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COMPARATIVE INHIBITION STUDIES OF THE PHOSPHOTRANSFERASE AND GLYCEROPHOSPHATE ACYLATION SYSTEMS IN MEMBRANE VESICLES OF *ESCHERICHIA COLI*

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SUMMARY

Purified membrane vesicles were treated with various reagents specific for different amino acid side-chains. Titration of sulfhydryl groups with specific reagents shows that the sulfhydryl content of membrane vesicles as estimated directly is similar to that found by treating spheroplasts or cells and then isolating the membrane vesicles. The blocking of sulfhydryl groups specifically inhibits the α-methylglucoside transport system (phosphotransferase system), whereas the glycerophosphate acylation system is not affected. The kinetics of inhibition of the first system show that a high reactivity of the sulfhydryl groups is involved. Inhibition of the acyltransferase activity by sulfhydryl reagents occurs only on partial denaturation of the membranes induced by mild sonication, heat or toluene treatment. The inhibition is at the level of the glycerol 3-phosphate:acyl thioester acyltransferase.

The effects of sonication and/or sulfhydryl reagents were measured by sulfhydryl titration, by assays of NADH oxidase and D-lactate dehydrogenase activities, as well as by 1-anilino-8-naphthalene sulfonate binding. The results support the hypothesis that the acyltransferase system is embedded within the membrane and that the readily accessible permease system is closer to (or at) the surface of the membrane.

INTRODUCTION

The phosphotransferase system of anaerobic and facultative anaerobic bacteria[1], which is involved in sugar transport, has been thoroughly investigated in *Escherichia coli* both in an intact form, *i.e.* in membranous vesicles[2], and in a system of dissociated components[3,4]; however, it is still uncertain at which level in the membrane the system is located.

It has recently been shown that the key enzymes of phospholipid synthesis, and particularly the glycerophosphate acylation system, are associated with the cytoplasmic membrane of *E. coli*[5,6], however, the degree to which it penetrates into the

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; PCMBS, *p*-chloromercuriphenylsulfonic acid; ANS, 1-anilino-8-naphthalene sulfonate; DFP, diisopropylfluorophosphate.

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membrane has not been investigated. Furthermore, the acyl carrier protein, which plays a key role both in fatty acid synthesis and in phosphatidic acid synthesis[7,8], has been localized near (or at) the inner side of the cytoplasmic membrane[9].

Thus it seemed conceivable that the glycerophosphate acylation system might be located close to the acyl carrier protein. Using purified membrane vesicles of *E. coli* we have compared the phosphotransferase and the glycerophosphate acylation (acyltransferase) systems in an inhibition study. The results reported below suggest that the permease system is located near (or at) the surface of the membrane, and that the acyltransferase system is within the membrane.

MATERIALS AND METHODS

Bacterial strain and media

E. coli ML 308-225 ($i^-z^-y^+$) donated by Dr H. R. Kaback, was used. Cells were grown on medium A (ref. 10) containing 0.5% glucose and harvested at 3/4 log phase. Spheroplasts were prepared by the method described by Kaback[2].

Membrane preparations

Membrane vesicles were usually prepared according to Kaback[2] or for some experiments according to Schnaitman[11]. Contamination by cell walls was checked both by 2-keto-3-deoxyoctonate determination[12] using *N*-acetylneuraminic acid as a standard, and by electron microscopy.

Chemical determinations

Protein was determined by the Lowry method[13] after precipitation with 10% trichloroacetic acid, using bovine serum albumin as a standard. Sulhydryl groups of cells, spheroplasts, and membrane vesicles were determined by the method of Ellman[14] using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) or by using *N*-[^{14}C]ethylmaleimide in the following way: preparations containing 1.5 mg protein/ml were incubated with 1 mM *N*-[^{14}C]ethylmaleimide for 15 min at 27 °C in 0.03 M Tris–HCl buffer, pH 8.0. The reaction was stopped by adding a 10-fold molar excess of 2-mercaptoethanol. The cells were centrifuged, washed twice at 4 °C and resuspended in the same Tris–HCl buffer containing 20% (w/v) sucrose at 12.5 mg cells (wet wt)/ml.

After the lyzozyme treatment as described for membrane preparations[2], the spheroplasts were centrifuged. The supernatant was exhaustively dialyzed in the cold against 0.01 M potassium phosphate buffer, pH 6.6, containing 5 mM 2-mercaptoethanol. This fraction corresponds to the periplasm. Control experiments performed with ^{14}C-labelled acyl carrier protein as a marker of cytoplasmic proteins[9] showed less than 10% contamination of the periplasmic proteins by the cytoplasmic ones. The pellet of spheroplasts was lysed and the membranes prepared in the usual way. The supernatant obtained by centrifugation of the lysate, which corresponds to the cytoplasmic fraction, was dialysed as above. Samples of the three fractions were counted for covalently bound *N*-[^{14}C]ethylmaleimide.

Determination of sulfhydryl groups in spheroplasts: spheroplasts prepared by the lyzozyme method were incubated at a cell concentration equivalent to 1.5 mg protein per ml with *N*-ethylmaleimide under conditions identical to those used for cells. Membranes and cytoplasm were obtained as described above.

Determination of sulfhydryl groups with DTNB was as follows: cells were treated with lyzozyme and separated into three aliquots. The first aliquot was centrifuged and the supernatant, corresponding to the periplasmic fraction, was assayed for sulfhydryl groups after 30 min incubation at 27 °C with DTNB.

The second aliquot was directly incubated with DTNB and then centrifuged. The absorbance measured for this supernatant corresponds to the titration of all the cellular sulfhydryl groups.

The difference between the values obtained on the first and second aliquots corresponds to the value for membrane *plus* cytoplasm. Finally, the value for the cytoplasmic fraction was obtained with a third aliquot by lysing the spheroplasts, centrifuging the lysate, and incubating the supernatant with DTNB. The value for the membranes was calculated from the difference between the second and the third aliquots. The values reported for cells, spheroplasts, and membrane vesicles were obtained by treating with DTNB at room temperature until the plateau of absorbance at 412 nm was reached, and by treating with *N*-[^{14}C]ethylmaleimide followed by Millipore filtration (0.45 μm) and counting of the radioactivity of the filters.

Enzyme assays

The α-methylglucoside phosphotransferase system was assayed according to Kaback[2]. The α-methylglucoside 6-phosphate present in the vesicles was recovered by Millipore filtration (0.45 μm) of the vesicles; the product that had leaked out of the vesicles was recovered in the filtrates according to Gachelin[15]. The radioactive barium salt was recovered on Millipore filters and counted by liquid scintillation[16]. (*sn*)-Glycerol 3-phosphate:acyl-CoA acyltransferase(s) was assayed in a final volume of 0.1 ml containing: 0.1 M Tris–HCl buffer, pH 8.4, 0.4 M KCl, 5 mM $MgCl_2$, 8 mM 2-mercaptoethanol, 0.05 mM palmityl-CoA, 0.4 mM (*sn*)-[^{14}C]glycerol 3-phosphate (2.5 Ci/mole) and from 25 to 100 μg of membrane protein per assay. After 10 min at 30 °C, the phospholipids formed were determined according to the disc assay method described by Goldfine[17]. The conditions of the spectrophotometric assay of the acyltransferase system (final volume: 0.2 ml) were identical, except that potassium phosphate buffer, pH 7.0, replaced Tris–HCl buffer and that bovine serum albumin (1 mg/ml) and 1 mM DTNB were added.

D-Lactate dehydrogenase was assayed according to Barnes and Kaback[18] and NADH oxidase according to Osborn *et al.* (Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J., unpublished). The cytochrome b_1 content was determined by the method of Miura and Mizushima[19].

Chemical modifications of membrane vesicles

Sulfhydryl modifications. Suspensions of vesicles (2–4 mg membrane protein per ml) in 0.1 M Tris–HCl buffer, pH 8.0 were incubated at 27 °C in the presence of various concentrations of either of the sulfhydryl reagents for different lengths of time. The reaction was stopped either by centrifugation at 4 °C (20 min at 37000 × *g*) and/or by addition of a 10-fold molar excess of 2-mercaptoethanol.

Membranes were washed with cold 0.1 M potassium phosphate buffer, pH 6.6, and finally resuspended in the same buffer at 10 mg protein per ml. *N*-Benzyl-, *N*-hexyl- and *N*-decylmaleimide were also tested as sulfhydryl reagents. Because of their low solubilities the first two compounds were dissolved in either water or methyl-

cellosolve, and the third in methylcellosolve. The final concentration of the solvent was 2.5% in the incubation mixture.

Acetylation. In some experiments the sulfhydryl groups were blocked by treating with 1 mM PCMB for 15 min. at 27 °C before acetylation; after elimination of the excess reagent by centrifugation, the membrane suspension (4 mg protein per ml) was treated with acetic anhydride dissolved in methylcellosolve for 10 min at 0 °C, and the pH maintained at 8.0 by the addition of NaOH. The final concentration of methylcellosolve was 1%.

Membranes were then washed in 0.1 M 2-mercaptoethanol and finally suspended as previously described in order to regenerate free sulfhydryl groups.

Nitration. After protection of the sulfhydryl groups by PCMB, the membranes (1 mg/ml) were resuspended in 0.1 M Tris–HCl buffer, pH 8.0. The nitration procedure was that of Vincent *et al.*[20]. The final concentration of ethanol was 1%. After a 20-min incubation at 35 °C the membranes were freed of excess tetranitromethane and resuspended as usual. Sulfhydryl groups were regenerated as described above.

DFP treatment. When needed, a preliminary protection of sulfhydryl groups was performed. Then the membranes were suspended in 0.1 M Tris–HCl buffer, pH 8.0, for 30 min at 27 °C with DFP (2–4 mg membrane proteins per ml); the final concentration of isopropanol was 1%. Subsequent handling of the membranes was as previously described.

The number of acetyl groups as well as the number of *N*-ethylsuccinimyl groups incorporated per mg of membrane proteins were determined by using [^{3}H]acetic anhydride and *N*-[^{14}C]ethylmaleimide. The number of nitrotyrosine residues was determined after solubilization of the membranes at pH 12 by the measurement of absorbance at 428 nm, using a molar extinction coefficient $\varepsilon = 4100$ for nitrotyrosine.

Dansylation. In some experiments, membranes were pre-treated with PCMB. The membranes were suspended (1 mg/ml) in 0.1 M potassium phosphate buffer, pH 6.6, and treated with dansyl chloride dissolved in acetone for 10 min at 27 °C. The final concentration of acetone was 1%. Sulfhydryl groups were regenerated as described above. In our conditions, less than 10% of the dansyl groups incorporated were found in the phospholipids (mainly represented by phosphatidylethanolamine), by titration with [^{14}C]dansyl chloride.

Sonication of membrane vesicles

Suspensions having a protein concentration of 0.5 mg/ml in 0.1 M Tris–HCl buffer, pH 8.0, were used. Sonication was carried out at 0 °C with a Branson sonifier (setting 3) for 3 × 10 s (separated by 1-min intervals for cooling). Membranes were concentrated by high-speed centrifugation and resuspended in 0.1 M potassium phosphate buffer, pH 6.6, at 10 mg protein per ml.

Spectrofluorimetric studies

A Fica fluorescence spectrometer which gave corrected emission spectra was used. The excitation wavelength was 380 nm. Emission spectra were recorded from 400 to 600 nm for 1-anilino-8-naphthalene sulfonate (ANS) alone and from 400 to 520 nm for ANS bound to membranes.

Chemicals

The radioactive chemicals were the products of Commissariat à l'Energie

Atomique (France) and the Radiochemical Center, Amersham (England). *N*-ethylmaleimide, maleic anhydride, benzylamine, hexylamine, and decylamine were purchased from Fluka. The *N*-maleimide derivatives were synthesized according to Tsou *et al.*[21]. PCMB and PCMBS were Sigma products. ANS (Eastman-Kodak) was purified according to McClure and Edelman[22]. Phosphoenol pyruvate, NADH, and lysozyme were purchased from Boehringer, and ribonuclease and deoxyribonuclease from Worthington. DTNB was a product of the Aldrich Chemical Company, and *N*-acetylneuraminic acid was a product of Koch-Light Laboratories.

RESULTS

Number of sulfhydryl groups in the subcellular fractions of E. coli

As will be shown below, most experiments deal with the role and the localization of essential sulfhydryl groups in the phosphotransferase and the acyltransferase systems. Thus the distribution of these groups was investigated in cells, spheroplasts and membrane vesicles. The results of Table I, expressed in nanoequivalents of –SH per mg of proteins, show the high values obtained for the periplasmic proteins, using DTNB and *N*-ethylmaleimide as specific reagents.

TABLE I

DETERMINATION OF SULFHYDRYL GROUPS IN SUBCELLULAR FRACTIONS OF *E. COLI*

See Materials and Methods for details.

Subcellular fraction	*Proteins (% of total)*	*nequiv –SH groups per mg protein*		*–SH groups* (% of total)*
		With 0.5 mM DTNB	*With 1 mM N-[^{14}C]ethylmaleimide*	
Cells				
Periplasm	7***	130	110	59
Membranes	15	15	17	16
Cytoplasm	77	4.8	4.5	25
Membranes	16		19.5	63
Spheroplasts				
Cytoplasm	84		2.2	37
Membrane vesicles				
Native**		13	12.7	
Sonicated**		27	12 75	
Solubilized**		27	25	

* Based on the average value of DTNB and *N*-ethylmaleimide determinations.

** Membranes prepared according to Kaback[2]; solubilization was obtained with 1% sodium dodedyl sulphate.

*** Lyzozyme content subtracted.

The similarity of the values obtained with both DTNB and *N*-ethylmaleimide indicates that *N*-ethylmaleimide as well as DTNB reacted with a high degree of specificity for sulfhydryl groups of membrane and cytoplasmic proteins. The labelling of cytoplasmic proteins by *N*-[^{14}C]ethylmaleimide clearly indicates that this reagent

enters both intact cells and spheroplasts, since the DTNB values were obtained by titration after separation of membranes from cytoplasm.

Interestingly enough, the sulfhydryl content of isolated membranes is similar (with *N*-ethylmaleimide) and almost identical (with DTNB) to that of membranes as determined by direct titration of intact cells; thus the treatment for preparing vesicles must not have significantly altered their structure; the unmasking of sulfhydryl groups represents a good criterion for denaturation, since the sulfhydryl content as assayed with *N*-ethylmaleimide and DTNB doubles when membrane proteins are solubilized and denatured by treatment with sodium dodecyl sulfate. Sonication of the native vesicles does not lead to an increased number of sulfhydryl groups as determined with *N*-ethylmaleimide. However, when DTNB is used, the value obtained is that of denatured vesicles. Thus the latter reagent has access to all the sulfhydryl groups.

TABLE II

COMPARATIVE INHIBITION OF THE PHOSPHOTRANSFERASE AND THE ACYLTRANSFERASE SYSTEMS BY CHEMICAL REAGENTS

Chemical modifications are described in Materials and Methods. With the sulfhydryl reagents, the membranes were incubated for 15 min at 27 °C, and the reaction stopped by centrifugation at 4 °C. For each experiment, the percentage of inhibition refers to a control without inhibitor and treated under identical conditions. No inhibition (100% activity) corresponds to 0.04–0.06 nmoles of α-[^{14}C]methylglucoside 6-phosphate formed and 1–1.8 nmoles of [^{14}C]glycerol 3-phosphate incorporated per min per mg of protein for the phosphotransferase and the acyltransferase systems, respectively. Values in parentheses indicate the number (nmoles) of acetyl, nitro, *N*-ethylsuccinimyl and dansyl groups incorporated per mg of protein using [^{3}H]acetic anhydride, tetranitromethane, *N*-[^{14}C]ethylmaleimide and [^{14}C]dansyl chloride, respectively.

Reagent	*% residual activity of phosphotransferase*		*% residual activity of acyltransferase*	
	–SH protected and regenerated	*–SH not protected*	*–SH protected and regenerated*	*–SH not protected*
Acetic anhydride, 10^{-4} M	80 (1.2)		100	
10^{-3} M	75 (9)	30	100	73
10^{-2} M	24 (55)	0	50	45
Tetranitromethane 10^{-4} M	74 (62)		100	
10^{-3} M	2 (110)		37	
DFP, 10^{-2} M	100	21	100	100
N-Ethylmaleimide, 10^{-4} M	100	0 (8.5)		100
PCMB, 10^{-4} M		0		100
Dansyl chloride, $5 \cdot 10^{-5}$ M	54 (23.4)	9 (18.8)	96	90.5
10^{-4} M		1.3		49.5

Comparative inhibition of phosphotransferase and acyltransferase systems by different chemical reagents

Table II gives the results of treatment of membrane proteins with acetic anhydride, tetranitromethane, DFP, two sulfhydryl reagents and dansyl chloride. In each case the inhibition is greater for the phosphotransferase system than for the acyltransferase system after a preliminary incubation of the membrane vesicles with PCMB in order to protect the essential sulfhydryl groups of both systems (*vide infra*). It must be pointed out that, in these experiments as well as in the subsequent ones, the values for phosphotransferase activities refer to total phosphorylating activities *i.e.*, α-[^{14}C]methylglucoside 6-phosphate recovered both in vesicles and in filtrates. The ratio between the two numbers is a measure of leakage. Leakage remained constant in all experiments whatever was the residual activity.

In the absence of sulfhydryl protection, the same general pattern is observed but the increased inhibition is likely to be less specific since, at the concentrations of reagents used, acetylation, nitration, diisopropylphosphorylation and dansylation could affect the -SH groups.

Treatment of the membranes with *N*-ethylmaleimide and PCMB at 10^{-4} M produces a complete inhibition of the phosphotransferase and no inhibition of the acyltransferase. Regeneration of sulfhydryl groups after PCMB treatment restores complete activity of the transport system.

Inhibition of the phosphotransferase by sulfhydryl reagents.

In Table III are given the results of inhibition by sulfhydryl reagents of different

TABLE III

INHIBITION OF PHOSPHOTRANSFERASE, D-LACTATE DEHYDROGENASE, AND NADH OXIDASE BY DIFFERENT SULFHYDRYL REAGENTS

The conditions were as described in Materials and Methods. The activities were estimated in relation to reference controls treated identically but with no inhibitor present. Unless otherwise stated, the incubation time was 15 min.

Sulfhydryl reagent	*% residual activity of*								
	Phosphotransferase					*D-Lactate dehydrogenase*		*NADH oxidase*	
	10^{-5} M	$5 \cdot 10^{-5}$ M	10^{-4} M	$5 \cdot 10^{-4}$ M	10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M
PCMB	40		0		0				
PCMBS	0		0		0				
Mercuriphenyl acetate					0				
Iodoacetamide			98		74				
DTNB				62	35				
DTNB (30 min)				0	0				
N-Ethylmaleimide	45		0		0	100	65	77	52
N-Benzylmaleimide			77	0					
N-Hexylmaleimide		33							
N-Decylmaleimide		37							

chemical structure. The best inhibitor is PCMBS, whereas iodoacetamide is a very poor one. All reagents give complete inhibition at 10^{-3} M. It must be stressed that none of these inhibitors (up to 10^{-2} M) inactivates the acyltransferase to any extent. It is conceivable that, because of their polarity, they do not have access to sulfhydryl groups embedded in a more hydrophobic environment. For this reason, *N*-benzyl-, *N*-hexyl-, and *N*-decylmaleimide were tested: no inhibition whatsoever of the acyltransferase occurs, whereas the transport system is weakly inhibited. D-Lactate dehydrogenase and NADH oxidase activities with 10^{-4} M *N*-ethylmaleimide are comparable to the activities of control experiments performed in the absence of inhibitor, and the inhibition only becomes apparent at 10^{-3} M. No inhibition of the acyltransferase system in isolated membranes was observed when cells or spheroplasts were broken in the French press, or when spheroplasts were lysed in the presence of 10^{-2} M *N*-ethylmaleimide.

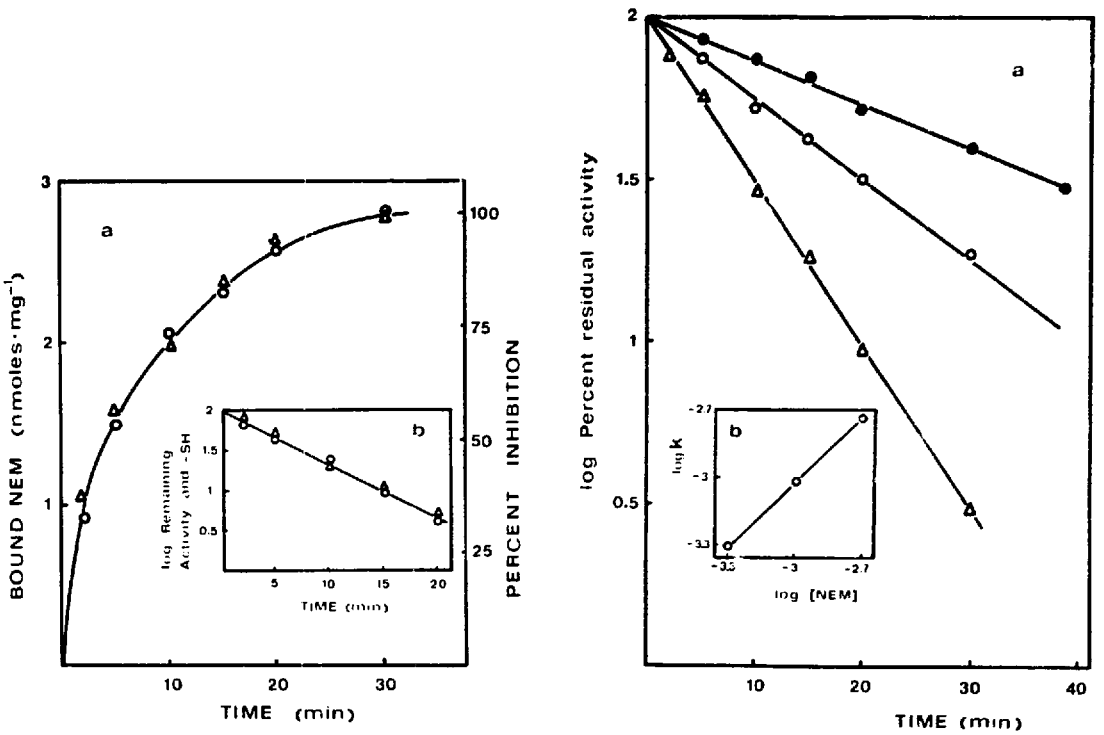

Fig. 1. Kinetics of inhibition of the phosphotransferase system by *N*-ethylmaleimide (NEM). 0.3 mg protein per ml were incubated in 0.1 M Tris–HCl buffer, pH 8.0, at 27 °C with 10^{-5} M *N*-ethylmaleimide. Aliquots were withdrawn at different intervals of time, and the reaction stopped by adding a 10-fold molar excess of 2-mercaptoethanol, centrifuged, washed with 0.1 M potassium phosphate, pH 6.6, centrifuged and resuspended at 10 mg/ml for phosphotransferase assays. For determining covalently bound *N*-ethylsuccinimyl groups, *N*-[^{14}C]ethylmaleimide was used under identical conditions except that, after 2-mercaptoethanol addition, membrane vesicles were extensively washed on Millipore filters (0.45 μm) and the filters directly counted. (a) Direct curves of inhibition (expressed in %) (○) and of *N*-ethylmaleimide binding (△). (b) Semi-log plot of the direct curves (same symbols).

Fig. 2. (a) Rate of inhibition of the phosphotransferase system with varying concentrations of *N*-ethylmaleimide at pH 6.6. 2 mg protein per ml were incubated in 0.1 M potassium phosphate buffer, pH 6.6, at 27 °C with $5 \cdot 10^{-4}$ M (●), 10^{-3} M (○) and $2 \cdot 10^{-3}$ M (△) *N*-ethylmaleimide. The experimental procedure was described in Fig. 1. (b) Determination of the order of the reaction with respect to *N*-ethylmaleimide (NEM).

Kinetics of inhibition by N-ethylmaleimide of the phosphotransferase system

Fig. 1a clearly demonstrates that at pH 8.0 (10^{-5} M *N*-ethylmaleimide) the kinetics of inhibition closely follows the kinetics of *N*-ethylmaleimide covalent binding to membrane vesicles. Plots (Fig. 1b) of the percentage of the remaining phosphotransferase activity and of the percentage of remaining accessible sulfhydryl groups (taking 2.8 nequiv –SH per mg protein as 100% of accessible groups) as functions of time on a semi-log scale indicate that the curves coincide, and that the inhibition is pseudo first order ($k = 2.6 \cdot 10^2$ l·mole^{-1}·s^{-1}).

Fig. 2a also gives semi-log plots obtained at pH 6.6 with $5 \cdot 10^{-4}$ M, 10^{-3} M and $2 \cdot 10^{-3}$ M *N*-ethylmaleimide for the phosphotransferase activity. The value calculated for k is 0.925 l·mole^{-1}·s^{-1}. When (Fig. 2b) the log of the pseudo first order constant of inactivation is plotted *versus* the log of *N*-ethylmaleimide concentration a straight line is obtained with a slope of 1.0, which indicates that only one molecule of inhibitor reacts with one type of sulfhydryl group[23].

Inhibition of the acyltransferase system by sulfhydryl reagents

Results of Table IV (part A) show that inhibition of the acyltransferase occurs

TABLE IV

INHIBITION OF ACYLTRANSFERASE BY DIFFERENT REAGENTS PRESENT DURING OR AFTER SONICATION

(A) After sonication, membrane suspensions were left for 10 min at 0 °C with acetic anhydride, and for 15 min at 27 °C with the other reagents, and assayed for activity by the disc assay method. (B) The time of incubation was 15 min in the experiments using *N*-ethylmaleimide and 30 min in those using DTNB. Control experiments without inhibitor were run in parallel. After centrifugation for 20 min at 40000 × *g* at 4 °C, the membranes were resuspended at 10 mg/ml and assayed for acyltransferase activity by the disc assay method. Values in parentheses correspond to the number of sulfhydryl groups per mg protein titrated by each specific reagent.

Treatment of membrane vesicles			*% residual activity*
A. Unsonicated			100
Sonicated			100
Sonicated;	10^{-2} M	DFP	71
	10^{-3} M	acetic anhydride	0
	10^{-3} M	PCMBS	100
	10^{-2} M	PCMBS	100
	10^{-3} M	PCMB	40
	10^{-2} M	PCMB	12
	10^{-3} M	PCMB followed by 10^{-1} M 2-mercaptoethanol	59
	10^{-2} M	PCMB followed by 10^{-1} M 2-mercaptoethanol	23
B. Unsonicated;	10^{-3} M	*N*-ethylmaleimide	100 (12.7)
	10^{-2} M	*N*-ethylmaleimide	100 (20.3)
Sonicated;	10^{-3} M	*N*-ethylmaleimide	55 (12.75)
	10^{-2} M	*N*-ethylmaleimide	35 (26.5)
Sonicated;	then 10^{-2} M *N*-ethylmaleimide		79
	addition of 10^{-2} M *N*-ethylmaleimide 15 min later		100 (21)
Sonicated;	$5 \cdot 10^{-4}$ M DTNB		37 (27)
	then $5 \cdot 10^{-4}$ M DTNB		10 (27)
	addition of $5 \cdot 10^{-4}$ M DTNB 15 min later		12 (27)

when the membrane vesicles are sonicated in the presence of the different sulfhydryl reagents. On the one hand, the percentage inhibition increases with increasing *N*-ethylmaleimide or PCMB concentration; a slight reversal of the PCMB inhibition is apparent when sulfhydryl groups are regenerated by incubation with 10^{-1} M 2-mercaptoethanol. On the other hand, PCMBS has no action. There is therefore a sharp contrast in the sensitivities of the two systems to this reagent (Table III). It should be recalled that sulfhydryl groups are known to have a role in the acyltransferase activity of eucaryotic cells[24,25].

Mild sonication by itself has no effect on acyltransferase activity or on D-Lactate dehydrogenase and NADH oxidase activities; phosphotransferase activity, on the other hand, is completely lost and cannot be recovered by recombination of the sonicated vesicles obtained by high-speed centrifugation with the resulting supernatant.

When membrane vesicles, prepared according to Schnaitman[11], or spheroplasts were sonicated in the presence of *N*-ethylmaleimide or PCMB, identical inhibitory effects of the acyltransferase were obtained. The cytochrome b_1 content, as well as NADH oxidase, D-lactate dehydrogenase, and acyltransferase activities of such vesicles were similar to those of membrane vesicles prepared according to Kaback[26]; only the 2-keto-3-deoxyoctonate content was higher in the latter case (42 against 9 nmoles/mg protein).

Results of Table IV also show (part B) that, as expected, sonication of the vesicles (and of the spheroplasts, which are not shown) in the presence of 10^{-3} to 10^{-2} M *N*-ethylmaleimide does inhibit the acyltransferase.

However, as demonstrated with membrane vesicles, this inhibition disappears as the time interval between sonication and the addition of *N*-ethylmaleimide increases. This effect is not observed with DTNB, which has access to all the sulfhydryl groups.

TABLE V

EFFECT OF SONICATION ON THE *sn*-GLYCEROL 3-PHOSPHATE: ACYL-COA ACYLTRANSFERASE(S)

The conditions are similar to those described in Table IV. Liberation of CoASH was estimated by the spectrophotometric assay, and [^{14}C]glycerol 3-phosphate incorporation by the disc assay method using identical experimental conditions. CoA release in the absence of glycerol 3-phosphate was substracted from the reported values.

Expt	*nmoles CoASH liberated per min per mg protein*	*nmoles [^{14}C]-glycerol 3-phosphate incorporated per min per mg protein*	*Products*	*CoASH / Glycerol-3-P ratio*
Control (unsonicated)	3.9	2.6	Lysophosphatidate and phosphatidate	1.5
Control (sonicated)	2.45	3.0	Lysophosphatidate	0.82
N-Ethylmaleimide, 10^{-3} M (unsonicated)	4.57	3.0	Lysophosphatidate and phosphatidate	1.52
N-Ethylmaleimide, 10^{-3} M (sonicated)	1.45	1.35	Lysophosphatidate	1.07

Inhibition of the acyltransferase involved in the first esterification of glycerol 3-phosphate

Results reported in Table V show again that treatment of the membrane either by sonication or by incubation with 10^{-3} M *N*-ethylmaleimide does not decrease the total acyltransferase activity (as usually measured by [^{14}C]glycerol 3-phosphate incorporation). However, determination of the ratio (CoASH liberated)/(glycerophosphate incorporated) which indicates the nature of the products formed, showed that inactivation of the second enzyme of glycerophosphate acylation occurs during sonication. The greater lability of the second enzyme compared to the first enzyme has already been observed with an enzyme system of *Clostridium butyricum*[27].

Thus it is the first enzyme involved in glycerophosphate esterification that is inhibited by sonication in the presence of 10^{-3} M *N*-ethylmaleimide as indicated in Table V.

Variation of ionic strength from 0.05 to 2.15 (with NaCl) and pH from 6.5 to 9.5 in the presence of 10^{-2} M *N*-ethylmaleimide does not lead to any inactivation of the acyltransferase without sonication.

In non-sonicated membranes this enzyme system can be 30–50% inhibited with *N*-ethylmaleimide by increasing the temperature to 50 °C. This observation suggests that some denaturation of many membranes proteins occurs, thus making it possible to inhibit the acyltransferase by *N*-ethylmaleimide without sonication. Similar conclusions can be drawn from the inhibition obtained by "toluenization" of the membrane vesicles. At 22 °C for 30 min in the presence of 0.3% toluene, 93 and 98% inhibition are observed with $2 \cdot 10^{-3}$ M *N*-ethylmaleimide and 10^{-3} M PCMB, respectively. In the latter case, 100% recovery of the activity is obtained by subsequent addition of 10^{-1} M 2-mercaptoethanol. Thus a full recovery of the acyltransferase activity is obtained.

ANS binding to membrane vesicles

Fig. 3 shows the fluorescence intensity of different concentrations of ANS bound to increasing amounts of *E. coli* membrane vesicles. In the double reciprocal plot, straight lines are obtained at all concentrations of ANS. The extrapolated intercept on the ordinate at infinite amount of membranes (when all the ANS is membrane bound) yields the maximum value of fluorescence.

It is clear from Fig. 3 that, at a given concentration of ANS, sonication induces an enhancement of ANS fluorescence, and that the same maximum values of fluorescence are obtained for sonicated and unsonicated membranes.

The latter result seems to exclude the possibility that sonication causes a change in quantum yield, *i.e.* that sonication allows ANS to bind more apolar sites than is possible without sonication.

It is possible, with a given amount of either unsonicated or sonicated membranes, to assay for ANS fluorescence by increasing ANS concentration. The difference between the maximal calculated values and those obtained at the same ANS level gives the values for free ANS, and thus for bound ANS, at each concentration of fluorescent probe.

Fig. 4 represents Scatchard plots drawn for sonicated and unsonicated membranes. The intercept on the abscissa gives n, the number of ANS molecules bound per mg of membrane protein. Sonication clearly increases the number of bound molecules, but the calculated binding constants ($K_A = 7.7 \cdot 10^4$ M^{-1} and $8 \cdot 10^4$ M^{-1}

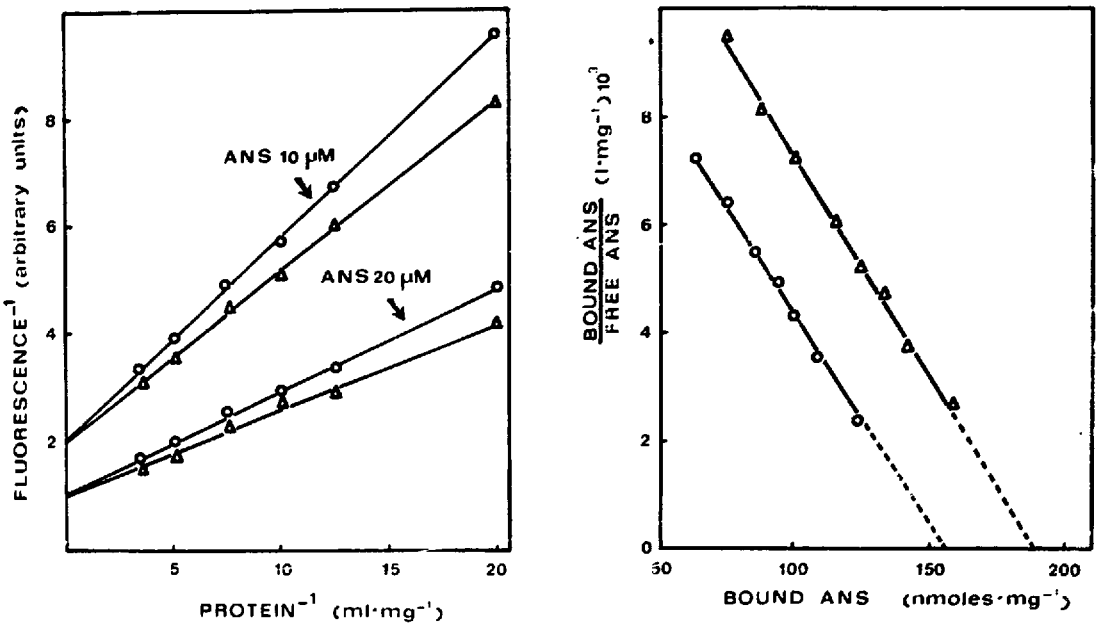

Fig. 3. Binding of ANS to membrane vesicles. Determination of saturation fluorescence intensity, by plotting 1/fluorescence *versus* 1/membrane protein concentration. Membranes were suspended in 0.1 M sodium phosphate buffer, pH 7.1, and fluorescence intensities measured at 21 °C at 480 nm; O—O, native vesicles; △—△, sonicated vesicles.

Fig. 4. Scatchard plot for ANS binding with native and sonicated membrane vesicles. 0.1 mg membrane protein per ml was used, with ANS concentrations ranging from 5 to 40 μM. Experimental conditions were as described above. Same symbols as in Fig. 3.

for unsonicated and sonicated membranes, respectively) indicate that the free energy of binding $\Delta F = -RTL_nK_A$ (−6.6 kcal/mole) is not affected. From all the above results it is possible to conclude that sonication leads to a small increase in the number of binding sites for ANS, and that these new binding sites have an average hydrophobicity identical to the sites present on the native membrane.

DISCUSSION

From the figures reported in Table I for cells and spheroplasts, it can be concluded that *N*-ethylmaleimide penetrates the cytoplasm and thus could inhibit the acyltransferase system if it were located on the inner side of the cytoplasmic membrane in the vicinity of the acyl carrier protein which participates in phospholipids synthesis[7,8]. This conclusion is further supported by the lack of inhibition of the acyltransferase system when cells or spheroplasts are broken in the presence of 10^{-2} M *N*-ethylmaleimide. Thus its difficult accessibility to inhibition could be related to a localization inside the membrane.

The fact that the numbers of sulfhydryl groups reacting with *N*-ethylmaleimide in membrane vesicles and in intact cells and spheroplasts are comparable indicates that no significant amount of denaturation of vesicles had occured, as judged by the unmasking of sulfhydryl groups, since treatment with such a denaturing agent as

sodium dodecyl sulfate causes a 2-fold increase in the number of reactive sulhydryl groups.

A 2-fold increase is also obtained during sonication with or without DTNB present: thus sonication does cause some structural changes. However this increase in sulfhydryl group number is not observed with 10^{-3} M *N*-ethylmaleimide, which should have a more limited access to the interior of the membrane as compared to DTNB.

The results reported in Table II show that the phosphotransferase system is more easily inactivated by different chemical reagents than the acyltransferase system, with or without a pre-treatment with PCMB to protect the sulfhydryl groups. The good accessibility of the reagents to some component(s) of the permease system, compared to their poor accessibility to the acyltransferase, strongly suggests a difference in the localization of the two systems in the membrane.

The results in Tables III and IV, as well as the curves of Fig. 1 give the following additional information:

(a) The comparative inhibition of the phosphotransferase and the acyltransferase systems by different chemical reagents, as well as the inhibition of phosphotransferase by different sulfhydryl reagents, show that the transport system is completely inactivated by low concentrations of different sulfhydryl inhibitors. PCMBS, which is known as a polar inhibitor that does not enter erythrocyte ghosts[28], behaves as the most potent reagent.

(b) The kinetics of inhibition by *N*-ethylmaleimide presented in Fig. 1 shows that a few covalently bound *N*-ethylsuccinimyl groups (approximately 3 nmoles per mg of membrane protein) lead to a complete inhibition of the phosphotransferase system. The calculated rate constants at pH 8.0 and at pH 6.6 are higher than the rate constants reported for soluble enzymes with essential sulfhydryl groups at their active site[23,29,30]. Since it is known that Enzyme I described by Kundig and Roseman[3] of the phosphotransferase system is the only component inhibited by this sulfhydryl reagent, it is likely that Enzyme I has an essential sulfhydryl group near or at the surface of the membrane. The localization of the transport systems near (or at) the surface seems quite general, since the D-lactate-stimulated amino acid and thiomethylgalactoside transports are completely inhibited by PCMB and *N*-ethylmaleimide (refs 31,32, and R. Négrel and G. Ailhaud, unpublished); under the conditions used (Table III), almost no inhibition of the D-lactate dehydrogenase and NADH oxidase activities can be observed, although they are somewhat inactivated at the higher concentration of 10^{-3} M *N*-ethylmaleimide.

As Table IV shows, a permanent inhibition of the acyltransferase by a sulfhydryl reagent is observed when DTNB is used during sonication; the latter compound, unlike *N*-ethylmaleimide, still behaves as an inhibitor after sonication. This result is in agreement with the fact that all the sulfhydryl groups are blocked by DTNB after sonication, as shown in Table I. Thus, although sonicated vesicles do not show any increase in the number of sulfhydryl groups with 10^{-3} M *N*-ethylmaleimide (Table I) and although most membrane activities remain intact (except the transport system), these results indicate that the conformation of some membrane proteins may have been altered.

The results of inhibition of the acyltransferase system all support the idea of a deeper localization in the membrane. The essential sulfhydryl group(s) of the glycerol

3-phosphate:acyl-CoA acyltransferase is accessible to sulfhydryl reagents only when structural changes are brought about by sonication and, to some extent, by denaturation of the membranes induced by heat or by toluene addition. Furthermore, inhibition by PCMB after toluene treatment is fully reversed by 2-mercaptoethanol. Finally, the lack of inhibition by sonication in the presence of PCMBS also supports this hypothesis. The formation of an acyl-*S*-enzyme intermediate is known to occur with the best studied enzymes, using thiolesters as substrates[33-35]. Although it is likely that the sulfhydryl groups involved in this inhibition belong to the active site of the glycerol 3-phosphate:acyl-CoA acyltransferase, no definite conclusion can be drawn since vesicles sonicated in the presence of sulfhydryl reagent, either after prior protection with acyl-CoA or with acyl-CoA in the sonicated mixture, become denatured. In both cases the denaturation is due to the detergent properties of fatty acyl-CoA which can not be eliminated in the first case even after careful washing. Inactivation of the second enzyme involved in the acylation of glycerol 3-phosphate, brought about by sonication, indirectly supports the genetic proofs for the existence of two separate enzymes[36,37].

The effect of sonication on the conformation of membrane proteins must be a transient one owing to the very high cooperativity of these structures, since sonicated membranes have normal levels of acyltransferase, D-lactate dehydrogenase, and NADH oxidase activities.

It should be pointed out that the sonication of the membrane vesicles was performed under very mild conditions: less than 10% of proteins were released; under more drastic conditions a complete inhibition of all the tested activities was observed with a loss of up to 50% of the membrane proteins. The transient effect brought about by sonication is clearly shown by the lack of inhibition by *N*-ethylmaleimide which is observed when the time between sonication and the addition of the reagent is increased. The unmasking of new sites (quantitatively estimated as a 22% increase) as a consequence of the sonication can be assessed by fluorescence studies: these new sites are apparently qualitatively similar to the previous ones, as far as the average hydrophobicity of membranes is concerned. The small increase observed for bound ANS after sonication is likely to be due to the increased area offered by vesicles as a consequence of the decrease of the average diameter brought about by this treatment. This hypothesis implies that ANS, with amphipatic properties similar to those of PCMBS, will in no case enter the membrane vesicles, and will thus bind near the surface of the membrane.

Our results strongly suggest that, unlike the permease systems that have some component(s) near or at the surface of the membrane, the first enzyme involved in phospholipid synthesis is located deep in the membrane, where a higher concentration of lipids is expected whatever the membrane model proposed[38].

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