genesis was as high (22.64 ± 2.93 μmoles × 30 min^{-1} × 10^8 cells^{-1}, $n = 4$) as with 1 mM oleate. Since octanoate is not esterified by the liver [13] it appears that not only esterification but rather the overall metabolic state of liver cells rapidly utilising free fatty acids is responsible for the observed labilization of endogenous triglycerides. The details of the mechanism(s) involved remain to be elucidated. They could provide valuable information pertaining to the situation in the perfused liver of diabetic rats which produces ketone bodies at a high rate, regardless whether fatty acids are present in the perfusion medium or not [14]. It is possible that in this situation, as in the experiments reported here, the continuous increased supply of fatty acids to the liver *in vivo* would not only sustain the high concentration of hepatic triglycerides but also accelerate their turnover.

Acknowledgements—The authors thank Miss D. Duyckaerts and Mr. L. Govaert for excellent technical assistance. This work was supported by a grant from the Fonds voor Geneeskundig Wetenschappelijk Onderzoek.

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REFERENCES

1. G. Mannaerts, L. J. Debeer and P. J. De Schepper, *Biochem. Pharmac.* **23,** 75 (1974).
2. L. J. Debeer, G. Mannaerts and P. J. De Schepper, *Biochem. Pharmac.* **24,** 1035 (1975).
3. R. N. Zahlten, N. M. Kneer, F. W. Stratman and H. A. Lardy, *Archs Biochem. Biophys.* **161,** 528 (1974).
4. R. Christiansen, B. Borrebaek and J. Bremer, *FEBS Letters* **62,** 313 (1976).
5. D. H. Williamson and J. Mellanby in *Methods of Enzymatic Analysis* (Editor, H. U. Bergmeyer) p. 459. Academic Press, New York (1965).
6. H. U. Bergmeyer und E. Bernt, *Enzym. Biol. Clin.* **5,** 65 (1965).
7. J. D. McGarry, M. J. Guest and D. W. Foster, *J. biol. Chem.* **245,** 4382 (1970).
8. W. Huth, C. Dierich, V. von Oeynhausen and W. Seubert, *Z. phys. Chem.* **354,** 635 (1973).
9. A. Hasselblatt, *Naunyn-Schmiederbergs Arch. exp. Path. Pharmak.* **262,** 152 (1969).
10. L. J. Debeer, J. Thomas, G. Mannaerts and P. J. De Schepper, *J. clin. Invest* in press (1977).
11. J. A. Ontko, *J. biol. Chem.* **247,** 1788 (1972).
12. L. J. Debeer, G. Mannaerts and P. J. De Schepper, *Eur. J. Biochem.* **47,** 591 (1974).
13. A. Kornberg and W. E. Pricer, *J. biol. Chem.* **204,** 345 (1953).
14. D. R. Van Harken, C. W. Dixon and M. Heimberg, *J. biol. Chem.* **244,** 2278 (1969).

Biochemical Pharmacology, Vol. 26, pp. 791–793. Pergamon Press, 1977. Printed in Great Britain.

Effects of local anesthetics and cholesterol on the ($Na^+ + K^+$)-dependent ATPase

(*Received* 18 *August* 1976; *accepted* 27 *September* 1976)

The ($Na^+ + K^+$)-dependent ATPase represents the biochemical basis for transporting Na^+ and K^+, the sodium pump, and is present *in vivo* in the plasma membrane [1]. Among the consequences of this localization is the sensitivity of the enzyme to membrane structure. Early studies showed that alternations in membrane lipids (such as enzymatic digestion or solvent extraction) drastically decreased activity, and that activity could be restored by replacing lipids [2–5]. Nevertheless, disagreements about the relative importance of various lipid classes have been difficult to resolve. For example, Wheeler and Whittam [4] obtained optimal restoration of ATPase activity only when phosphatidyl serine was added, and Noguchi and Freed [5] found that the presence of cholesterol was crucial, whereas Hilden and Hokin [6] were able to restore activity when the only lipid present was phosphatidyl choline.

The experiments presented here are concerned with another aspect of enzyme–lipid interactions, the effects on enzyme activity of agents known to interact with the lipid bilayer: cholesterol and two local anesthetics, procaine and benzyl alcohol (one largely ionized at the pH used and the other unionized). These agents were studied in terms of their effects on cation activation both of the ($Na^+ + K^+$)-dependent ATPase and of the related K^+-dependent phosphatase reaction, which appears to reflect the terminal hydrolytic steps of the overall ATPase reaction [1]. The data demonstrate differences between the three agents in effects on several kinetic parameters, as well as generally greater effects at an incubation temperature below the lipid phase transition temperature of the membrane.

The enzyme preparation used was obtained from rat brain microsomes after treatment with deoxycholate and NaI, as described previously [7]. For addition of cholesterol, a suspension of 0.2 mM cholesterol in 60 mM histidine-HCl/Tris (pH 7.8) was sonicated to translucence, and then mixed with an equal volume of the enzyme preparation 10 min before initiating the assay. The final concentration of cholesterol in the incubation medium was 0.01 mM. Controls without cholesterol were run concurrently.

($Na^+ + K^+$)-dependent ATPase activity was measured in terms of the production of P_i, as previously described [7]. The standard medium contained 30 mM histidine-HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP (as the Tris salt), 90 mM NaCl, 10 mM KCl, and the enzyme preparation (0.1 mg protein/ml). Activity in the absence of Na^+ and K^+ was measured concurrently; such activity averaged only a few per cent of the ($Na^+ + K^+$)-dependent ATPase activity [7], and was subtracted from the total activity in the presence of Na^+ and K^+ to give the ($Na^+ + K^+$)-dependent activity. Because of variations in the absolute activity of different enzyme preparations, enzyme velocities are expressed relative to the ($Na^+ + K^+$)-dependent ATPase activity of the concurrent control incubation in the standard medium, defined as 1.0. K^+-dependent phos-

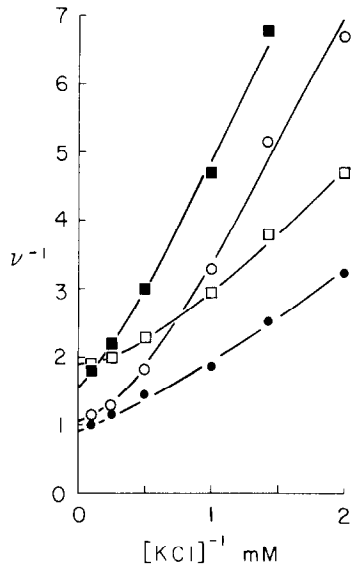

Fig. 1. Effect of KCl on $(Na^+ + K^+)$-dependent ATPase activity at 37°. The enzyme preparation was incubated in media containing 30 mM histidine-HCl/Tris (pH 7.8), 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and the concentrations of KCl, plus other additions as noted: none (●), procaine (○), benzyl alcohol (■), or cholesterol (□). Data are presented in double-reciprocal Lineweaver–Burk form, with velocity expressed relative to that in the presence of 10 mM KCl (and the absence of experimental agents) defined as 1.0.

phatase activity was measured in terms of the production of *p*-nitrophenol after incubation with *p*-nitrophenylphosphate, as previously described [8]. The standard medium contained 30 mM histidine-HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM *p*-nitrophenylphosphate (as the Tris salt), 10 mM KCl, and the enzyme preparation (0.1 mg protein/ml). Activity in the absence of added KCl was measured concurrently; such activity averaged only a few per cent of the K^+-dependent phosphatase activity under optimal conditions [8], and was subtracted from the total activity in the presence of KCl to give the K^+-dependent activity. As with the ATPase, velocities are expressed relative to the K^+-dependent phosphatase activity of a concurrent control incubation in the standard medium, defined as 1.0. In these experiments, the incubation was initiated by adding the enzyme to the complete medium. Preliminary studies indicated that under control conditions and in the presence of 3 mM procaine and 60 mM benzyl alcohol the liberation of product increased linearly with time during the standard incubation periods used, 8 min at 37° and 25 min at 16°; to obtain consistent results with added cholesterol, however, it was necessary to preincubate the enzyme with the suspension for 10 min, as specified above. For both activities the data presented represent the averages of four or more experiments performed in duplicate. Statistical significance was calculated by the procedure of Dunnett [9].

The $(Na^+ + K^+)$-dependent ATPase was inhibited during incubations with 3 mM procaine and 60 mM benzyl alcohol, and after preincubation with 0.1 mM cholesterol. The degree of inhibition, however, varied not only between inhibitors but also with the concentration of monovalent cations and incubation temperature (Fig. 1; Tables 1 and 2). To quantitate such effects, both the $K_{0.5}$ for the cations (the concentration for half-maximal activation) and V_{max}

Table 1. Kinetic parameters for K^+*

Temperature	Additions	ATPase		Phosphatase	
		V_{max}	$K_{0.5}$ (mM)	V_{max}	$K_{0.5}$ (mM)
37	None	1.06 ± 0.01	1.18 ± 0.12	1.10 ± 0.04	1.46 ± 0.16
	Procaine	1.00 ± 0.09	1.62 ± 0.12	1.10 ± 0.02	1.44 ± 0.07
	Benzyl alcohol	0.59 ± 0.07†	1.14 ± 0.04	0.52 ± 0.03†	6.84 ± 0.75†
	Cholesterol	0.55 ± 0.06†	0.76 ± 0.07†	1.02 ± 0.04	2.19 ± 0.14†
16	None	0.90 ± 0.04	0.59 ± 0.09	1.13 ± 0.03	0.45 ± 0.03
	Procaine	0.90 ± 0.02	1.17 ± 0.11	0.92 ± 0.04†	0.74 ± 0.04†
	Benzyl alcohol	0.79 ± 0.03	0.87 ± 0.09	0.41 ± 0.03†	4.23 ± 0.77†
	Cholesterol	0.77 ± 0.14	0.46 ± 0.07	0.94 ± 0.03†	1.02 ± 0.06†

* ATPase and phosphatase activities were measured at 37° and 16°, as described in the text. V_{max} was obtained from Lineweaver–Burk plots and $K_{0.5}$ from least squares lines of Hill plots of the individual experiments [7].
† $P < 0.05$.

Table 2. Kinetic parameters for Na^+*

Temperature	Additions	ATPase	
		V_{max}	$K_{0.5}$ (mM)
37	None	1.02 ± 0.04	5.35 ± 0.37
	Procaine	1.08 ± 0.11	6.00 ± 0.38
	Benzyl alcohol	0.44 ± 0.03†	7.38 ± 0.37†
	Cholesterol	0.73 ± 0.03†	5.48 ± 0.43
16	None	0.97 ± 0.02	5.64 ± 0.25
	Procaine	0.88 ± 0.02†	4.73 ± 0.45
	Benzyl alcohol	0.67 ± 0.01†	11.32 ± 0.61†
	Cholesterol	0.88 ± 0.01†	3.47 ± 0.31†

* V_{max} and $K_{0.5}$ were obtained as in Table 1.
† $P < 0.05$.

were determined in the presence and absence of the agents Data are presented for these parameters in Tables 1 and 2; the experiments measuring K^+-activation of the ATPase are also illustrated in Fig. 1.

Although procaine, at the concentration used, had little effect on the V_{max} of the ATPase reaction, benzyl alcohol and cholesterol reduced V_{max} significantly (Table 1). On the other hand, cholesterol did not alter the V_{max} of the related K^+-dependent phosphatase reaction, whereas benzyl alcohol reduced it to the same extent as with the ATPase.

With regard to the $K_{0.5}$ values for the cations activating the ATPase reaction, procaine increased the $K_{0.5}$ for K^+ 2-fold at 16° (with a smaller but non-significant increase at 37°), although procaine did not affect the $K_{0.5}$ for Na^+ at either incubation temperature. By contrast, benzyl alcohol did not alter significantly the $K_{0.5}$ for K^+, but increased the $K_{0.5}$ for Na^+. Cholesterol also had little effect on the $K_{0.5}$ for K^+, but significantly reduced the $K_{0.5}$ for Na^+ at 16°.

Effects on K^+-activation of the phosphatase activity were also distinctive, and differed from effects on K^+-activation of the ATPase as well. Benzyl alcohol produced a 5- to 9-fold increase in $K_{0.5}$ (although it did not significantly alter the $K_{0.5}$ for K^+ of the ATPase). Cholesterol, which if anything tended to decrease the $K_{0.5}$ for K^+ in the ATPase reaction, significantly increased the $K_{0.5}$ for K^+ in the phosphatase reaction. As before, effects were generally greater at 16° than at 37°.

Although lipid–enzyme interactions are incompletely understood, certain qualitative descriptions are possible, and the effects of the local anesthetics and of cholesterol may be considered in this context. Local anesthetics apparently increase disorder within the lipid bilayer of the membrane, thereby producing a more fluid state [10, 11]. Cholesterol also dissolves in the hydrophobic portion of the membrane, but through intercalations between the phospholipid molecules may decrease fluidity [12]. Kimelberg [13] found a rise in energy of activation of the $(Na^+ + K^+)$-dependent ATPase associated with an increase in membrane cholesterol, and Farias *et al.* [14] attributed an increased cooperativity for fluoride-inactivation of the ATPase in enzyme preparations from rats fed a high cholesterol diet to tighter packing of membrane lipids.

The effect of temperature on the response to these agents also bears on lipid–enzyme interactions, and is consistent with actions of the agents through the lipid matrix. Arrhenius plots of the $(Na^+ + K^+)$-dependent ATPase show a break near 20° [15, 16]; this change in energy of activation is sensitive to the lipid composition of the enzyme preparation [17], and is attributed to lipid phase transitions [18]. In accord with this, the effects of these agents were generally greater at 16°, below the lipid phase transition, than at 37°, above the transition temperature.

Finally, the disparate effects of these agents on K^+-activation of the ATPase and phosphatase reactions also relate to the nature of the K^+-sites of the enzyme. Although in both reactions of this enzyme K^+ activates a dephosphorylation, the K^+-sites for each have been shown to differ in terms of several criteria, including apparent affinity for K^+, relative efficacy of other cations as substitutes for K^+, and sensitivity to pH [19]. The agents studied here reflect a further distinction between the higher affinity β-sites seen in the ATPase reaction, and the lower affinity α-sites seen in the phosphatase reaction [19], the latter being far more sensitive.

Acknowledgement—This work was supported by USPHS research grant NS-05430, and was taken in part from a thesis submitted by G. A. M in partial fulfillment of the requirements for the M.S. degree at the State University of New York.

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REFERENCES

1. J. Dahl and L. E. Hokin, *A. Rev. Biochem.* **43**, 327 (1974).
2. R. Tanaka and K. P. Strickland, *Archs Biochem. Biophys.* **111**, 583 (1965).
3. K. Taniguchi and Y. Tonomura, *J. Biochem., Tokyo* **69**, 543 (1971).
4. K. P. Wheeler and R. Whittam, *J. Physiol., Lond.* **207**, 303 (1970).
5. T. Noguchi and S. Freed, *Nature New Biol.* **230**, 148 (1971).
6. S. Hilden and L. E. Hokin, *Biochem. biophys. Res. Commun.* **69**, 521 (1976).
7. J. D. Robinson, *Biochemistry* **6**, 3250 (1967).
8. J. D. Robinson, *Biochemistry* **8**, 3348 (1969).
9. C. W. Dunnett, *J. Am. statist. Ass.* **50**, 1096 (1955).
10. J. C. Metcalfe, P. Seeman and A. S. V. Burgen, *Molec. Pharmac.* **4**, 87 (1968).
11. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
12. J. Kroes, R. Ostwald and A. Keith, *Biochim. biophys. Acta* **274**, 71 (1972).
13. H. K. Kimelberg, *Biochim. biophys. Acta* **413**, 143 (1975).
14. R. Farias, B. Bloy, R. Morero, F. Sineriz and R. Trucco, *Biochim. biophys. Acta* **415**, 231 (1975).
15. N. Gruener and Y. Avi-Dor, *Biochem. J.* **100**, 762 (1966).
16. J. D. Robinson, *J. Neurochem.* **14**, 1143 (1967).
17. R. Tanaka and A. Teruya, *Biochim. biophys. Acta* **323**, 584 (1973).
18. C. M. Grisham and R. E. Barnett, *Biochemistry* **12**, 2635 (1973).
19. J. D. Robinson, *Biochim. biophys. Acta* **384**, 250 (1975).