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FURTHER STUDIES ON F_1-ATPase INHIBITION BY LOCAL ANESTHETICS

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We have measured the inhibitory potencies of several local anesthetics (procaine, lidocaine, tetracaine and dibucaine) and related compounds (chlorpromazine, procainamide and propranolol) on the ATPase activities of bovine heart submitochondrial particles and purified F_1 extracted from these particles. All of these agents cause inhibition of ATPase in F_1 as well as in submitochondrial particles. A linear relationship is found between the log of the octanol/water partition coefficients and the log of the concentrations required for 50% inhibition of F_1. Sedimentation velocity ultracentrifugation and polyacrylamide gel electrophoresis showed that 1.0 mM tetracaine caused partial dissociation of the F_1 complex. Complete reversibility of the enzyme inhibitory effects was demonstrated, however. This work shows that local anesthetics can affect protein structure and enzyme activity without the mediation of lipid.

Introduction

The question 'What is the mechanism of action of anesthetics?' may be taken either in the narrow sense to mean how anesthetics cause nerve block (local anesthetics) or narcosis (general anesthetics), or it may understood more generally to mean how anesthetics affect physiological systems, with the neural mechanism being only one aspect of the general question. If it were true that anesthetics are highly specific agents affecting only nerve, this distinction would be irrelevant. This is not the case, however, and we are therefore pursuing the general question, with an emphasis at this point on the scope and mechanism of anesthetic effects on enzymes [1–3]. We have previously shown that various mitochondrial electron-transport enzymes are inhibited by anesthetics [1,2]; now we are carrying out similar studies on water-soluble enzymes.

It is well known that anesthetics both perturb the properties of membrane lipids and alter the activities of membrane enzymes (e.g., see Refs. 4 and 5). Arguments have been advanced by various workers to support each side of the question of whether membrane enzyme effects are due to direct interactions of anesthetics with proteins, or rather to indirect effects resulting from lipid perturbation [6–12].

In a recent note [3] we reported that tetracaine and *n*-butanol caused inhibition of F_1-ATPase isolated in a water-soluble, lipid-free state from beef heart mitochondria. The action of *n*-butanol on F_1 was similar to that which had been previously reported for the inhibition of oligomycin-insensitive ATPase activity in intact mitochondria [8,9]. ** This result demonstrated that direct protein effects by anesthetics are indeed possible, giving additional credence to the arguments previously ad-

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** Parenti-Castelli et al. [9] also observed the inhibition of F_1 by *n*-butanol in the lipid-free state.

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vanced favoring a direct mechanism of interaction of anesthetics with membrane proteins [1,2,6,12].

We have continued work on F_1 using tertiary amine local anesthetics and related compounds, and now show that not only tetracaine, but also a whole series of these agents give inhibition of ATPase activity. The inhibitory potencies are similar in F_1 and submitochondrial particles. Furthermore, the inhibitory potencies are directly correlated with the octanol/water partition coefficients. This means that the relative affinities of these anesthetics for F_1 are related by the same hydrophobic correlation which has for years been taken as evidence for a lipoidal site of primary action of anesthetics.

If it is true that the anesthetics affect protein structure as suggested by the enzyme inhibition studies, one would hope to be able to obtain direct evidence for this by physical characterization methods. In this paper, we show that both the shape of the sedimentation velocity Schlieren peak of F_1 and the electrophoretic pattern in polyacrylamide gels are changed by the presence of 1 mM tetracaine. These experiments demonstrate that low concentrations of tetracaine do indeed alter the macromolecular structure of F_1.

Materials and Methods

Mitochondrial preparations. Sonic submitochondrial particles were derived from bovine heart mitochondria as previously described [2,3,13,14]. Soluble F_1-ATPase was prepared according to the method of Beechey et al. [15]. The purity of the product so obtained was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis [16]; three bands were visible on normally loaded gels, and these had the apparent molecular weights expected for F_1 subunits [15]. The F_1 was stored at room temperature and could be used for up to 3 days after preparation without appreciable loss of activity.

Enzyme assays. An ATP-regenerating system [17] was used in the spectrophotometric assay of ATPase activity in F_1 or submitochondrial particles. The assay mixture consisted of 50 mM Tris-acetate (pH 7.5), 4 mM $MgCl_2$, 0.4 μM rotenone, 2.5 mM phospho*enol*pyruvate, 0.5 mM ATP, 0.35 mM NADH (incorrectly stated as 3.5 mM NADH in our previous paper [3]), 5 μl of an ammonium sulfate suspension containing 150 units of lactic dehydrogenase and 130 units of pyruvate kinase (Sigma, lactic dehydrogenase type I), and an aliquot of F_1 solution or submitochondrial particle suspension. The enzyme reaction was initiated by the final addition of ATP; this followed a 2 min preincubation at the desired temperature. An aliquot of a concentrated solution of the chosen anesthetic was added just prior to the 2 min preincubation. The final volume was 2 ml. The rate was determined by following the absorbance change at 355 nm in a Cary 219 spectrophotometer. (This wavelength was used since most of the tertiary amines employed absorb strongly at 340 nm.) The spectrophotometer was equipped with a water-jacketed cell holder. Each determination was performed in duplicate or triplicate, and the results averaged.

Particular attention was given to the possible effects of the anesthetic agents on the lactic dehydrogenase-pyruvate kinase ATP-regenerating system. Since there is some inhibition of these auxiliary enzymes by the anesthetics (unpublished observations), they were added in a sufficiently large excess to ensure by empirical test that the ATPase reaction was indeed the rate-limiting step for all anesthetic concentrations employed.

Ultracentrifugation. Sedimentation velocity experiments on F_1 in the absence and presence of tetracaine were carried out using a Beckman Model E analytical ultracentrifuge equipped with electronic speed control. All runs were at 48000 rpm in an AN-D rotor, with the temperature maintained at 20°C and the phase plate set at 60°. The time at which the rotor achieved two-thirds of the set speed was defined as time zero. The stock F_1 solution was concentrated for the ultracentrifugation studies by passing it through a Diaflow PM-10 membrane under nitrogen pressure. The concentrated supernatant was dialyzed at room temperature against 10 mM Tris-sulfate buffer containing 1 mM EDTA at pH 7.6, which served to remove the sucrose that remained from the isolation procedure. The final protein concentration was determined by the method of Lowry et al. [28] to be 3 mg/ml. 0.4 ml of this solution was placed in the sample cell, and tetracaine was also added when desired. The enzyme was in the presence of

the tetracaine for about 30 min before time zero of the ultracentrifugation run.

Electrophoresis. The method of Ornstein [18] and Davis [19] was adapted for electrophoretic studies on F_1. A 6% polyacrylamide gel with 1% cross-linking was employed. Electrophoresis was carried out in 0.6 × 7 cm gels, at constant voltage. The buffer in both reservoirs was 10 mM Tris-sulfate, pH 7.6. The gels were stained with Coomassie brilliant blue R in water/acetic acid/isopropanol (65:25:10, v/v). The F_1 solutions to be electrophoresed were incubated in the desired concentration of tetracaine for about 25 min before being applied to the gels. Electrophoresis was begun promptly after the samples were applied.

Source of materials. All anesthetics and biochemical reagents were obtained from Sigma. The beef heart mitochondria were generously supplied by Dr. D.E. Green of the University of Wisconsin-Madison.

Results

Anesthetic inhibition of ATPase. The relative activity of F_1-ATPase is given in Fig. 1 as a function of the concentrations of several anesthetics and related compounds. Similar data are given in Fig. 2 for the ATPase activity of submitochondrial particles derived from beef heart mitochondria. Comparison of these figures shows there is a high degree of similarity between the effects of the various agents on F_1 and submitochondrial particles. The concentrations required for 50% inhibition are given in Table I. It can be seen in Table I that for most anesthetics the submitochondrial particles are somewhat more sensitive to inhibition than F_1. Chlorpromazine is the most potent agent tested, and also shows the greatest difference in effect, with 26 and 72 μM giving 50% inhibition in submitochondrial particles and F_1, respectively. These chlorpromazine results are in reasonable agreement with earlier reports [20,21].

The shapes of the curves in Figs. 1 and 2 are worthy of note. First, they are hyperbolic rather than sigmoidal, as found for inhibition of mitochondrial electron transport by the same agents [2]. Second, with several agents there is a residual activity of 20–40% of the original at high

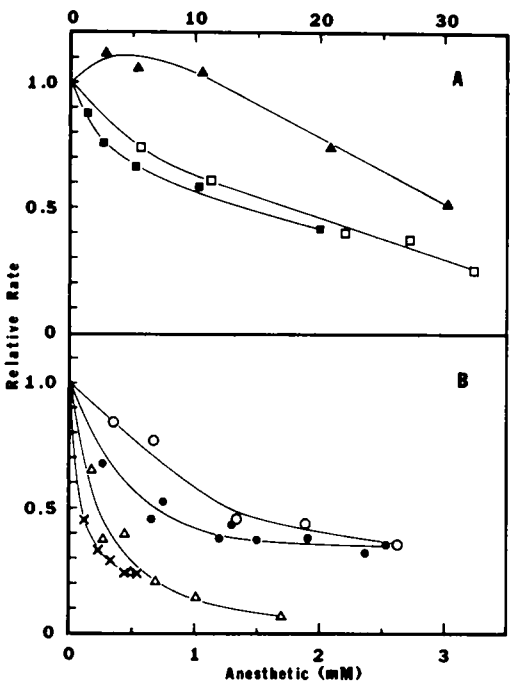

Fig. 1. Inhibition of F_1-ATPase by local anesthetics and related compounds, at 25°C. A, procainamide (▲), procaine (□), lidocaine (■); B, propranolol (○), tetracaine (●), dibucaine (△), and chlorpromazine (×). Note that the concentration axis for A (given at top) is 10-fold greater than that for B.

anesthetic concentration. The exceptions are dibucaine, which gives close to complete inhibition in both F_1 and submitochondrial particles, and chlorpromazine, which gives nearly full inhibition in submitochondrial particles, but shows a plateau with F_1. Finally, the exception to both of the above generalizations is procainamide, which alone gave activation at low concentrations followed by inhibition as its concentration was increased.

Temperature dependence of inhibition. The concentrations of the various agents required for 50% inhibition of F_1 are given in Table I for 10 and 35°C, in addition to 25°C. The results are best summarized by saying that there is no pronounced or uniform effect for all anesthetics over the range studied. For some agents (i.e., procaine, lidocaine and dibucaine), potency appears to increase with temperature, whereas with others (i.e., tetracaine, chlorpromazine, propanolol and procainamide) it decreases. This is in contrast to inhibition of electron transport in which a strong temperature dependence was observed for most agents, with

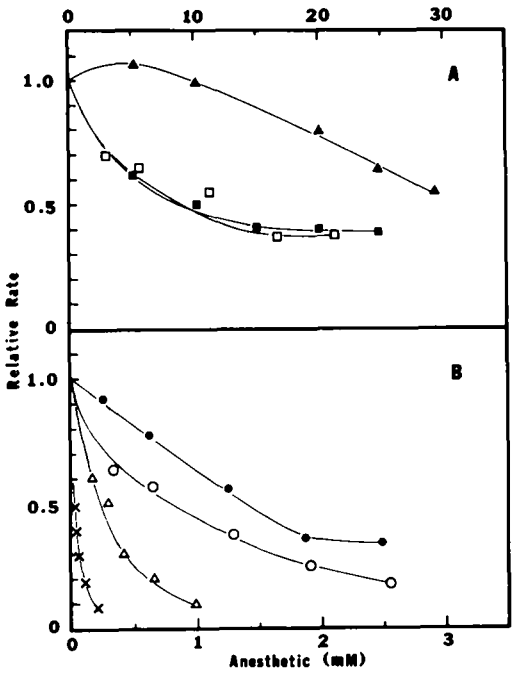

Fig. 2. Inhibition of ATPase activity of bovine heart submitochondrial particles by local anesthetics and related compounds, at 25°C. The symbols have the same meanings as in Fig. 1.

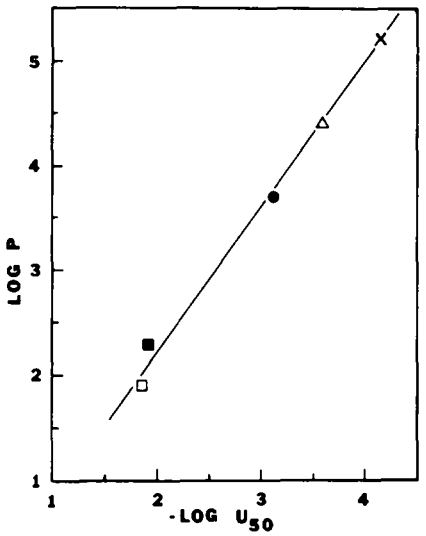

Fig. 3. Logarithmic plot of the octanol/water partition coefficients [22] vs. the negative of the logarithms of U_{50}, the concentrations of anesthetic required for 50% inhibition of F_1-ATPase at 25°C. See the legend to Fig. 1 for the meanings of the symbols. The numerical values for log P and U_{50} are given in Table I.

potency increasing as the temperature was raised [2].

Hydrophobicity correlation. Fig. 3 is a logarithmic plot of the octanol/water partition coefficients [22] vs. the concentrations required for 50% inhibition of F_1. Propranolol and procainamide are not shown here, since their partition coefficients could not be located. The least-squares best-fit line has a slope of 1.4 and a correlation coefficient of 0.995. This linear correlation may be interpreted to mean the hydrophobicity of the molecules employed is a major determinant of their relative affinity for F_1. A similar plot is obtained for the inhibition of ATPase in submitochondrial particles by these agents.

TABLE I

CONCENTRATIONS OF ANESTHETIC AGENTS REQUIRED FOR 50% INHIBITION OF ATPase ACTIVITY

P, octanol/water partition coefficient [22]; SMP, submitochondrial particles; n.d., not determined. Values expressed in mM.

Substance	log *P*	F_1			SMP (25°C)
		10°C	25°C	35°C	
Procaine	1.9	17	16	15	9.5
Lidocaine	2.3	16	13	12	10
Tetracaine	3.7	n.d.	0.70	0.83	1.4
Dibucaine	4.4	0.50	0.25	0.19	0.26
Chlorpromazine	5.3	0.050	0.072	0.10	0.026
Propranolol		0.87	1.3	1.4	0.84
Procainamide		17	31	35	31

Reversibility. A detailed study was carried out to determine whether the anesthetic inhibition is reversible. Tetracaine and dibucaine were used for these experiments. Reversibility experiments can be performed either by physically removing the inhibitory agent, as by dialysis, or by decreasing its concentration, as by dilution. We chose the latter method since it is more rapid and less liable to error from undetermined sources than the former. The experiments were carried out on the following manner. First, control experiments were run on the particular F_1 preparation to determine the degree of inhibition given by 1.5 and 0.75 mM tetracaine, or 0.5 and 0.25 mM dibucaine, under normal assay conditions. Samples were then prepared at normal assay volume but containing twice the usual concentration of F_1 and the other components of the assay system (but excluding ATP). Tetracaine or dibucaine was added to a concentration of 1.5 or 0.5 mM, respectively. Following incubation for 5 min at 25°C, the tubes were diluted to twice normal volume by the addition of buffer; this dilution reduced the anesthetic concentration by half, but returned the concentration of F_1 and the other components to their normal values. ATP was added and the enzyme rate measured. At most, 15 s were allowed for reversal of the inhibition by the dilution. The results of these experiments are shown in Table II. It can be seen that for both tetracaine and dibucaine, the activity following dilution was close to that given by the lower inhibitor concentration. Thus, reversal of the additional inhibition given by the higher anesthetic concentration was, within experimental error, complete.

While the results given in Table II are for 5 min incubation of the enzyme in the presence of anesthetic, a similar degree of reversal was found after 20 min of incubation. Also, the activity of the enzyme in the presence of anesthetic remained constant for at least 30 min. This is further evidence that the inhibition observed is truly revesible, and not a time-dependent irreversible process. (We make this claim only for the tertiary amine anesthetics; experiments with normal alcohols, not reported here, suggest that their perturbational effects may be more drastic, and not necessarily reversible.)

Ultracentrifugation. Fig. 4 shows the Schlieren patterns observed in a sedimentation velocity experiment with F_1 in anesthetic-free buffer and in the presence of 1.0 mM tetracaine. A single sedimenting band was observed in the absence of anesthetic having a sedimentation coefficient of 13.95 S. This value compares favorably with previously determined sedimentation values for F_1, e.g., 14.1 [15], 12.9 [23] and 13.05 S [24]. In the presence of tetracaine, the shape of the main band changed from having a smooth gaussian shape to a sharpened profile, and in addition a slowly sedimenting band with a coefficient of about 3.95 S appeared. The main band had a coefficient of 13.05 S in the presence of tetracaine. In some experiments a rapidly sedimenting fraction was also seen when tetracaine was present. Since hypersharp peaks are indicative of a heterogeneous system [25], and since additional bands were seen in the presence of tetracaine, it appears that tetracaine partially disrupted the multisubunit structure of F_1.

Electrophoresis. A photograph of the electrophoretic pattern given by F_1 on polyacrylamide gels in the absence and presence of tetracaine is shown in Fig. 5. The three gels shown were run simultaneously and were identical in all ways except for the presence of the indicated amounts of

TABLE II

REVERSAL OF ANESTHETIC INHIBITION OF F_1-ATPase BY DILUTION

The experiment was carried out as described in the text. Quadruplicate determinations were performed on each sample. The ATPase activity is given relative to that observed in the absence of anesthetic. The dilution was performed after 5 min incubation at the higher concentration, and the spectrophotometric assay of activity was begun within 15 s of the dilution.

Anesthetic	% activity
Tetracaine (mM)	
0	100
0.75	52 ± 4
1.5	37 ± 4
Dilution: 1.5 to 0.75	48 ± 2
Dibucaine (mM)	
0	100
0.25	38 ± 8
0.5	24 ± 6
Dilution: 0.5 to 0.25	42 ± 3

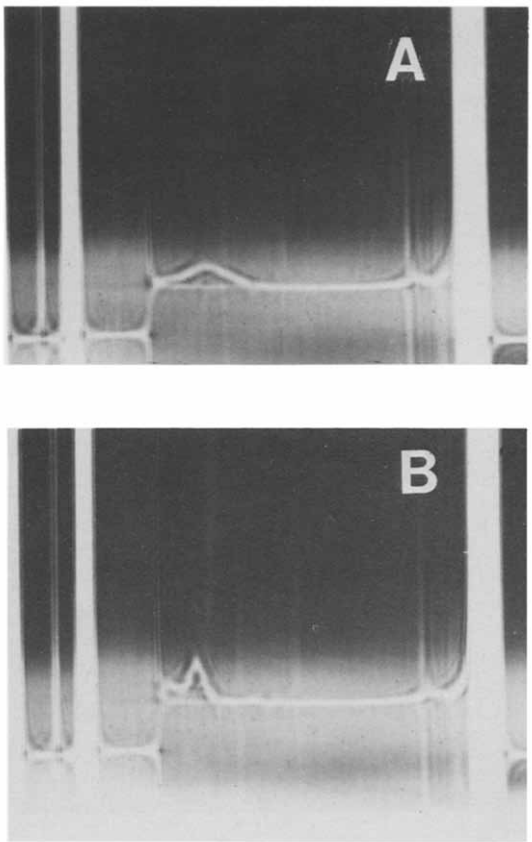

Fig. 4. The Schlieren patterns for F_1 obtained in sedimentation velocity ultracentrifugation runs in the absence (A) and presence (B) of 1.0 mM tetracaine, with all other conditions identical. These photos were taken at 8.5 and 8.0 min after the start of the runs, respectively. The conditions were as described in the text.

tetracaine in the samples applied. A single intense band was seen in the absence of tetracaine (Fig. 5A), which confirms the essential homogeneity of the preparation. Addition of 1.0 or 2.0 mM tetracaine to the F_1 solution caused the mobility of the intense band to decrease and also caused the production of diffuse bands of greater mobility than the intense band (Fig. 5B and C). In each case there was also a small fraction of staining material which failed to enter the gel.

For the experiments shown in Fig. 5, tetracaine was added only to the F_1 solution and not to the gel itself or to the reservoir buffers. In a separate series of experiments (not shown), 1.0 mM tetracaine was included in the reservoir buffers and in

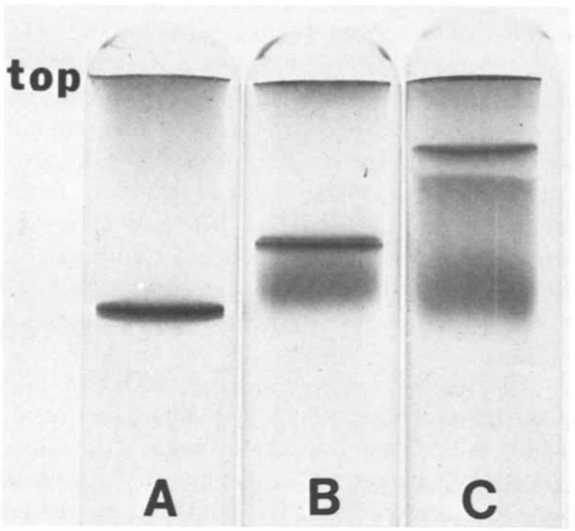

Fig. 5. Polyacrylamide gel electrophoresis patterns of F_1 in the absence of anesthetic (A), or following incubation for 25 min in the presence of 1.0 mM (B) or 2.0 mM (C) tetracaine. See text for the details of the conditions employed.

the gels. Under these conditions all of the protein material had very low mobility. We attribute this diminished mobility to a change in net charge on the protein, resulting from binding of the positively charged tetracaine molecules to the protein.

The electrophoresis results confirm the interpretation offered for the ultracentrifugation pattern, viz., that tetracaine causes a partial disruption of the multisubunit structure of F_1.

Discussion

The results presented here show clearly that the tertiary amine anesthetics have a pronounced effect on the activity of mitochondrial ATPase in both the membrane-bound and water-soluble states. At the pH of our experiments, the tertiary amines employed are expected to be in a predominantly cationic, monomeric state, since the pK_a values range from 7.85 for lidocaine to 8.95 for procaine [4], and since the critical micellar concentration of tetracaine (for example) is 61 mM at pH 6.5 [26]. The physical changes observed by electrophoresis and ultracentrifugation demonstrate that structural modifications of F_1 are induced by tetracaine. The results of both of these

methods suggest that tetracaine induces the dissociation of one or more subunits from the holoenzyme. The portion of F_1 which remains intact must still have some enzymic activity, however, in order to account for the plateau of 20–40% residual activity found at high concentrations of anesthetic. Furthermore, the demonstration that the tetracaine and dibucaine inhibition is reversible implies that the subunit(s) which apparently dissociate in the presence of these agents are capable of reassociation upon decreasing the concentration of the anesthetic.

It is clear from the results presented here and in our earlier papers [1–3] that local anesthetics inhibit many aspects of mitochondrial function. We showed previously [2] that the mitochondrial electron-transport chain is inhibited at several points or regions by alcohols and tertiary amine anesthetics. Now we see that the mitochondrial ATPase activity is likewise inhibited. The concentrations required for ATPase inhibition are close to those reported to give nerve block (e.g., 0.7 and 0.45 mM tetracaine and dibucaine, respectively, for nerve block [27]; as compared to 0.7 and 0.25 mM, respectively, for F_1 at 25°C, Table I). The net result of these mitochondrial effects in anesthetized tissue will be a slowing down of ATP production and hence also of energy-requiring processes in general. This could hardly be considered a desirable characteristic of these widely used drugs, but their redeeming feature is that their inhibitory effects appear to be readily reversible, both under clinical conditions and in vitro.

Since it is now evident that membrane-bound as well as soluble enzymes can be inhibited by local anesthetics in the same concentration range as gives nerve block, it is plausible to suggest that the structure and activity of one or more neural enzymes or membrane-transport proteins (e.g., the sodium 'gate' [5]) are altered as a result of a direct interaction with the anesthetic molecules. This idea is compatible with the 'degenerate protein perturbation hypothesis' of Richards et al. [6] for the mechanism of anesthetic action.

Acknowledgments

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References

1 Vanderkooi, G., Chazotte, B. and Biethman, R. (1978) FEBS Lett. 90, 21–23
2 Chazotte, B. and Vanderkooi, G. (1981) Biochim. Biophys. Acta 636, 153–161
3 Vanderkooi, G., Shaw, J., Storms, C., Vennerstrom, R. and Chignell, D. (1981) Biochim. Biophys. Acta 635, 200–203
4 MacDonald, A.G. and Wann, K.T. (1978) Physiological Aspects of Anesthetics and Inert Gases, Academic Press, London
5 Richards, C.D. (1978) International Review of Biochemistry, Biochemistry of Cell Walls and Membranes II, vol. 19 (Metcalfe, J.C., ed.), pp. 157–219, University Park Press, Baltimore
6 Richards, C.D., Martin, K., Gregory, S., Keightley, C.A., Hesketh, T.R., Smith, G.A., Warren, G.B. and Metcalfe, J.C. (1978) Nature 276, 775–779
7 Lenaz, G., Curatola, G., Mazzanti, L., Parenti-Castelli, G. and Bertoli, E. (1978) Biochem. Pharm. 27, 2835–2844
8 Lenaz, G., Parenti-Castelli, G. and Sechi, A.M. (1975) Arch. Biochem. Biophys. 167, 72–79
9 Parenti-Castelli, G., Sechi, A.M., Landi, L., Cabrini L., Mascarello, S. and Lenaz, G. (1979) Biochim. Biophys. Acta 547, 161–169
10 Lee, A.G. (1976) Nature 262, 545–548
11 Trudell, J.R. (1977) Anesthesiology 46, 5–10
12 Eyring, H., Woodbury, J.W. and D'Arrigo, J.S. (1973) Anesthesiology 38, 415–424
13 Blair, P.V. (1967) Methods Enzymol. 10, 78–81
14 Gregg, C.T. (1967) Methods Enzymol. 10, 181–185
15 Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D. and Munn, E.A. (1975) Biochem. J. 148, 533–537
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
17 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
18 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321–349
19 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
20 Low, H. (1959) Biochim. Biophys. Acta 32, 11–18
21 Penefsky, H.S., Pullman, M.E., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3330–3336
22 Kunze, H., Nahas, N., Traynor, J.R. and Wurl, M. (1976) Biochim. Biophys. Acta 441, 93–102
23 Penfesky, H.S. and Warner, R.C. (1965) J. Biol. Chem. 240, 4694–4702
24 Warshaw, J.B., Lam, K.W., Nagy, B. and Sanadi, D.R. (1968) Arch. Biochem. Biophys. 123, 385–396
25 Tanford, C., (1961) Physical Chemistry of Macromolecules, John Wiley, New York, p. 379
26 Fernandez, M.S. (1981) Biochim. Biophys. Acta 646, 27–30
27 Goldstein, A., Aronow, L. and Kalman, S.M. (1968) Principles of Drug Action, Harper and Row, New York, p. 766
28 Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275