granules resulted in a red shift of both its absorption and fluorescence emission peaks (Table II). The shifts were concentration-dependent with maximal figures of 20.5 and 16 nm for absorption and fluorescence, respectively, obtained at high granule concentrations under conditions in which most of the probe was bound. As expected, the addition of ATP which increased the amount of bound oxonol-V also increased the red shifts, but only at low granule concentrations. At high concentrations, identical limiting values of the red shifts were obtained for the resting and the energized granules.

The binding of oxonol-V to resting granules also resulted in an increased fluorescence efficiency (Fig. 6, curve 4). A double-reciprocal plot of the quantum yield as a function of granule concentration gave the 'limiting quantum yield', which was 6.5 times that of free oxonol-V in the same buffer. On the other hand, energized granules at moderate concentrations (less than 0.01 mg protein/ml) induced a quenching of oxonol-V fluorescence (Fig. 6, curve 3). The corresponding limiting quantum yield was calculated to be 45% of that of the free dye. It has to be pointed out that the reciprocal plot was not linear and that no quenching was observed at higher granule concentrations (Fig. 6, curve 5).

Kinetics of the oxonol-V chromaffin granule interaction. To follow the kinetics of interaction the differential absorption, $A_{620} - A_{700}$, was measured in a dual-wavelength spectrophotometer equipped with a stopped-flow device. The half-lifetimes derived from semilogarithmic plots of the absorption changes (reflecting the red shift associated with binding of the probe to either resting or energized granules) are presented in Table III. The probe interacted rapidly with resting granules ($\tau_{1/2} = 0.3$ s at 2 μM oxonol-V, or apparent $k_2 = 1.1 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) and slowly with energized granules ($\tau_{1/2} = 9.8$ s at 2 μM oxonol-V, or apparent $k_2 = 3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$), thus suggesting binding on different sites. Ex-

TABLE II

OXONOL-V SPECTRAL CHANGES INDUCED BY BINDING TO GRANULES

Experimental conditions were as in Fig. 8. Oxonol-V was 2 μM. Temperature was 25°C.

Chromaffin granules protein (mg/ml)	Fluorescence emission peak (nm)		Absorption peak (nm)	
	Resting granules	Energized granules	Resting granules	Energized granules
0	635.5	635.5	603.5	603.5
0.025	640.5	642	—	—
0.05	643	648	607.5	610
0.10	649	651.5	614	621
0.20	650.5	652.5	621	624.5
0.50	651	652.5	624	624.5
1.00	651.5	652.5	—	—

TABLE III

KINETIC PARAMETERS OF THE OXONOL-V ABSORPTION CHANGES

Differential absorption changes ($A_{620} - A_{700}$) were followed after mixing in the stopped-flow apparatus. Incubation medium was as in Fig. 1. Final concentrations were: oxonol-V, 2 μM; ATP, 1.25 mM; $MgSO_4$, 0.625 mM; CCCP, 5 μM. Association of oxonol-V with pre-energized granules (experiment 2) was followed after various times of preincubation with ATP (1—10 min) and found to be independent of the preincubation time.

Experiment	Medium 1	Medium 2	Half-lifetime (s)
1	granules	oxonol-V	0.3
2	granules, ATP and $MgSO_4$	oxonol-V	9.8
3	granules, oxonol-V	ATP and $MgSO_4$	16.0
4	granules, ATP and $MgSO_4$, oxonol-V	CCCP	4.0

periment 2 was performed with granules preincubated for 1—10 min with ATP and the kinetics of interaction were independent of the preincubation time. When ATP was added to granules preincubated with oxonol-V (experiment 3) the lifetimes observed were higher than in experiment 2. Therefore, energization by ATP of the granules appears to be the rate-limiting process and its kinetics can normally be followed by the probe. Nevertheless, it may be noted that the kinetics of experiment 3 were altered at high oxonol-V concentrations, $\tau_{1/2}$ being 14.6, 16 and 19 s at 1, 2 and 7.5 μM oxonol-V, respectively.

Effect of oxonol-V on catecholamine uptake by chromaffin granules. Oxonol-V had no lytic effect on chromaffin granules. However, at high concentrations it inhibited the ATP-dependent noradrenaline uptake by ghosts. The inhibition was 16 and 50% at 2 and 5 μM oxonol-V, respectively. This inhibition is not related to a dye-ATPase interaction, since oxonol-V at up to 10 μM did not affect ATPase activity. Most of the experiments described in this communication have been performed at 2 μM oxonol-V.

Discussion

Energetic properties of chromaffin granules

Chromaffin granules maintain a resting potential attributed to proton diffusion in a Donnan equilibrium. Electrogenic H^+ ionophores, such as CCCP, generate only a slight hyperpolarization in spite of the pH gradient, thus indicating some permeability of the membrane to protons and the presence of impermeant anions inside the vesicles. The second condition is certainly met since ATP and the acidic protein, chromogranin A, are major matrix components [29]. The permeability of the granule membrane to protons is slight [22], but it has to be pointed out that most measurements were performed in the presence of internal impermeant anions, thus allowing only electrogenic H^+ fluxes. Furthermore, in other systems the F_0-complex of the ATPase itself has been shown to act as an H^+ ionophore [30].

Our measurements confirm the electrogenic nature of chromaffin granule ATPase. In sucrose medium at pH 6.6, ATP addition generated a positive potential in the vesicle interior without affecting the internal pH measured by [^{14}C]methylamine distribution [12,16]. The proton motive force, $\Delta\bar{\mu}H$, of the ATPase can be calculated as the sum of the pH gradient at pH 6.6 (0.46 pH unit, corresponding to 26 mV, data not shown) and the transmembrane potential existing after ATP addition (28 mV). A figure of 54 mV or 1.25 kcal has been obtained, in accordance with our direct estimation of the total potential shift induced by ATP. Higher values of $\Delta\bar{\mu}H$, 150 and 124 mV in sucrose and KCl media, respectively, have been reported recently for the proton motive force of ghost ATPase at pH 7.1 at 37°C [13,16].

From the kinetic data, we conclude that the oxonal-V response is fast enough to follow energization of the granule by ATP. Experiments performed at low probe concentration gave a half-lifetime of energization of 16 s. This value is supported by rough calculations estimating the half-lifetime required to charge a leaky condensor to 50 mV with a proton pump. A value of 13 s has been derived assuming an ATPase activity of 10 nmol ATP hydrolyzed/min per mg protein at 22°C [31], 1-5 H^+ transported/ATP hydrolyzed [14], a capacity of 6 $\mu F/cm^2$ for the granule membrane, a membrane surface area of 1450 cm^2/mg protein and a proton leakage proportional to transmembrane potential.

Utilization of oxonol-V to measure transmembrane potentials

The dye, oxonol-V, has been proposed as a useful probe of membrane-related phenomena [6,11]. A correlation between absorption changes and membrane potentials was described together with more complicated fluorescence changes [11]. In the chromaffin granule system, we confirm the existence of correlations between both absorption and fluorescence changes and transmembrane potential. The observation was extended by showing that, under controlled conditions, oxonol-V can provide quantitative data as shown by the calibration curve (Fig. 4) associated with the ATP-induced polarization of chromaffin granules. Polarization by K^+ influx in the presence of valinomycin also gave a reproducible calibration curve (Fig. 5). In this case, the mechanism of the oxonol-V response was certainly complex, since the half-lifetime of the potential was shorter than that of the optical effect. This phenomenon, which has not been investigated, is likely to be due to the dye-antibiotic interaction, but it has to be pointed that, whatever the mechanism, the slow optical changes were related to the transmembrane potential measured independently. In addition, it should be noted that the usual calibration based on the potential derived by the Nernst equation from the ratio of external and internal K^+ concentrations gave erroneous results [32—34]. This technique assumes a constant internal K^+ concentration, a condition which is not met in the chromaffin granule case. It has been calculated (using an SO_4^{2-} passive permeability of $3 \cdot 10^{-10}$ cm/s, half of the Cl^- value [23]) and verified by osmotic swelling experiments, that the internal K^+ increase was not due to electrogenic K^+ transport but to neutral K_2SO_4 transport during the slow oxonol-V response (15 min). This explanation also accounts for the increased internal volume at high K_2SO_4 concentrations.

Certain precautions should be taken in the utilization of the probe. The dye-to-membrane ratio should be optimized (Fig. 2). It is interesting to note that the ATP-induced fluorescence quenching was observed for ratios exceeding 20 and 80 nmol/mg protein of granules and ghosts, respectively, and that figures exceeding 20 nmol/mg protein have been reported to induce a quenching of the dye bound to submitochondrial particles or pigeon heart mitochondria [11]. The experiment should be performed at pH > 6. The pK_a of oxonol-V is 4.2 in ethanol [11]. Although the pK_a has not been determined in aqueous media, the loss of probe sensitivity at pH < 6 might be related to the existence of the insoluble neutral species at acidic pH. Another limitation to the use of oxonol-V is that it detects only positive potentials. Thus, negative potential changes will induce fluorescence and absorbance increases only if the starting potential is sufficiently positive. The behaviour of oxonol-V in chromaffin granules is thus different to that of the cyanine dyes in red cells [7] or the merocyanine in squid giant axons [1], which are sensitive to positive and negative potentials. Other instances of dyes responding differently to positive and negative potentials have been reported [1]. Absorption changes associated only with hyperpolarization have been described in squid axon labelled from the inside with an oxonol dye [4].

Mechanism of action of oxonol-V

Three different types of mechanism have been proposed to explain the mode of action of potential-sensitive probes. In the first type, changes in the fluorescence of the cyanine dyes in response to potential changes in red blood cells and phospholipid vesicles were explained on the basis of a potential-dependent partition between the inside and outside of cells and vesicles, assuming that the dye behaves as a permeant cation [7]. Non-fluorescent aggregates of dye were formed at the high internal concentration of the free monomer form, which was then driven into the vesicles by the equilibrium distribution gradient. The oxonol-V fluorescence changes cannot be explained by this type of mechanism for the following reasons. A positive polarization of 28 mV at pH 6.6 would concentrate the dye only 3-fold, thus leading to an internal dye concentration of 6 μM under the usual conditions. Now, it has been reported that no dimer nor aggregate formation could be detected in aqueous solution at concentrations of up to 26 μM [11]. Another argument is that such an hypothesis cannot account for the spectral red shifts which do not exist in the cyanine case [7]. Moreover, the asymmetrical potential effects observed in the oxonol-V case are not explained by a partition hypothesis.

According to the second type of explanation, it is the electric field associated with the transmembrane potential which shifts the absorption of the probe [33,35]. Such an electrochromic effect has been proposed in the case of oxonol-V [11]. Nevertheless, we rule it out because the absorption red shift is dependent on the probe-to-granule ratio. Moreover, a response time of 10 s is not compatible with such an effect.

In the third type of mechanism, the transmembrane potential determines the binding of the probe to the membrane and the optical changes originate in the differences between free and bound dye [2,7,9]. The binding of oxonol-V to resting chromaffin granules induced a red shift of its absorption peak (20.5 nm) and, as expected, of its fluorescence emission peak. Binding was also associated with a 7-fold increase in the fluorescence yield. A decrease in the solvent polarity gave similar spectral changes. For instance in *n*-hexanol, absorption and fluorescence shifts were 19.5 and 17.5 nm, respectively, with respect to water whereas the fluorescence yield was doubled. Since the same changes were observed with ghost preparations, the probe is likely to be associated with hydrophobic sites on the granule membrane. The nature of these binding sites is unknown, but on resting granules they are less abundant than those of the amphiphilic 8-anilino-1-naphthalenesulfonic acid on submitochondrial particles, 6 and 56 μmol/g protein, respectively [9]. The rates of association of oxonol-V and 8-anilino-1-naphthalenesulfonic acid with these sites are comparable, the apparent k_2 values being $1.1 \cdot 10^6$ and $2.8 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, respectively [9].

Titration experiments have shown that polarization of the granules resulted in an increase in the number of oxonol-V binding sites (Fig. 8). The binding constant of these new sites is not different to that of the resting ones, but the association rate constants differed by two orders of magnitude. The two types of site must then be regarded as belonging to different classes. Similar observation have been made with 8-anilino-1-naphthalenesulfonic acid on submitochondrial particles [9]. The 'slow' sites were interpreted as indicating penetration of the probe into the membrane. Some potential-dependent motion of membrane components could well have induced the formation of these new sites. Alternatively, they might have been a result of surface potential changes. In any case, if the response of the anionic oxonol-V probe is linked to unmasking of these new sites, we understand why it is sensing only positive potentials.

That the optical changes are only related to the binding of the dye is shown by the following experiment. In two cuvettes containing the same amount of oxonol-V, granule concentrations were adjusted in such a manner as to have the same amount of bound dye either with resting or with energized granules. The spectral shifts (absorption and fluorescence) and the absorption spectra of both samples were identical. This experiment also suggests an explanation of the ATP-induced fluorescence quenching, which is quite paradoxical since binding should result in a fluorescence enhancement, as observed in 8-anilino-1-naphthalenesulfonic acid. The 40% fluorescence decrease is not accounted for by the red shift of the absorption peak which causes only a 10% absorption decrease, but by a considerable increase in the dye concentration on the membrane. At the usual concentrations of 2 μM oxonol-V and 0.1 mg granule protein/ml and assuming a membrane surface area of 1450 cm^2/mg protein derived from the specific internal volume measurements, the mean distance of oxonol-V molecules bound to the membrane is 40 Å when 85% of the dye is bound. This binding may bring the dye molecules into sufficient proximity to induce quenching by inner filter effect and reabsorption processes [11]. A similar observation has been made in the case of pigeon heart mitochondria and submitochondrial particles where a ratio of dye-to-protein exceeding 20 nmol oxonol-V/mg protein caused a quenching of fluorescence [11]. The large diversity of the oxonol-V fluorescence changes observed in various systems may then be a result of the magnitude of the potential and the binding parameters of the membrane. Additional binding will give rise either to an enhancement or to a quenching of fluorescence.

After completion of this manuscript, our attention has been drawn to a different interpretation of our results. According to the proposed model, the membrane would have two classes of binding site, internal and external sites.

The equilibrium constant (K_o) for distribution of dye between the aqueous phase and the exterior sites is voltage-independent and the rate constant (k_o) for this binding is large. The equilibrium constant (K_i) and rate constant (k_i) for distribution of dye between the intravesicular medium and the inner membrane sites are probably similar, that is, $k_i \approx k_o$, but the distribution of dye between the intravesicular space and the exterior medium is slow: $k(V) < k_o \approx k_i$ and voltage-dependent $K(V)$. A similar model has been proposed by Bashford et al. [36] to explain the mechanism of the potential-dependent absorption changes of oxonol dyes in illuminated chromatophores of *R. rubrum.* Such a model is interesting since it accounts for differences in the kinetics of dye binding to resting or energized vesicles, without postulating unmasking of binding sites or movement of proteins. Nevertheless, the evidence presented in this report does not allow discrimination between the two models. Experiments on the permeability of the chromaffin granule membrane to oxonol-V would probably settle this question since this property is only required in the second model.

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References

1 Cohen, L.B., Salzberg, B.M., Davila, H.V., Ross, W.N., Landowne, D., Waggoner, A.S. and Wang, C.H. (1974) J. Membrane Biol. 19, 1—36
2 Ross, W.N., Salzberg, B.M., Cohen, L.B., Grinvald, A., Davila, H.V., Waggoner, A.S. and Wang, C.H. (1977) J. Membrane Biol. 33, 141—183
3 Conti, F. (1975) Annu. Rev. Biophys. Bioenerg. 4, 287—310
4 Tasaki, I. and Warashina, A. (1976) Photochem. Photobiol. 24, 191—207
5 Ross, W.N., Salzberg, B.M., Cohen, L.B. and Davila, H.V. (1974) Biophys. J. 14, 983—986
6 Chance, B. and Baltscheffsky, M. (1975) Biomembranes 7, 33—59
7 Sims, P.J., Waggoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315—3330
8 Jasaitis, A.A., Kuliene, U.V. and Skulachev, V.P. (1971) Biochim. Biophys. Acta 234, 177—181
9 Brocklehurst, J.R., Freedman, R.B., Hancock, D.J. and Radda, G.K. (1970) Biochem. J. 116, 721—731
10 Waggoner, A.S., Wang, C.H. and Tolles, R.L. (1977) J. Membrane Biol. 33, 109—140
11 Smith, J.C., Russ, P., Cooperman, B.S. and Chance, B. (1976) Biochemistry 15, 5094—5105
12 Casey, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1977) Biochemistry 16, 972—977
13 Phillips, J.H. and Allison, Y.P. (1978) Biochem. J. 170, 661—672
14 Flatmark, T. and Ingebretsen, O. (1977) FEBS Lett. 78, 53—56
15 Pollard, H.B., Zinder, O., Hoffman, P.G. and Nikodejevic, O. (1976) J. Biol. Chem. 251, 4544—4550
16 Holtz, R.W. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5190—5194
17 Morris, S.J. and Schovanka, I. (1977) Biochim. Biophys. Acta 464, 53—64
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Henry, J.P. and Ninio, M. (1978) Biochim. Biophys. Acta 504, 40—59
20 Phillips, J.H. (1974) Biochem. J. 144, 311—318
21 Flatmark, T., Terland, O. and Helle, K.B. (1971) Biochim. Biophys. Acta 226, 9—19
22 Johnson, R.G. and Scarpa, A. (1976) J. Gen. Physiol. 68, 601—631
23 Phillips, J.H. (1977) Biochem. J. 168, 289—297

24 Waggoner, A.S. (1976) J. Membrane Biol. 27, 317—334
25 Liberman, E.A. and Skulachev, V.P. (1970) Biochim. Biophys. Acta 216, 30—42
26 Skulachev, V.P. (1971) Curr. Top. Bioenerg. 4, 127—190
27 Johnson, R.G. and Scarpa, A. (1976) J. Biol. Chem. 251, 2189—2191
28 Bashford, C.L., Casey, R.P., Radda, G.K. and Richtie, G.A. (1976) Neuroscience 1, 399—412
29 Winkler, H. (1976) Neuroscience 1, 65—80
30 Okamoto, H., Sone, N., Hirata, H., Yoshida, M. and Kagawa, Y. (1977) J. Biol. Chem. 252, 6125-6131
31 Bashford, C.L., Radda, G.K. and Richtie, G.A. (1975) FEBS Lett. 50, 21—23
32 Hoffman, J.F. and Laris, P.C. (1974) J. Physiol. 239, 519—552
33 Evans, E.H. and Crofts, A.R. (1974) Biochim. Biophys. Acta 333, 44—51
34 Sone, N., Yoshida, M., Okamoto, H., Hirata, H. and Kawaga, Y. (1976) J. Membrane Biol. 30, 121-134
35 Platt, J.R. (1961) J. Chem. Phys. 34, 862—863
36 Bashford, C.L., Chance, B. and Prince, R.C. (1979) Biochim. Biophys. Acta 545, 46—57