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TRANSPORT OF LOCAL ANAESTHETICS ACROSS CHROMATOPHORE MEMBRANES

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Summary

1. Both simple amines and tertiary amino local anaesthetics give rise to an accelerated decay of the absorption change of added pH indicator dyes and a decelerated decay of the endogenous carotenoid absorption band shift, following short flash excitation of *Rhodopseudomonas sphaeroides* chromatophores.

2. With increasing medium pH, lower concentrations of amine or local anaesthetics are effective.

3. The order of potency of the local anaesthetics concurs with their reported membrane/buffer partition coefficients and concentrations required for action potential blockade in nerve fibres.

4. The data are taken as evidence for rapid transport of the free base across the chromatophore membrane and relatively slow penetration of the protonated local anaesthetic. Protolytic reactions complete the effective dissipation of the trans-membrane pH gradient.

5. Benzocaine, with its unusually low pK_a and the quaternary derivative, chlorpromazine methiodide do not display this type of behaviour.

6. In the presence of membrane potential-collapsing agents, such as valinomycin/K^+ or thiocyanate ions, local anaesthetics decelerate the decay of the cresol red change but have no effect on the carotenoid shift decay. It appears that transport of the unprotonated local anaesthetic although electrically neutral, requires the presence of a membrane potential.

7. In contrast, the non-anaesthetic amines act independently of the membrane potential.

8. Ca^{2+} interferes with the mechanism of local anaesthetic deceleration of the cresol red change decay in the presence of valinomycin/K^+ or thiocyanate but not with other anaesthetic or amine reactions.

Introduction

The mechanism by which the amine local anaesthetics block the propagation of action potentials in nerve membranes is not fully understood. There appears to be a general consensus that the locus of action of the anaesthetic is at the sodium gate on the inside interface of the axon membrane [1—4]. Whether the anaesthetic lowers sodium conduction through binding and direct interference with the carrier molecule [1,2] or indirectly through modification of the physicochemical properties of the membrane or membrane interface [5—8] is still a matter for debate. Many different model systems have been used to study the effects of local anaesthetics. The effects are varied and may be peculiar to the system under investigation (see ref. 5).

We have studied the effects of local anaesthetics on ion transport in bacterial chromatophores, closed vesicular structures which possess an active, light-induced proton pump and ion-impermeable membranes. Following a short flash of light, an electric potential (positive inside) and pH gradient (acid inside) are rapidly developed across the chromatophore membranes [9,10]. These potentials relax within a few seconds after flash excitation. To a first approximation, the generation and decay of the chloroplast or chromatophore membrane potential may be followed from changes in the absorption of endogenous carotenoid molecules [9,11—14]. The uptake and release of protons by chromatophores may be reliably measured from absorbance changes of externally added pH indicator dyes [10,15,16].

The addition of ion-transporting compounds such as uncoupling agents, ionophores and lipid-soluble ions to chromatophore suspensions predictably modifies the decay rates of the carotenoid band shift and pH indicator change [9,16,17]. It has been proposed that local anaesthetics cross biological membranes as the free base, the protonated form of the anaesthetic being relatively impermeable [1,6,7]. This type of behaviour should have simple consequences on the carotenoid shift and pH indicator decays and this was the starting point of our investigations. The chromatophore system also affords the opportunity for studying the possible interactions between local anaesthetics and ion-transporting agents, in a biological membrane across which membrane potentials and H^+ movements may be measured with relative ease.

Methods

Rhodopseudomonas sphaeroides strain 2.4.1 was grown photosynthetically in completely filled, stoppered bottles at 30°C in a succinate medium [18]. The cells were harvested in late exponential phase and used immediately. Chromatophores were prepared by ultrasonic disintegration (3 bursts of 30 s at 150 W) and the differential centrifugation prodecures [14] in 50 mM Tricine, 50 mM NaCl, 8 mM $MgCl_2$ (pH 7.4) at 4°C. The preparations were stored in ice and used within 5 days. Bacteriochlorophyll was determined from the in vivo absorption at 850 nm using an extinction coefficient, $\epsilon = 95\ mM^{-1} \cdot cm^{-1}$ [19].

The kinetics of the carotenoid shift were followed at 523—509 nm in a double-beam spectrophotometer. The measuring beams were 90° apart at the 1 × 1 cm path-length cuvette and were detected by separate photomultipliers,

screened with 1 cm of saturated $CuSO_4$. The signals were backed off with a DC offset voltage, amplified, subtracted and stored in a Datalab DL 4000 signal averager. Saturating 20 μs half-peak width flash excitation was provided with a xenon tube, filtered through 2 layers Wratten 88A gelating filter from the underside of the sample cuvette. The flash tube was triggered periodically (minimum dark time, 35 s) and averaged signals, stored in the DL 4000 were displayed on a DL 450 microprocessor unit. The electronic time constant of the system was set to 100 μs and further smoothing of the waveforms was performed with the microprocessor after averaging.

Changes in the absorption of indicator dyes added to unbuffered chromatophore suspensions were monitored at wavelengths around 580 nm on the same instrument, operating as a single beam. The exact wavelength was determined for each series of experiments using a chromatophore sample in heavily buffered medium containing indicator. It was chosen such that background absorbance changes due to chromatophore pigment were negligible compared with the signal in unbuffered medium.

Local anaesthetics were purchased from the Medical Pharmacy, University of Birmingham. Chlorpromazine methiodide was a generous gift from Dr. Massey Stewart (Smith, Kline and French, Welwyn Garden City, U.K.). Antimycin A, FCCP and valinomycin were obtained from Sigma London. Dianemycin was a gift from Dr. C.L. Hamill (Lilly Research Laboratories, Indianapolis, U.S.A.). Other chemicals used were of the highest purity commerically available.

Experimental rationale

The carotenoid shift and pH indicator decays as diagnostic indicators of transport mechanisms. It should be emphasised that the ion fluxes that we have studied are passive processes. The single turnover burst of electron transport and accompanying, active H^+ translocation were effectively complete before our measurements began, i.e. within 1 ms in the presence of antimycin A, but in any case were not modified by the concentrations of ionophores, amines and local anaesthetics that we employed.

At pH 7.4 the decays of the carotenoid shift and cresol red absorbance change following flash activation of *Rps. sphaeroides* chromatophores show similar kinetics ([16] and Fig. 1). This suggests that the membrane potential, generated by the light-driven electron transport reactions, is dissipated by an outward flux of H^+. This flux is consequent upon the difference in the electrochemical potential of H^+ ($\Delta\bar{\mu}_{H^+}$) between the inside and outside aqueous phases of the chromatophores. That both electrical ($\Delta\psi$) and chemical potential (ΔpH) differences contribute to the driving force may be concluded from experiments in which either $\Delta\psi$ or ΔpH is rapidly dissipated. For instance the addition of valinomycin to the chromatophore suspension leads to rapid, electrophoretic K^+ efflux following the flash and therefore an acceleration of the carotenoid shift decay [9]. The increased rate of decay of the $\Delta\psi$ component of $\Delta\bar{\mu}_{H^+}$ leads to a decreased rate of H^+ efflux and decay of the pH indicator signal [16]. Conversely, chromatophores treated with dianemycin or nigericin, which exchange H^+/K^+ in an electrically neutral manner [20], show an accelerated decay of the pH indicator change and, owing to the rapid dissi-

pation of the ΔpH component of $\Delta\bar{\mu}_{H^+}$ a deceleration of the carotenoid shift is observed [9,16] (see Table I). Uncoupling agents, such as FCCP, which have rapid diffusional mobilities of both the anionic ($FCCP^-$) and protonated FCCPH) species within the membrane [21], are able to effectively dissipate both $\Delta\psi$ and ΔpH and therefore induced increased decay rates of both the carotenoid shift and pH indicator change [17].

Results

Local anaesthetics cross the chromatophore membrane as the free base

The addition of $1 \cdot 10^{-5}$—$1 \cdot 10^{-4}$ M tetracaine gives rise to an increase in the rate of decay of the cresol red change and a small but significant decrease in the decay rate of the carotenoid shift (Figs. 1 and 2). This behaviour is characteristic of all the tertiary amine anaesthetics (except benzocaine, see below) that we have studied, of ammonium chloride, a variety of primary, secondary and tertiary amines and of certain ionophores such as nigericin and dianemycin (Table I). The explanation is clear: all those agents catalyse an electrically neutral dissipation of the pH gradient produced by the flash. The model shown in Fig. 3 is similar to that which has been proposed to interpret the pH dependence of action potential blockade by local anaesthetics added to the bathing medium of nerve fibres [3,4]. It differs slightly from the model proposed by Crofts [22] to account for amine uncoupling of chloroplast photophosphorylation in that the protolytic reactions of local anaesthetics are assumed to take place at the membrane interface [6,23]. The increased acidity of the chromatophore internal space following the flash would tend to protonate internal amines. This would lead to inward displacement of the neutral amine species and dissociation of H^+ on the outside.

The order of potency of different local anaesthetics in producing an accelerated cresol red change decay is similar to the order of the clinical potency of the anaesthetic and concurs with their membrane/buffer partition coefficients (Table I). The concentration of individual anaesthetics required to double the decay rate of the cresol red change is similar to the concentration which, in nervous tissue gives rise to action potential blockade (Table I).

The ability of the anaesthetics and the non-anaesthetic amines to act as electrically neutral proton carriers increases with pH (Figs. 4 and 5). Various pH indicators were used in these experiments. All these indicators, except phenol violet, have been previously shown to be reliable indicators of the external pH of chromatophores suspensions [10]. Phenol violet with a conveniently high pK_a (9.6) adequately meets the requirements of reliability [24]. Taking the reciprocal of the decay half-time of the indicator changes as proportional to the membrane proton conductance, the ΔpH-collapsing efficiency of tetracaine does not show a simple correlation with the concentration of either protonated or unprotonated forms of the anaesthetic. Quantitative effects of pH are difficult to interpret since the properties of the chromatophores may vary in a manner which effects anaesthetic adsorption and translocation; properties such as membrane surface charge density, internal buffering capacity and ionic permeability of the membrane. It may be noted however than only Reaction 1 in the anaesthetic transport processes described in Fig. 3 will

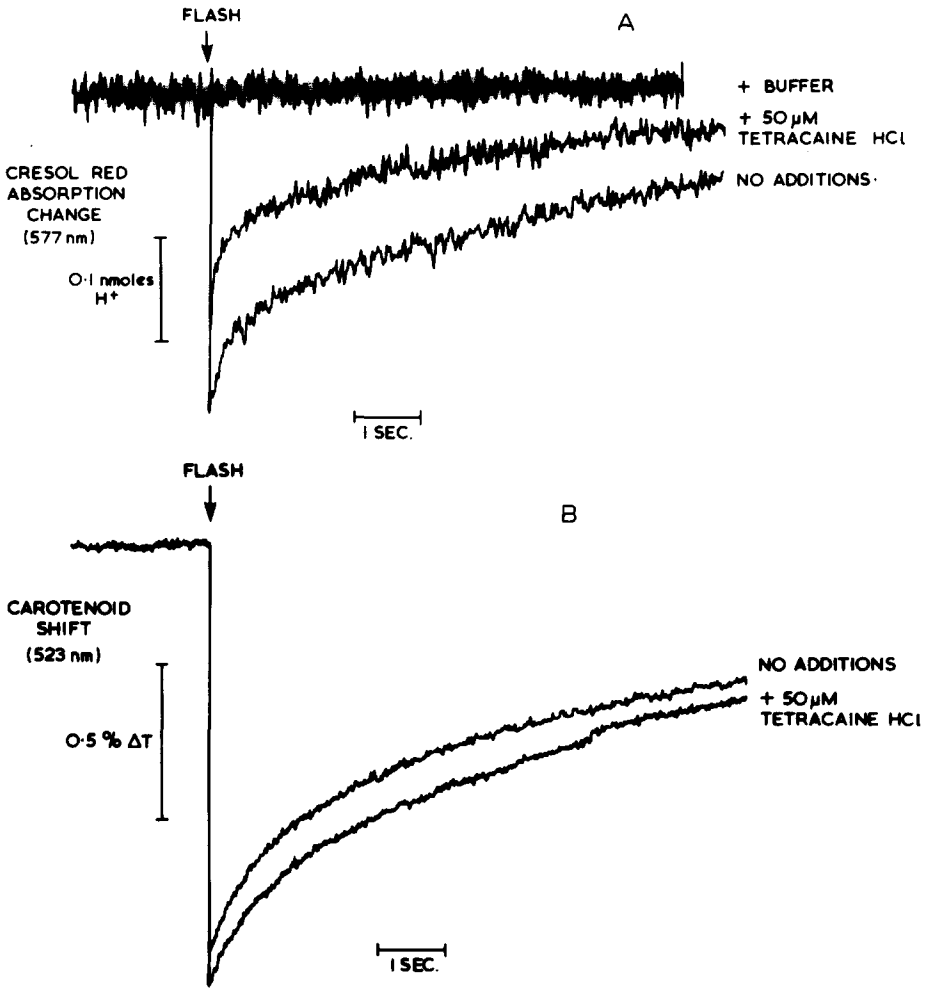

Fig. 1. The effect of tetracaine on the decay kinetics of the carotenoid shift and cresol red absorption change. Chromatophores (bacteriochlorophyll concentration $1.0 \cdot 10^{-5}$ M) were suspended in $5 \cdot 10^{-2}$ M KCl, pH adjusted to 7.4 using dilute KOH. $4 \cdot 10^{-5}$ M cresol red and $5 \cdot 10^{-6}$ M antimycin A were also present. The traces obtained were an average of 32 recordings spaced 50 s apart. $5 \cdot 10^{-5}$ M tetracaine present where indicated. (A) Cresol red absorption changes measured at 577 nm where no background changes were apparent as indicated from recordings in the presence of $5 \cdot 10^{-2}$ M Tricine, pH 7.4. (B) Carotenoid shift measured simultaneously with the cresol red absorption changes at 523 nm.

increase with increasing pH, which suggests that H^+ association with the free base at the internal interface of the chromatophore membrane is rate limiting.

The fact that the low concentrations of anaesthetic may accelerate the cresol red change decay but not the carotenoid shift decay suggests that the protonated forms of the anaesthetic (R_1-R_2-R_3-NH^+ or (R_1-R_2-R_3-N)$_2H^+$) may not move across the membrane at a significant rate. At higher concentrations however, the anaesthetics and the non-anaesthetic amines shown in Table I do begin to increase the rate of decay of the pigment shift (Fig. 2 and Table I). The acceleration of the carotenoid shift decay characteristically takes place in two stages. The time taken for the carotenoid signal to decay by 50% shows a

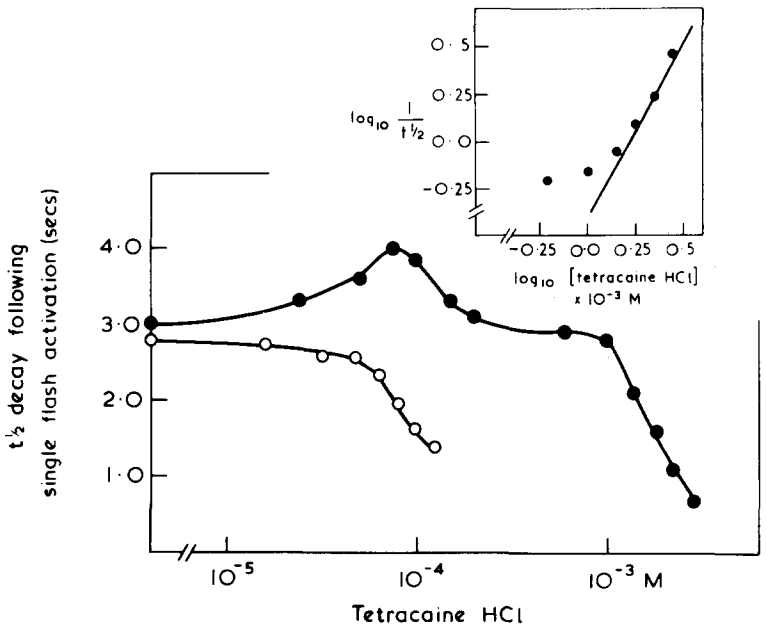

Fig. 2. Concentration dependence of tetracaine on the decay half-times of the carotenoid shift and cresol red absorption change. Chromatophores (bacteriochlorophyll concentration of $1.8 \cdot 10^{-5}$ M) were suspended in $5 \cdot 10^{-2}$ M NaCl, $8 \cdot 10^{-3}$ M $MgCl_2$, pH 7.4, with either no added buffer (○———○) or in the presence of $5 \cdot 10^{-2}$ M Tricine. The decay half-times were measured from traces obtained from 16 recordings spaced 40 s apart. ○———○, decay half-time of the cresol red absorption change measured at 575 nm; •———•, decay half-time of the carotenoid shift measured at 532 nm. The inset shows the effect of high concentrations of tetracaine on the reciprocal of the carotenoid shift decay half-time.

quadratic dependence (inset, Fig. 2) on the anaesthetic concentration across a limited range. McLaughlin [6] described a similar dependence for anaesthetic-induced conductance across artificial lipid bilayers. McLaughlin concluded that the electrophoretic species of the anaesthetic has the structure $(R_1\text{-}R_2\text{-}R_3\text{-}N)_2H^+$. Alternatively, the high concentration of amine within the membrane may have a disruptive effect and thus render the chromatophores generally permeable to ions.

At all values of external medium pH the concentration of anaesthetic required to produce an accelerated decay of the carotenoid shift is higher than that required to increase the rate of H^+ efflux following a short flash. The membrane potential-dissipating effect is also observed at progressively lower concentrations of anaesthetic as the pH of the external medium is raised from pH 6.0 to 9.0 (Fig. 5).

As with other properties discussed so far, NH_4Cl and the range of simple, organic amines (listed in Table I) show qualitatively similar behaviour with varying medium pH. Chlorpromazine, although used clinically as a tranquilizer, effectively blocks action potentials in nerves and modifies chromatophore proton transport in the manner of local anaesthetics. Its quaternary derivative, chlorpromazine methiodide, however, is unable to collapse the ΔpH generated across chromatophore membranes. The derivative is capable of membrane potential collapse, the phenomenon associated with the high concentration range of the

TABLE I

	Concentration required to halve the $t_{1/2}$ of the cresol red decay at pH 7.4 (C_{50})	[$t_{1/2}$ carotenoid shift decay at C_{50}]/[$t_{1/2}$ carotenoid shift decay (no additions)]	Concentration required to accelerate the carotenoid shift decay at pH 7.4 by a factor of 2	Oil or alcohol/buffer partitioh coefficient of free base	Nerve-blocking concentration	pK_a
Tetracaine	$1.0 \cdot 10^{-4}$ M	1.5	$2.0 \cdot 10^{-3}$ M	273 *	$1.0 \cdot 10^{-4}$ M **	8.5 *
Lidocaine	$8.0 \cdot 10^{-4}$ M	1.2		225 *	$1.0 \cdot 10^{-3}$ M **	7.9 *
Procaine	$4.0 \cdot 10^{-3}$ M	1.2	$5.0 \cdot 10^{-2}$ M	45 *	$4.6 \cdot 10^{-3}$ M **	8.9 *
Benzocaine	$2.0 \cdot 10^{-4}$ M	—	$5.0 \cdot 10^{-5}$ M	41 *	$1.2 \cdot 10^{-3}$ M	2.6 *
Chlorpromazine	$1.2 \cdot 10^{-5}$ M	1.2	$2.0 \cdot 10^{-4}$ M	1600 **	$1.0 \cdot 10^{-5}$ M **	8.2 ***
Chlorpromazine methiodide	—	—	$1.0 \cdot 10^{-3}$ M	—	—	—
Ammonium chloride	$8 \cdot 10^{-4}$ M	1.3		—	—	9.5
Tri-*n*-butylamine	$2.8 \cdot 10^{-5}$ M	1.2	$2.0 \cdot 10^{-3}$ M	—	—	10.8
N,N-Dimethylaniline	$2.2 \cdot 10^{-4}$ M	1.4	$5.0 \cdot 10^{-3}$ M	—	—	5.2
1-Amino-2-phenylethane	$1.6 \cdot 10^{-5}$ M	1.1	$1.0 \cdot 10^{-2}$ M	—	—	—
Dianemycin	10^{-8} g	1.3	10^{-5} g	—	—	—

* See Ref. 31 for source of data.

** See Ref. 5 for source of data.

*** See Ref. 32.

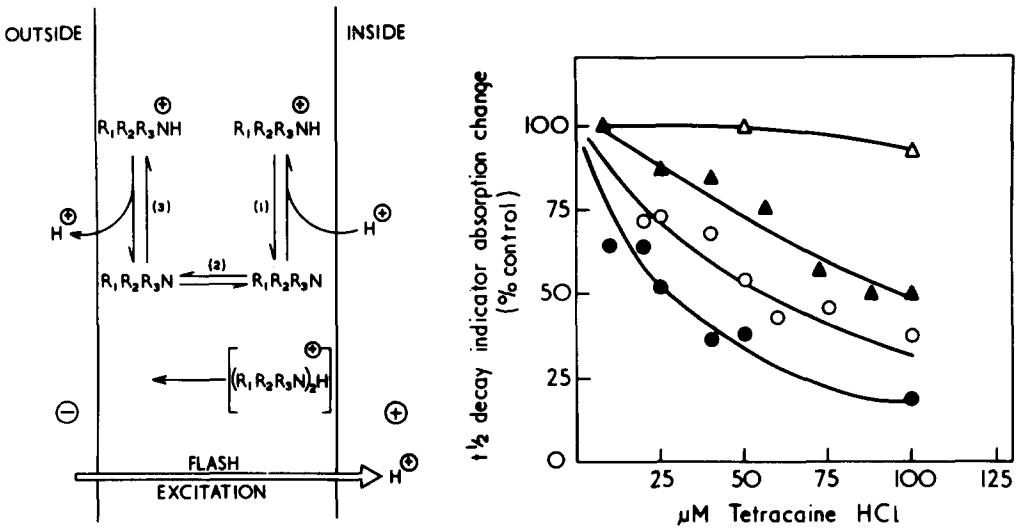

Fig. 3. Local anaesthetic-catalysed proton transport. Short flash excitation is shown to give rise to an electrochemical proton gradient across the membrane. Protolytic reactions at the membrane interfaces and diffusion of uncharged local anaesthetic serve to dissipate ΔpH as described in the text.

Fig. 4. The effect of tetracaine on the pH indicator absorption change decay at various suspending medium pH values. Conditions as for Fig. 1 except that $4 \cdot 10^{-5}$ M bromocresol purple, or $4 \cdot 10^{-5}$ M phenol red, or 14 μg/ml phenol violet replaced $4 \cdot 10^{-5}$ M cresol red at external medium pH values of 6.0 (△——△), 7.0 (▲——▲) and 9.0 (●——●), respectively. ○——○, Cresol red absorption changes measured at pH 8.0. The decay half-times are expressed as percent control in the absence of tetracaine. Control half-times obtained at pH values of 6.0, 7.0, 8.0 and 9.0 were 1.0 s, 1.6 s, 1.0 s, and 0.78 s, respectively.

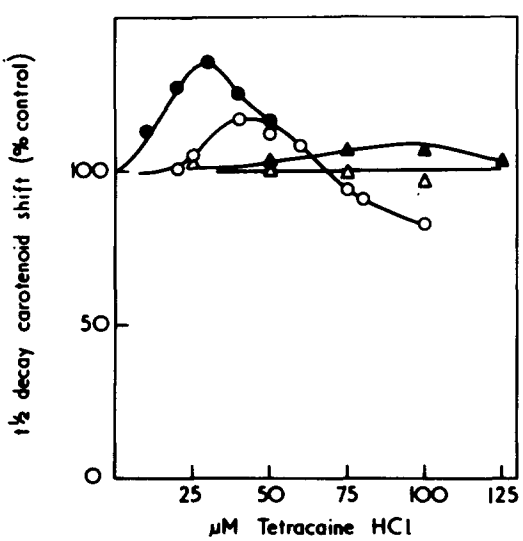

Fig. 5. The effect of tetracaine on the carotenoid shift decay at various suspending medium pH values. Conditions as for Fig. 4. Control half-times obtained at pH values of 6.0, 7.0 and 9.0 were 2.6 s, 2.3 s, 1.2 s and 0.9 s, respectively. Symbols as for Fig. 4.

tertiary amine anaesthetics. Benzocaine is an unusual local anaesthetic in that its pK_a is very low and consequently the molecule exists predominantly as the neutral species at physiological pH values. This compound also fails to accelerate the decay of the cresol red change after a flash without simultaneously stimulating the carotenoid shift decay. The failure of both chlorpromazine methiodide and benzocaine to catalyse an electrically neutral H^+ transport is not inconsistent with the model shown in Fig. 3 since the normal sequence of protonation and deprotonation cannot occur with either of these compounds.

Effective membrane concentration of local anaesthetic

An electron microscope analysis of our *Rps. sphaeroides* chromatophore preparations has shown that the average vesicle diameter is 360 Å and that on average, each chromatophore possesses about 1000 molecules of bacteriochlorophyll [14]. Assuming that the thickness of the hydrocarbon shell of the membrane is 30 Å, then the membrane volume of a chromatophore suspension containing 25 nmol bacteriochlorophyll is about 0.15 μl.

At pH 8.0, 50 μM tetracaine (i.e. 12 μM free base) is sufficient to decrease the decay half-time of the cresol red change by 50%. Since the oil/buffer partition coefficient of tetracaine is 273 (referenced in Table I), the membrane concentration of the free base should be about 3 mM. In 0.15 μl this amounts to 0.47 nmol, i.e. approx. 20 molecules of anaesthetic/chromatophore.

Inconsistencies arise when comparative calculations are made at different pH values; it appears that with decreasing medium pH, lower membrane concentrations of the free base are effective in catalysing the decay of the pH gradient. In reality, the calculation is complicated by the unknown degree of adsorption of the protonated form of the anaesthetic to the membrane and the effective pK of the anaesthetic at the interface. However the approximation shows that low membrane concentrations of local anaesthetic are able to neutralise transient pH inequalities across the membrane. In comparison, approx. 0.3 molecules of FCCP or 0.6 molecules of valinomycin/chromatophore are sufficient to decrease by 50% the half-time of decay of the cresol red absorption change and the carotenoid band shift decay, respectively, in our preparations (Packham, N.K., unpublished observations).

The influence of local anaesthetics on electrophoretic ion flux catalysed by ionophores or by lipid-soluble anions

Valinomycin in the presence of K^+ [9], uncoupling agents such as FCCP [9,17] or lipid-soluble anions such as thiocyanate induce an accelerated decay of the carotenoid band shift elicited by flash activation. The effect of thiocyanate has not previously been described but even at concentrations higher by five orders of magnitude is not nearly so pronounced as that due to FCCP or valinomycin. These results may be interpreted to indicate electrophoretic charge transfer (either K^+ or H^+ expulsion, or slower, SCN^- uptake) in response to the flash-induced charge separation [9].

Local anaesthetics have been shown to inhibit the steady-state flux of valinomycin-mediated K^+ transport across mitochondrial membranes [25,26] and artificial lipid bilayers [6]. Judging by the carotenoid response, local anaesthetics do not modify the transient flux of SCN^-, valinomycin/K^+ or FCCP/H^+

following flash excitation of chromatophore membranes. The carotenoid shift decay kinetics, accelerated by any one of these agents is not significantly affected by any of the local anaesthetics listed in Table I (data not shown).

Interference of local anaesthetic-catalysed H^+ transport by membrane potential collapsing agents

The inclusion of either valinomycin/K^+ or thiocyanate in the chromatophore suspension medium gives rise to a decelerated decay rate of the cresol red change after a flash (Figs. 6 and 7). The decelerated cresol red change decay is still sensitive to stimulation by ammonium chloride and non-anaesthetic amines completely in accordance with the model. Local anaesthetics such as tetra-

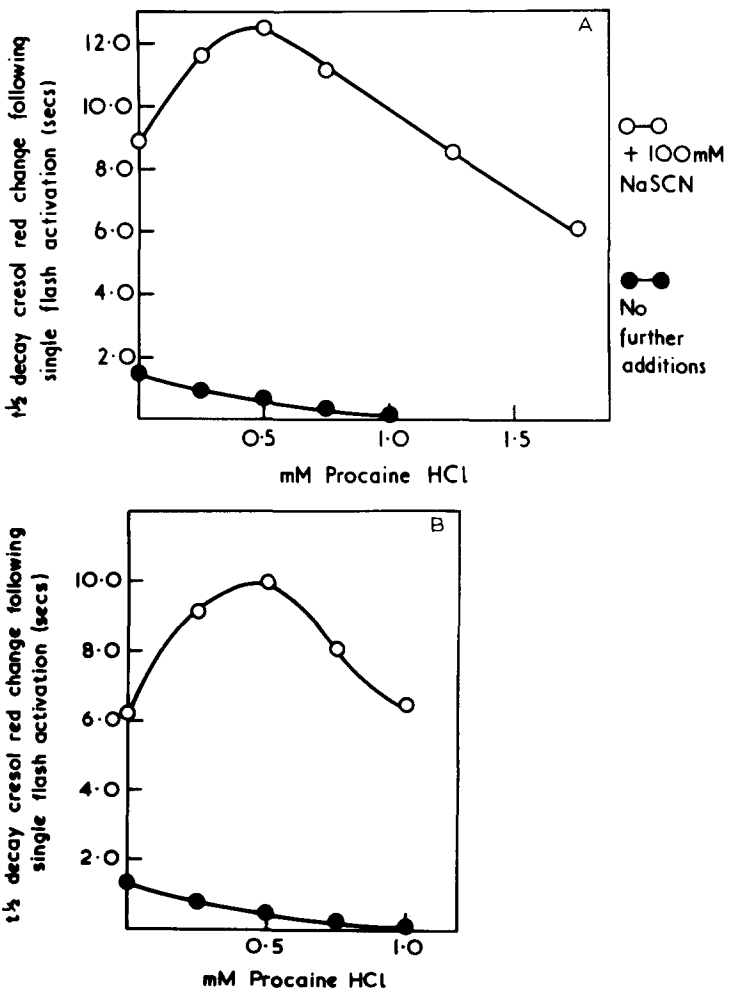

Fig. 6. The influence of procaine on the cresol red absorption change decay in the presence or absence of membrane potential-collapsing agents. Conditions as for Fig. 1 except that the external medium pH was adjusted to 8.0. Bacteriochlorophyll concentration was $1.9 \cdot 10^{-5}$ M. (A) Procaine added either in the absence (●———●) or presence (○———○) of 100 mM sodium thiocyanate, or (B) in the absence (●———●) or presence (○———○) of 30 nM valinomycin.

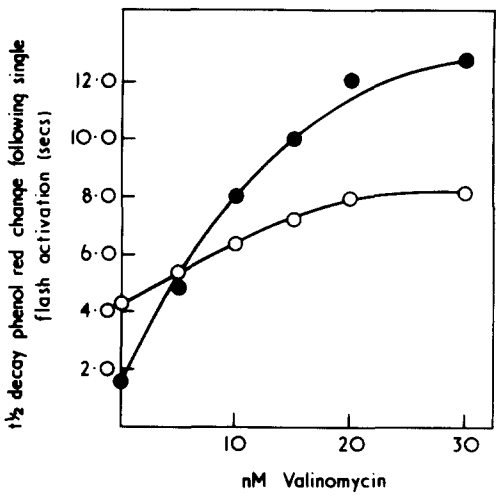

Fig. 7. The effect of chlorpromazine on the phenol red change decay in the presence and absence of valinomycin. Conditions as for Fig. 1 except for the absence of antimycin A and that $4 \cdot 10^{-5}$ M phenol red replaced the cresol red. The suspending medium pH was adjusted to 7.0. Valinomycin was added to chromatophore suspensions in the presence (●———●) or absence (○———○) of $1.6 \cdot 10^{-5}$ M chlorpromazine.

caine, lidocaine, procaine (and chlorpromazine) behave quite anomalously. Under conditions where the membrane potential is dissipated by electrophoretic flux of counter ions, low concentrations of anaesthetics further decelerate the rate of reappearance of H^+ into the external medium. This effect is shown in two different types of experiment in Figs. 6 and 7. In one approach it is shown that, whereas low concentrations of procaine give rise to an accelerated decay of the cresol red change in a standard chromatophore suspension medium, under conditions of membrane potential collapse, i.e. in the presence of valinomycin or 100 mM NaSCN, the half-decay time is prolonged (Fig. 6) by similar concentrations of anaesthetic. Higher concentrations of anaesthetic give rise to acceleration of the indicator decay in the presence or absence of lipophilic ion. Analogously, at a fixed low concentration of chlorpromazine, increasing concentrations of valinomycin exert a much more pronounced deceleration than in the absence of anaesthetic under otherwise identical conditions. (Fig. 7). Neither the unprotonated benzocaine nor the quaternary methiodide derivative of chlorpromazine display this type of behaviour. They accelerate the decay of the pH indicator in the manner of 'high' concentrations of anaesthetic.

The concentration of chlorpromazine required to give rise to the deceleration of H^+ reappearance after the flash in the presence of valinomycin decreases with increasing pH, i.e. with increasing proportion of the anaesthetic in the neutral form (Fig. 8). At each pH, the concentration of chlorpromazine required for this effect is the same as that which gives rise to a stimulated decay in the absence of valinomycin.

In chromatophores suspended in a K^+-free medium the indicator decay was scarcely modified by the addition of valinomycin. Further addition of local anaesthetic simply led to an increased decay rate. The decelerative effect of the

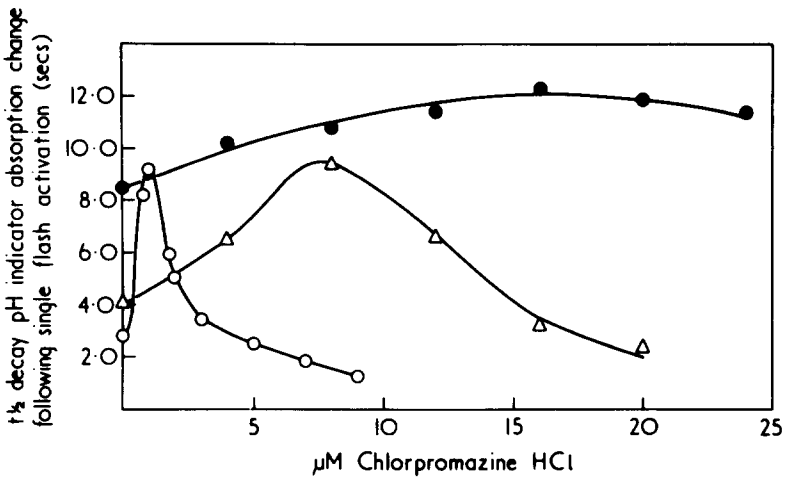

Fig. 8. The effect of chlorpromazine on the pH indicator change decay, in the presence of valinomycin and at various suspending medium pH values. Conditions as for Fig. 4 except for a final bacteriochlorophyll concentration of $2.0 \cdot 10^{-5}$ M and the absence of antimycin A. 30 nM valinomycin was present in all cases. External medium pH values were 7.0 (●———●), 8.0 (△———△) and 9.0 (○———○).

anaesthetic clearly requires the lipid-soluble species, valinomycin/K^+ but valinomycin alone is not effective.

The effect of calcium on local anaesthetic-catalysed reactions in chromatophores

The effects of local anaesthetics have been reported to be depressed by the presence of low concentrations of Ca^{2+} [6,27,28]. It is supposed that the calcium, by chemically binding to anionic phospholipids in the membrane interface, and through ionic 'screening', displaces the adsorbed anaesthetic [6,27]. The addition of $CaCl_2$ up to 15 mM has no effect on the decay rates of

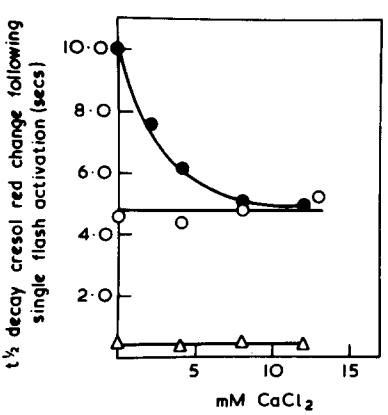

Fig. 9. The effect of $CaCl_2$ on the cresol red change decay in the presence and absence of procaine and valinomycin. Conditions as for Fig. 7 except that the external medium pH was kept at 8.0. The effects of added $CaCl_2$ in the presence of either $5 \cdot 10^{-4}$ M procaine (△———△), or $3 \cdot 10^{-8}$ M valinomycin (○———○), or in the presence of both $5 \cdot 10^{-4}$ M procaine and $3 \cdot 10^{-8}$ M valinomycin (●———●) are shown.

either the cresol red change or carotenoid shift in the presence or absence of valinomycin or thiocyanate; nor does it modify the behaviour of either simple amines or anaesthetic amines on these experimental parameters in the absence of membrane potential-collapsing agents. It does however, prevent the deceleration of the cresol red change decay produced by addition of local anaesthetic to a chromatophore suspension already containing either valinomycin/K^+ or thiocyanate. Fig. 9 shows that addition of $CaCl_2$ up to 15 mM does not modify the decay of the cresol red absorption change, accelerated by 0.5 mM procaine in the absence of valinomycin. On the other hand 10 mM $CaCl_2$ abolishes the decelerative effect produced by 0.5 mM procaine in the presence of valinomycin.

Discussion

With the exception of benzocaine and chlorpromazine methiodide, we may distinguish two concentration regimes for each of the local anaesthetics investigated in our experiments. Although the absolute concentrations may vary from one anaesthetic to another, apparently depending on the membrane/buffer partition coefficient, there is generally a marked difference in behaviour between 'low' and 'high' concentration ranges.

At low concentrations, the anaesthetic is unable to carry electrical charge across the chromatophore membranes viz. the failure to accelerate the decay of the carotenoid shift after a short flash. Under these conditions the passage of current by species such as R_1-R_2-R_3-NH^+ of $(R_1$-R_2-R_3-$N)_2H^+$ is minimal. The increased rate of H^+ reappearance after the flash must be an electrically neutral process corresponding to inward diffusion of R_1-R_2-R_3-N coupled to protolytic association and dissociation at the membrane interface (Fig. 3). These data therefore give some support for the use of the equations derived by Naharashi and coworkers [3,4] for the equilibrium distribution of anaesthetic with pH gradients across the axon membrane. As a corollary, the presence of local anaesthetics in nerve membranes would be capable of dissipating small gradients of pH between the axoplasm and bathing medium.

The data of Figs. 5—7 show that in the presence of lipid-soluble anions (SCN^-) or cations (valinomycin/K^+), low concentrations of local anaesthetics slow down the rate of H^+ re-release from the chromatophores following flash-induced H^+ uptake. The anaesthetics, however, do not appear to modify the electrophoretic transport of these ions as judged by the carotenoid response. This last observation merits after further attention since its has been shown that local anaesthetics inhibit the steady-state valinomycin-catalysed transport of K^+ across artificial membranes [6]. Also, in an interesting paper by Papa et al. [25] it was claimed that dibucaine decreased valinomycin and nigericin-catalysed K^+ flux in submitochondrial particles although these results may be partly reinterpreted in terms of dibucaine-catalysed H^+ transfer. In any case the experiments with lipid bilayers and mitochondrial membranes were essentially steady-state measurements of K^+ flux involving multiple turnovers of the valinomycin, whereas in our experiments we observe transient ionic flux after a burst of electron transport amounting to approx. 12 elementary charges/chromatophore vesicle [14].

We cannot account for the local anaesthetic-induced deceleration of H^+ re-release in the presence of SCN^- or valinomycin/K^+. The fact that either a lipophilic cation or anion is required argues against a mechanism of direct charge interaction with the local anaesthetic molecules. What seems more likely is that, although the process of local anaesthetic-catalysed H^+ transport does not result in charge transfer across the membrane, it is dependent upon the presence of a membrane potential. When the membrane potential is dissipated with valinomycin/K^+ or SCN^-, the reactions shown in Fig. 3 fail and other processes dominate. Both the free base and protonated forms of the anaesthetic are implicated from the pH dependence (Fig. 7), the effect of Ca^{2+} (Fig. 8) and the fact that neither benzocaine with its low pK not chlorpromazine methiodide show this behaviour. Most interesting, is the finding that non-anaesthetic amines listed in Table I may rapidly accelerate H^+ re-release in the presence or absence of lipid-soluble ions. Some of these compounds have quite similar pK_a values and are effective at similar concentrations to local anaesthetics (Table I). Several studies a number of years ago emphasised the importance of ester or amide function in the action of local anaesthetics [29,30]. In particular it was noted that the electronegativity of the carbonyl oxygen or amide nitrogen profoundly influences the potency of the anaesthetic. These functional groups must modify the translocational properties of the anasthetic from those of simpler amines. The presence of this extra dipole on the anaesthetic molecule may confine the reactions to the membrane interfaces where they would be more susceptible to changes in surface potential.

The relevance of this behaviour of tertiary amine anaesthetics to the mechanism of action potential blockade is not clear. A striking parallel, however, is afforded by the so-called 'use-dependent' or 'voltage-sensitive' inhibition in which local anaestethic (not quaternary analogues or benzocaine) blockage is potentiated by repetitive depolarization of the excitable membrane under voltage clamp [1,2,31]. Although these experiments were interpreted in terms of receptor-anaesthetic binding we suspect that the phenomenon may be more general and involve a voltage-dependent transport of local anaesthetic across the membrane.

The effect of 'high' concentrations of local anaesthetics and other amines on ion transport across chromatophore membranes appears to be less specific. The accelerated carotenoid shift decay indicates increased ionic permeability of the vesicles, which may be due to penetration by species such as $(R_1\text{-}R_2\text{-}R_3\text{-}N)_2H^+$ [6] since a dependence on the square of the anaesthetic concentration is observed across a narrow range. However, it is well known that concentrations of anaesthetic above those required for action potential blockade, render membranes generally leaky to ions and even proteins [5]. This can be seen as a fall in the resting potential of perfused axons, haemolysis of erythrocytes, etc. Such an effect of chromatophore membrane lysis may be produced by what we have called 'high' concentrations of anaesthetic. It is most pronounced at high pH owing probably to the greater anaesthetic solubility in the membrane and is not modified by the addition of lipid-soluble ions to the chromatophore suspension. The fact that benzocaine and chlorpromazine methiodide only display behaviour associated with 'high' concentrations of the other anaesthetics suggests that the increased membrane conductivity is consequent upon

increased permeability of the membrane to ions other than R_1-R_2-R_3-NH^+ or $(R_1$-R_2-R_3-$N)_2H^+$.

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