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INTERACTION OF BENZOIC ACID DERIVATIVES WITH THE TRANSPORT SYSTEM OF GLUCOSE IN HUMAN ERYTHROCYTES

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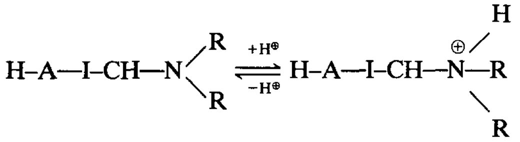
Abstract—The effect of benzoic acid derivatives on glucose uptake in human erythrocytes was studied. It was observed that all compounds competitively inhibit glucose transport and that the inhibition was a reversible process. The kinetic analysis revealed that benzoic acid derivatives form complexes with the transport system with either one or two molecules bound to the transport site. Elongation of the methyl group of the ester to the butyl group led to a higher affinity of the benzoic acid derivatives to the transport system. Their inhibitory potency (except some compounds with free COOH groups) correlated with their octanol–water partition coefficients, i.e. with their hydrophobicity. Comparing the inhibitory potencies of the local anesthetics with those of the benzoic acid derivatives it was found that the substituted NH_2 groups did not intensify the effect of the local anesthetics.

A wide variety of biological processes is influenced by local anesthetics [1–5], which are from a chemical point of view mostly secondary or tertiary amine compounds. Their molecules can be divided into three parts: a hydrophobic aromatic one (H–A), an intermediate chain (I–CH) and a substituted amino group $\left(-N\begin{smallmatrix}\nearrow R\\ \searrow R\end{smallmatrix}\right)$. The latter exists in a charged cationic or an uncharged form. The relation of these two forms depends on the p*K* value of the $-N\begin{smallmatrix}\nearrow R\\ \searrow R\end{smallmatrix}$ group and the pH of the bathing solution:



$$\text{H-A—I-CH—N}\begin{smallmatrix}\nearrow R\\ \searrow R\end{smallmatrix} \underset{-H^{\oplus}}{\overset{+H^{\oplus}}{\rightleftharpoons}} \text{H-A—I-CH—}\overset{\overset{H}{\oplus/}}{\underset{\underset{R}{\diagdown}}{N}}\text{—R}$$

The question whether the charged, the uncharged or both groups are responsible for the biological activities of local anesthetics is not yet clarified [6].

Recently we dealt with the significance of the hydrophobic part of the local anesthetics on glucose transport in human erythrocytes [7]. The present paper reports studies of the influence of compounds (benzoic acid derivatives), consisting only of the hydrophobic part and intermediate chains* of various lengths, on glucose movement across the erythrocyte membrane. They differ from the usual local anesthetics bearing no substituted NH_2 group in their molecule and consequently are uncharged. One representative of this group, benzocaine is known to exert a local anesthetic effect [8].

MATERIALS AND METHODS

Human blood of healthy donors was collected in ACD-solution (11 g sodium citrate, 35 g glucose, 4 g citric acid with aqua bidest, ad 1000 ml). After removal of the plasma by centrifugation, the erythrocytes were preloaded with 200 mM glucose by four washings in isotonic NaCl solution, containing 200 mM glucose. 150 μl of these preloaded cells were incubated at 20° in 10 ml medium of isotonic phosphate buffer with different concentrations of [^{14}C]glucose and benzoic acid derivatives (dissolved in 2% ethanol) as indicated in the various experiments. After five seconds the incubation was stopped by pouring the suspension into 80 ml ice-cold stopping solution (2 mM $HgCl_2$, 310 mM NaCl, 1.25 mM KJ). After centrifugation and rinsing the inside of the tube with the stopping solution at 0°, the erythrocytes were hemolysed in aqua bidest and the proteins precipitated according to Somogyi [9]. The radioactivity was determined by liquid scintillation counting. For details of the experimental procedure see Lacko *et al.* [10]. The kinetic analysis for the inhibition of the benzoic acid derivatives was carried out (with a correction for the effect of ethanol) as described previously [11].

The partition coefficients were determined as follows: the benzoic acid derivatives were dissolved in phosphate buffer pH 7.5 and shaken vigorously with a defined volume of octanol for five minutes. After centrifugation (5 min) the octanol was taken off. The concentrations of the benzoic acid derivatives and the aqueous phase were estimated from their absorbances, measured with a spectrophotometer (Zeiss

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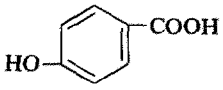
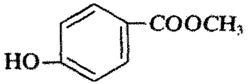
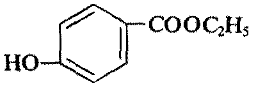
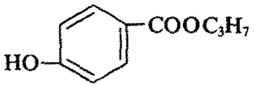
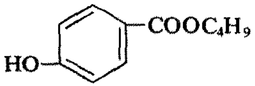
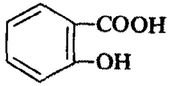
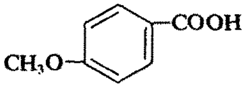
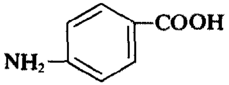
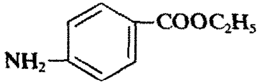
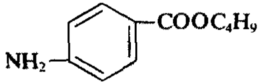
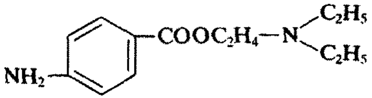
* Thus an 'intermediate chain' does not exist in these compounds; however, we kept this expression (justified for the local anesthetics) to avoid confusion when comparing the benzoic acid derivatives with the local anesthetics.

Table 1. Partition coefficients, inhibition potencies, K_i values and the mode of inhibition of some benzoic acid derivatives

Inhibitor	Structural formulae	Partition coefficient (P)	$\frac{1^*}{I_{50}}$	K_i^{LB} (mM)	K_i^{o} (mM)	K_i^{i} (mM)	Type of inhibition	Binding sites
p-HB		—	0.04	24.0	52.6	20.0	competitive	2
p-HBME		95.5	1.6	0.43	0.77	1.2	competitive	2
p-HBEE		277.0	4.2	0.20	—	—	competitive	1
p-HBPE		1015.0	6.2	0.11	0.15	1.1	competitive	2
p-HBBE		2077.0	18.5	0.05	—	—	competitive	1

o-HB (salicylic acid)		—	0.05	18.0	—	—	competitive	1
p-MOB (anisic acid)		0.14	0.03	32.5	71.4	30.0	competitive	2
p-AB		—	0.03	32.6	100.0	18.0	competitive	2
p-ABEE (benzocaine)		92.3	2.1	0.40	—	—	competitive	1
p-ABBE		1126.0	10.4	0.08	—	—	competitive	1
Procaine†		2.0	0.05	16.0	—	—	competitive	1

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* I_{50} (mM) calculated from the K_i values at the glucose concentration of 0.5 mM.

† The K_i value of procaine is from our previous paper [10].

PM 6). From the differences between the concentrations before and after shaking with octanol, the partition coefficients were calculated according to the relation:*

$$P = \frac{V_{H_2O} \cdot (c_1 - c_2)}{V_{oct.} \cdot c_2}.$$

The benzoic acid derivatives used in the experiments are commercial preparations from Sigma (München).

RESULTS

Reversibility of inhibition

Reversibility of inhibition was tested by incubation of erythrocytes, pretreated with benzoic acid derivatives, in glucose media without inhibitor. There was no difference between the velocities of glucose uptake in erythrocytes pretreated with benzoic acid derivatives and not pretreated ones. It can be concluded that the benzoic acid derivatives, bound to the erythrocyte membrane in the course of the preincubation, dissociate immediately from their binding sites during the main incubation and are lost to the medium. Due to their large dilution by the medium they exert no more influence on glucose uptake. It follows that the interaction of benzoic acid derivatives with the transport system is a reversible process, occurring very fast, in comparison to the incubation time of five seconds.

The inhibition kinetics

The inhibition kinetics of all benzoic acid derivatives, listed in Table 1, and the dissociation constants of their complexes with the transport system were determined as described previously [11]. Some typical examples of plots are shown in Figs 1–3. The Lineweaver–Burk plots of all benzoic acid derivatives reveal a competitive inhibition (as examples see Fig. 1a, b); the differences of the other two types of plots indicate that in some derivatives only one molecule of inhibitor interacts with one molecule of the transport system (Figs 2a, 3a). In other benzoic acid derivatives two molecules can be bound to one molecule of the transport system (Figs 2b, 3b). The results of the kinetic analysis for all tested benzoic acid derivatives are specified in Table 1.

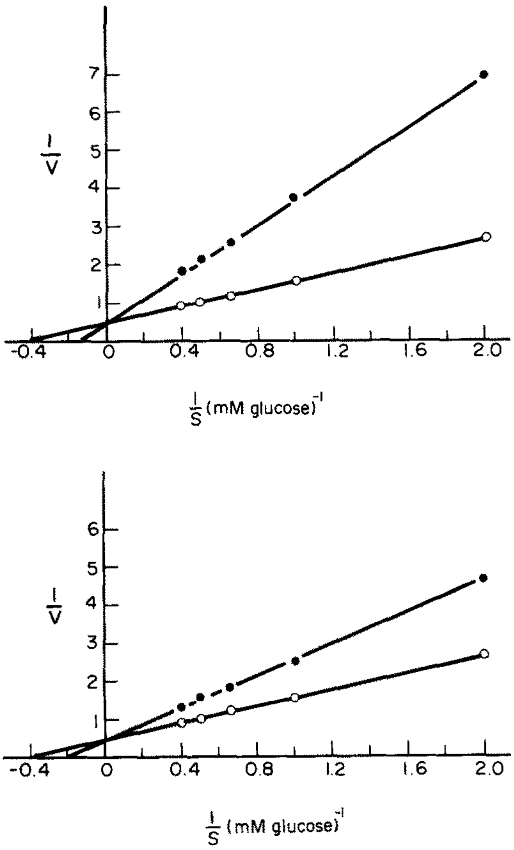

Fig. 1. Double-reciprocal plot of glucose uptake with *p*-ABEE or *p*-HB respectively in the medium. Erythrocytes were preloaded with 200 mM glucose in isotonic NaCl solution and incubated for five seconds at 20° in phosphate buffer at pH 7.5, containing five different [^{14}C]glucose concentrations between 0.5 and 2.5 mM, with (●) and without (○) the inhibitor. In (a) the inhibitor is 0.75 mM *p*-ABEE, in (b) the inhibitor is 24 mM *p*-HB. The reciprocal value of the glucose uptake velocity $1/v$ (sec mM^{-1}) is plotted against the reciprocal value of glucose concentrations.

Efflux of glucose into a medium containing different competitive inhibitors

Figure 4 shows the efflux of glucose from erythrocytes, preloaded with glucose, into a medium containing different competitive inhibitors (galactose, maltose, *p*-HBME)† in concentrations of multiples of their K_i values. (The K_i values of galactose and maltose were estimated for our experimental conditions; details are not described.) The action of the inhibitors was compared with that of dulcit (in similar concentrations as galactose) being ineffective on glucose transport. It can be seen that the inhibitors have different qualitative and quantitative effects: while galactose accelerates the glucose efflux, maltose and *p*-HBME inhibit it to a different extent.

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* List of symbols—P: partition coefficient (octanol/water); V_{H_2O}: volume of water; V_{oct}: volume of octanol; c_1: concentration of the drug in the aqueous phase before shaking; c_2: concentration of the drug in the aqueous phase after shaking; $[I]$: concentration of the inhibitor (benzoic acid derivatives) in the medium (mM); v_{I_1} and v_{I_2}: transport velocity of the reaction inhibited by the same inhibitor of the concentrations $[I_1]$ and $[I_2]$; K_m: Michaelis constant for glucose; K_i^{LB}: dissociation constant of the inhibitor/transport system complex, derived from the Lineweaver–Burk plot; K_i^0: dissociation constant of the inhibitor/transport system complex; K_i^1: dissociation constant of the second inhibitor molecule/transport system complex.

† Abbreviations—*p*-HB: *p*-hydroxy-benzoic acid; *p*-HBME: *p*-hydroxy-benzoic acid methylester; *p*-HBEE: *p*-hydroxy-benzoic acid ethylester; *p*-HBPE: *p*-hydroxy-benzoic acid propylester; *p*-HBBE: *p*-hydroxy-benzoic acid butylester; *o*-HB: *o*-hydroxy-benzoic acid (salicylic acid); *p*-MOB: *p*-methoxy-benzoic acid (anisic acid); *p*-AB: *p*-amino-benzoic acid; *p*-ABEE: *p*-amino-benzoic acid ethylester (benzocaine); *p*-ABBE: *p*-amino-benzoic acid butylester.

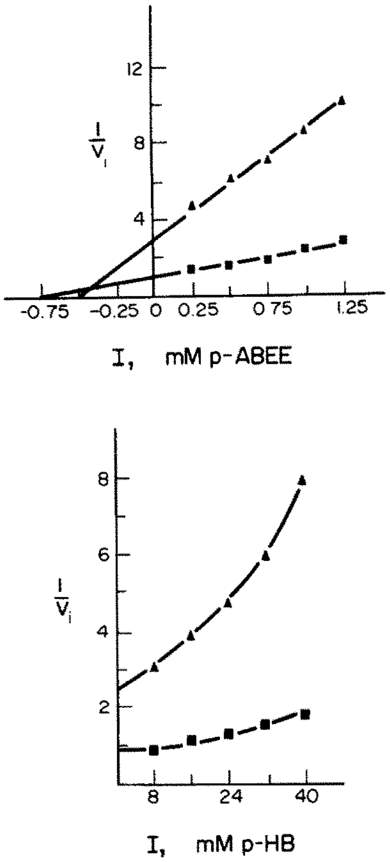


Fig. 2. Dependence of the glucose uptake on the inhibitor concentrations [I]. Erythrocytes, preloaded with 200 mM glucose in 0.9% NaCl solution were incubated for 5 sec at 20° in phosphate buffer at pH 7.5, containing two different glucose concentrations (▲ = 0.5 mM glucose and ■ = 2.5 mM glucose) and five different inhibitor concentrations: (a) *p*-ABEE between 0.25 and 1.25 mM; (b) *p*-HB between 8 and 40 mM. The reciprocal value of the glucose uptake velocity $1/v_i$ (sec mM^{-1}) is plotted against the inhibitor concentrations.

Inhibitory potencies and partition coefficients of benzoic acid derivatives

It has been found that the biological action of many compounds can be correlated with the equation:

$$\log \frac{1}{C} = a \log P + b; \text{ [12]}.$$

C is the molar concentration of the drug causing a standard biological response, a and b are characteristic constants of a given system and P is the octanol–water coefficient. We estimated the partition coefficients of the investigated benzoic acid derivatives (Table 1) and plotted the above relation in Fig. 5. As a standard biological response I_{50} the concentrations (mM) of the benzoic acid derivatives producing 50 per cent inhibition of glucose transport were taken. In Fig. 5 it can be found that the relationship is valid for seven benzoic acid derivatives. For some derivatives with free COOH groups the partition coefficients could not be estimated because

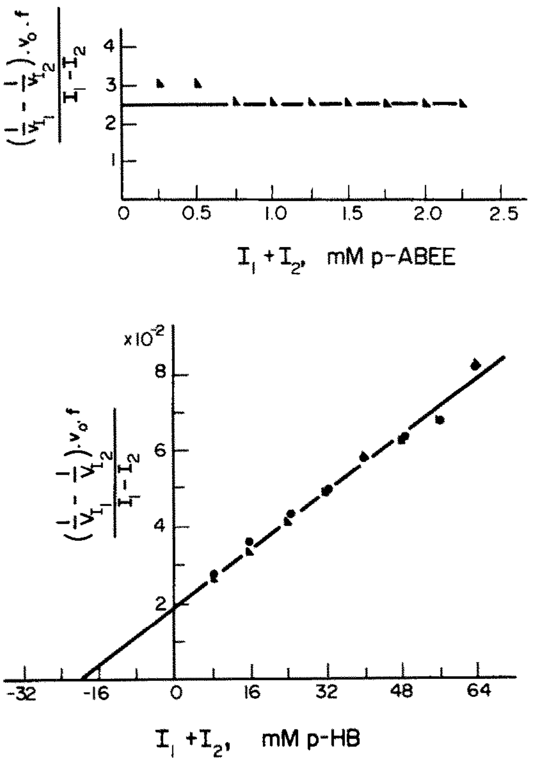


Fig. 3. Graphical evaluation to determine the dissociation constants of the inhibitor transport system complex. In (a) the inhibitor is *p*-ABEE and in (b) the inhibitor is *p*-HB. The glucose concentrations are 0.5 mM (▲) and 2.5 mM (●). [I_1] + [I_2] is the sum of the different concentrations of one inhibitor. f is a constant, calculated from $\frac{[S] + K_m}{K_m}$; it is 1.2 for 0.5 mM glucose and 2.0 for 2.5 mM glucose (for details see ref. 10).

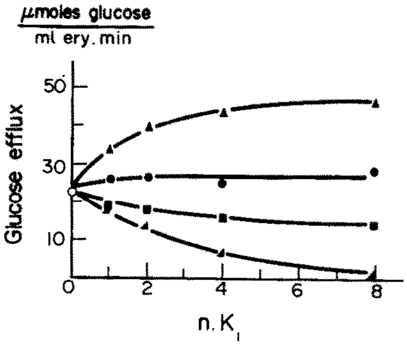


Fig. 4. Glucose efflux from erythrocytes. Erythrocytes, preloaded with 100 mM glucose in isotonic NaCl solution were preincubated for 30 min at 30° with 100 mM [^{14}C]glucose. The main incubation was carried out for one minute at 10° in a medium, containing phosphate buffer at 7.5, the three inhibitors galactose (▲) maltose (■), *p*-HBME (◢) and for comparison, dulcit (●). The glucose efflux (μmoles glucose/ml erythrocytes · min) is plotted against their concentrations between $1 \times K_i$ and $8 \times K_i$. (○) is the glucose efflux into a medium, consisting only of phosphate buffer.

their solubility in the octanol phase was very low; the decrease of concentration in the aqueous phase could not be detected by our method. However, according to their I_{50} values measurable partition coefficients should be anticipated. The estimation of partition coefficients of compounds ionized in the aqueous phase, but existing in form of ion pairs in the octanol phase may present difficulties [12].

DISCUSSION

Table 1 shows that the esters of *p*-HB and *p*-AB and even the acids inhibit glucose transport in erythrocytes. Thus the inhibitory effect, observed with the local anesthetics, is not due to their substituted NH_2 group adjacent to the intermediate chain. On the contrary: comparing the influence of procaine and benzocaine (the only difference being the presence of the substituted NH_2 group in the procaine molecule (see Table 1), it is obvious that the affinity of benzocaine to the transport system of glucose at pH 7.5 is much higher than that of procaine (see K_i values of Table 1). However, the K_i values of procaine depend on the pH because of the substituted NH_2 group, which can be charged or uncharged [13]. But even at pH 10, where the effect of procaine becomes maximal, its affinity is smaller (K_i = 1.1 mM, as calculated from the equation for the dependence of log K_i on the pH, [13]) than that of benzocaine (see Table 1). Elongation of the intermediate chain from the methyl- to the butyl group results in an increase of inhibition of glucose transport (Table 1). Compounds with an OH group in para position have higher affinity to the transport system than compounds where the OH group is substituted by a NH_2. If the OH group is in ortho position to the COOH group (as in the salicylic acid) the inhibitory effect is stronger than that of the *p*-HB. A similar finding was described for the inhibition of phosphate transport in erythrocytes [14]; the remainder of the molecule is supposed to become more lipophilic if the two hydrophilic groups (OH and COOH) are located nearer to each other.

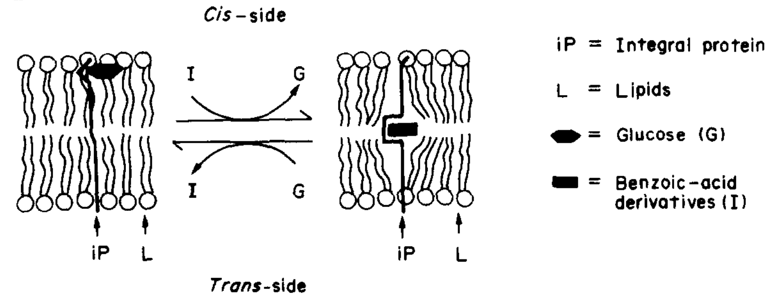
molecule. More homologous compounds would be required to confirm this finding.

It is interesting that the tested compounds with chemical structures dissimilar to glucose, when present on the *cis*-side of the erythrocyte membrane, inhibit the glucose uptake competitively. Their action does not differ from compounds similar to glucose as galactose and maltose [15]. However, if they are on the *trans*-side, they differently influence the efflux of glucose: while galactose accelerates it, due to a higher mobility of the carrier loaded with galactose (particularly at lower temperatures [16]), maltose (impermeable for the erythrocyte membrane [15]) inhibits the efflux; the investigated esters of the benzoic acid exert an even stronger inhibitory effect on glucose efflux.

Our observations show that the potencies of the investigated benzoic acid derivatives (except some compounds with free COOH groups) correlate with the partition coefficients (Fig. 5) in the manner mentioned by Hansch *et al.* [12].

The slope of the straight line ($tg\alpha = 0.66$) indicates that a small change of the lipophilicity of the compound leads to a great change of concentration, producing a 50 per cent inhibition. The high correlation coefficient ($r = 0.99$, explaining 99% of the variance in the data) points out that the inhibitory effect of these compounds correlates highly with their hydrophobicity and that the electronic and steric contributions are practically insignificant.

According to these findings we suppose that the inhibitory effect of glucose transport by benzoic acid derivatives may be connected to their interaction with a particular portion of lipids; these are thought to be intimately associated with the membrane transversing integral proteins, which are considered as transport sites [17]. A similar suggestion was put forward on the activities of some membrane bound enzymes [18]. The mechanism of competitive inhibition of glucose uptake by structurally dissimilar molecules like benzoic acid derivatives or local anesthetics can be explained by conformational change of transport sites, as shown in the schematic drawing:



Scheme 1.

By means of kinetic analysis [11] we found out that all tested compounds exhibited a competitive inhibition. The inhibition kinetics indicate that compounds with even-numbered CH_2 groups in the intermediate chain form a complex with the transport system to which only one inhibitor molecule is bound; compounds with uneven-numbered CH_2 groups form complexes with also a second inhibitor

According to this scheme the conformational change $A \rightleftharpoons B$ is caused by binding of glucose or inhibitor to the transport molecule (see also Krupka and Devés [19]).

Binding of glucose induces a conformation of the transport molecule lacking affinity to the benzoic acid derivatives; on the other hand, binding of benzoic acid derivatives results in a conformation of the

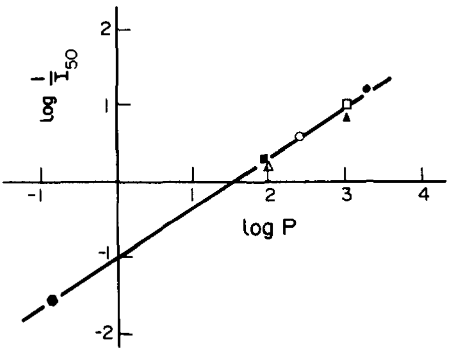


Fig. 5. Relationship between the potency of some benzoic acid derivatives in inhibiting glucose transport and their octanol–water partition coefficients P. I_{50} are the inhibitor concentrations in mM (calculated from their K_i values), where the relative inhibition of glucose transport is 50% and the glucose concentration 0.5 mM. Symbols for the different derivatives: (●) p-HBBE, (□), p-ABBE, (▲) p-HBPE, (○) p-HBEE, (■) p-ABEE, (△) p-HBME and (●) p-MOB. The regression line is: $y = -1.03 + 0.66\ x$; the correlation coefficient $r = 0.996$; $r^2 = 0.991$ (it explains 99% of the variance in the data).

transport molecule incapable of binding glucose molecules. Interaction of distant regions of one molecule can be explained according to the conception of Monod *et al.* [20]. Binding of the first inhibitor molecule of some compounds (p-HB, p-MOB, p-AB) to the transport molecule promotes binding the second one, as can be seen from their K_i-values (Table 1). In this case, interaction of the first molecule causes a conformation of the transport molecule having no affinity to glucose (antagonistic effect [20]) but a stronger affinity to a second molecule of the inhibitor (cooperative effect). It is still a matter of conjecture whether the conformational change of the transport site is brought about from the hydrophobic part of the protein or the adjacent lipid entity of the membrane.

REFERENCES

1. J. M. Ritchie and P. Greengard, *Ann. Rev. Pharmac.* **6**, 405 (1966).
2. P. Seeman, in *Permeability and Function of Biological Membrane* (Ed. L. Bolis), p. 40 North Holland Publishing Company, Amsterdam (1970).
3. R. B. Gunn and J. A. Cooper, *J. Membrane Biol.* **25**, 311 (1975).
4. H. Kunze, N. Nahas, J. R. Traynor and M. Wurl, *Biochim. biophys. Acta* **441**, 93 (1976).
5. I. Ueda, H. Kamaya and H. Eyring, *Proc. natn. Acad. Sci. U.S.A.* **73**, 481 (1976).
6. S. Ohki, Y. Shinagawa and CH. Gravis, *Biochim. biophys. Acta* **507** 395 (1978).
7. L. Lacko, B. Wittke and I. Lacko, *J. Cell. Physiol.* **100**, 169 (1979).
8. A. G. Lee, *Biochim. biophys. Acta* **448**, 34 (1976).
9. M. Somogyi, *J. biol. Chem.* **160**, 69 (1945).
10. L. Lacko, B. Wittke and I. Lacko, *J. Cell.Physiol.* **92**, 257 (1977).
11. L. Lacko, B. Wittke and I. Lacko, *Drug Res.*, **30**, 1849 (1980).
12. C. Hansch and W. R. Glave, *Molec. Pharmac.* **7**, 337 (1971).
13. L. Lacko, B. Wittke and I. Lacko, *Experientia* **34**, 26 (1978).
14. B. Deuticke and E. Gerlach, *Klin. Wschr.* **19**, 977 (1967).
15. L. Lacko and M. Burger, *Biochem. J.* **83**, 622 (1962).
16. L. Lacko, B. Wittke and H. Kromphardt, *Eur. J. Biochem.* **25**, 447 (1972).
17. M. Kasahara and P. C. Hinkle, *Proc. natn. Acad. Sci. U.S.A.* **73**, 396 (1976).
18. L. M. Gordon, R. D. Sauerheber, J. A. Esgate, I. Dipple, R. J. Marchmont and M. D. Houslay, *J. biol. Chem.* **255**, 4519 (1980).
19. R. M. Krupka and R. Devés, *Biochim. biophys. Acta* **598**, 127 (1980).
20. J. Monod, J. Wyman and J.-P. Changeux, *J. Molec. Biol.* **12**, 88 (1965).