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SOLUBILIZATION AND PROPERTIES OF FORMATE DEHYDROGENASES FROM THE MEMBRANE OF *ESCHERICHIA COLI*

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SUMMARY

1. *Escherichia coli* has two inducible formate dehydrogenases. One is induced by nitrate, whereas the other increases when formate is added to the growth medium. The first (N) reacts with methylene blue or phenazine methosulfate, whereas the other (H) reacts with viologen dyes.

2. Both enzymes are bound to the cell membrane and are solubilized in an active form by the non-ionic detergent, BRIJ-36T.

3. The enzymes differ in K_m for formate, optimum pH, optimum temperature, and mobility in agarose-gel columns. General properties were not affected significantly by solubilization.

4. The active peak of the H enzyme obtained from agarose-gel columns does not react with methylene blue or phenazine methosulfate.

5. It is suggested that the enzymes are different, even though it is not yet known whether they share some polypeptide subunit.

INTRODUCTION

It has been suggested that *Escherichia coli* contains two formate dehydrogenases, one particulate and one soluble[1,2]. The first one would be associated with the function of formate oxidase, and the second involved in H_2 evolution. Further, it has been suggested that particulate formate dehydrogenase is involved in nitrate reduction by the so called nitrate reductase complex[3,4], and that formate oxidase consists of formate dehydrogenase and two autooxidizable b_1 cytochromes. More recently we have obtained results which confirm these hypotheses[19]. It has been mentioned from genetic studies that possibly both formate dehydrogenases are expressions of the same structural gene and that the variations observed are due to their association with distinct electron transport components[4]. This same hypothesis has been put forward by Lester and De Moss[5].

Abbreviations: PMS, phenazine methosulfate; DCIP, dichlorophenolindophenol.

Recently we succeeded in solubilizing membrane-bound formate dehydrogenase[20] and undertook the investigation reported here to compare the localization of formate dehydrogenases and their kinetic properties, in order to gain information about the relationships between the two enzymes.

METHODS

Strain. E. coli HFrH was used in this study. It was obtained from J. A. De Moss (University of California, San Diego, U.S.A.).

Culture media. The strain was maintained on slants of nutrient agar (Difco). It was propagated in the low-citrate base medium from Sypherd and Straus[6] with the addition of 0.4% nutrient broth, 1% glucose and 5 μg/ml thiamine. In order to induce the formate dehydrogenase involved in nitrate reduction (N), 1% KNO_3 was included in the medium and for the induction of formate dehydrogenase associated with hydrogen-lyase (H), 0.5% formate was added to the medium instead of KNO_3.

Culture conditions. Erlenmeyer flasks containing 1 l of medium were inoculated with an overnight-grown culture. The medium was covered with a layer of mineral oil and incubated for 6 h at 37 °C. Cells were harvested by centrifugation, washed with 0.05 M potassium phosphate buffer, pH 7.3, and finally resuspended in 1 ml of Tris–Mg^{2+}–K^+ buffer (0.01 M Tris–HCl, pH 7.6, 0.01 M $MgSO_4$, 0.05 M KCl)[7] containing 20% sucrose and 2 mg of lysozyme. Cells were then frozen and kept at −20 °C until used.

Preparation of cell free extracts and isolation of cell envelopes. Cells were treated with lysozyme as described by Morris and De Moss[7], 9 ml of Tris–Mg^{2+}–K^+-buffer containing 10 μg/ml of deoxyribonuclease were added, and after the viscosity had decreased to a minimum, intact cells were eliminated by centrifugation at 2000 × *g* for 10 min. The supernatant was then spun at 40 000 × *g* for 20 min and both sediment and supernatant were saved. The sediment containing the cell membranes was washed three times with 0.02 M Tris, pH 7.3, containing 1 mM EDTA.

Nomenclature of formate dehydrogenases. In order to distinguish between both enzymes, the formate dehydrogenase which reacts with phenazine methosulfate (PMS) was named N enzyme (after nitrate reduction). The enzyme which reacts with benzyl viologen (BV) was named H enzyme (after hydrogen formation).

Solubilization of formate dehydrogenases. An aliquot of cell envelopes was mixed with an equal volume of 10 mM BRIJ-36T dissolved in 0.02 M Tris, pH 7.3, containing 1 mM EDTA. When formate dehydrogenase H was solubilized, the buffer solution contained in addition 1 mM dithioerythritol and 0.01 mM potassium selenite. The material was incubated at 4 °C for 60 min and centrifuged at 40 000 × *g* for 30 min. Activity was assayed in both sediment and supernatant.

Assay of formate dehydrogenases. Formate dehydrogenase N was assayed by a modification of the method of Ells[3]. Formate dehydrogenase H was measured by a method suggested to us by Lester and De Moss (personal communication; see also ref. 5). To a 3-ml spectrophotometer cuvette, a ground joint was sealed and a hollow stopper with two perforations was adapted to the joint. Through one of the holes a capillary tube was introduced. Through this capillary tube a fine stream of argon flowed into the cuvette at all times. The other perforation was used to make additions to the system. The assay was conducted as follows: 0.5 ml of 2.5 mM benzyl viologen

and variable amounts of enzymatic material were introduced into the cuvette. The volume was made up to 2.9 ml with the buffer described above. Argon (purified by passing through two succesive traps of alkaline pyrogallol) was bubbled for 3 min through the system and the capillary was withdrawn to just above the liquid level. 0.01 ml of 1 M sodium formate were placed over the capillary in case it dropped into the reaction mixture. After 2 min, the capillary was introduced again into the liquid and mixing was achieved by the argon stream. Finally, the capillary was withdrawn above the level of the liquid. The appearance of color due to the reduction of benzyl viologen was measured at 550 nm with a Maroc V (Jobin et Ivon) spectrophotometer coupled to a photovolt model 43 recorder. It was observed that normally there was a lag phase of 1 or 2 min before the reaction became linear. All attempts to reduce the lag phase were unsuccesful. The activity of formate dehydrogenase N is expressed as nmoles of formate oxidized per mg of protein per min of incubation. The activity of formate dehydrogenase H was expressed as the change in absorbance at 550 nm per min per mg of protein.

Other determinations. Protein was measured by the method of Lowry *et al.*[8] and total phosphorus by the method of Fiske and SubbaRow[9]. Determination of organic phosphorus was used as a measure of phospholipids.

Chemicals. BRIJ-36T, a non-ionic detergent is an ether of Lauric alcohol with 10 oxyethylene groups. It was a kind gift from C. Gitler of the C.I.E.A. of the Instituto Politécnico Nacional, México. Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden; phenazine methosulfate (PMS) from Sigma Chemical Co., dichlorophenol-indophenol (DCIP) and benzyl viologen from K & K laboratories. All other chemicals were of the highest purity available.

RESULTS

Location of formate dehydrogenase in the cell

In agreement with data existing in the literature[2,10,11] we found that the formate dehydrogenase which reacts with methylene blue or PMS is bound to the cell membrane (Table I). This enzyme is induced by nitrate[3] (see also Table I). On the other hand, it has been suggested that the formate dehydrogenase involved in gas formation is a soluble enzyme[1,2,12]. We have obtained evidence that this enzyme (formate dehydrogenase H) is also a membrane-bound enzyme (Fig. 1). Studies made with pleiotropic mutants[4], have shown that mutants lacking this enzyme are unable to form hydrogen from formate.

TABLE I

LOCATION OF FORMATE DEHYDROGENASE N IN THE CELL

Specific activity is expressed as nmoles of formate oxidized per mg of protein in 1 min.

Growth conditions	*Total protein (mg)*		*Specific activity*	
	Membrane	*Soluble*	*Membrane*	*Soluble*
Anaerobic	5.9	28	88.4	0
Anaerobic *plus* formate	4.4	18	21.4	0
Anaerobic *plus* nitrate	6.1	32	554	0

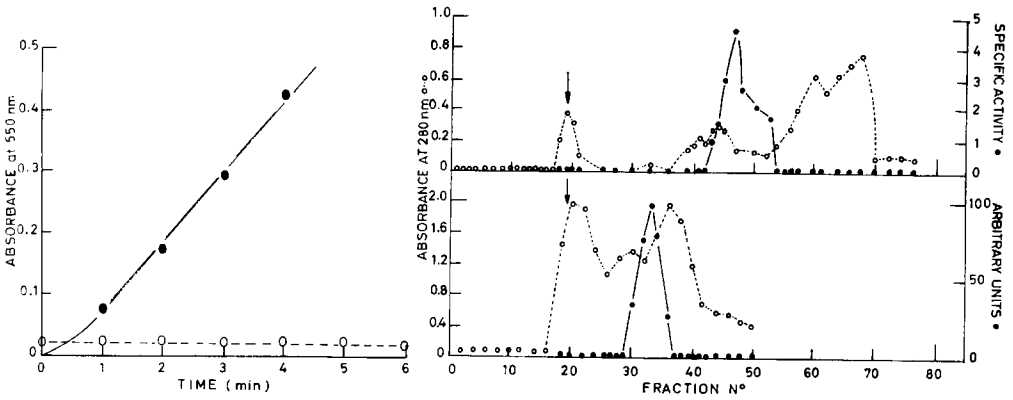


Fig. 1. Activity of formate dehydrogenase H in membrane and soluble fractions of *E. coli*. Bacteria were lysed with lysozyme and soluble and membrane fractions were isolated. Activity of formate dehydrogenase was measured using both fractions. ●—●, membrane fraction (0.127 mg protein); ○—○, soluble fraction (0.817 mg protein).

Fig. 2. Chromatography of solubilized membrane in agarose-gel columns. Bacteria were grown anaerobically in complex medium containing 1% KNO_3 (upper figure) or 0.5% formate (lower figure). They were lysed with lysozyme, the membrane fraction was recovered and solubilized with 5 mM BRIJ-36T. Solubilized material was subjected to chromatography in an agarose-gel column. Aliquots of 3 ml were recovered and the absorbance at 280 nm, and activity were measured. In the upper figure results for formate dehydrogenase N are shown, in the lower figure results for formate dehydrogenase H are recorded. In this case activity is recorded as Klett units measured after 3 h of incubation under a stream of argon. ○—○, absorbance at 280 nm ●—●, activity. Arrow indicates void volume.

Solubilization of formate dehydrogenases with BRIJ-36T

Treatment of membranes with BRIJ-36T under the conditions described in the experimental section resulted in the solubilization of 30–40% of the total protein from the membranes. All activity was solubilized without appreciable loss of activity No activity was detected in the insoluble residue. Enzymes were stable in BRIJ-36T and remained active for several days if kept at 0 °C.

Chromatographic separation of formate dehydrogenases

Solubilized material was resolved in an agarose-gel column (Sepharose 4B Pharmacia; Uppsala, Sweden). The size of the column was 2 cm × 50 cm and the flow was 10 ml per h. 3-ml aliquots were recovered. Protein elution was monitored by ultraviolet light absorption at 280 nm. Lipids and cytochromes were separated from formate dehydrogenase under these conditions. Typical results obtained are shown in Fig. 2. The enzymes behave differently on the column, formate dehydrogenase H having a faster mobility. It was observed that formate dehydrogenase H was almost completely inactivated when subjected to column chromatography. It is interesting to mention also that the active peak of formate dehydrogenase H had no activity when tested with methylene blue or PMS as electron acceptors; and that the active peak of the N enzyme does not reduce benzyl viologen. There was no formate dehydrogenase H activity present in any of the fractions obtained during chromatography of the solubilized membranes obtained from nitrate-grown cells. Also, there was no formate dehydrogenase N activity in the fractions obtained during chromatography of the solubilized membranes obtained from formate-grown cells.

TABLE II

KINETIC PROPERTIES OF FORMATE DEHYDROGENASE N AT DIFFERENT STAGES OF PURITY

Parameter	*Stage of purity of the enzyme*		
	Membrane-bound	*Solubilized*	*From column*
K_m for PMS	$1.67 \cdot 10^{-4}$ M	$6.66 \cdot 10^{-4}$ M	$3.33 \cdot 11^{-3}$ M
K_m for formate	$2.5 \cdot 10^{-5}$ M	—	$1.33 \cdot 10^{-5}$ M
Optimum temperature	30 °C	30 °C	30 °C
Optimum pH	7.3	7.3	7.3

Kinetic properties of formate dehydrogenase N

The effect of the concentration of diclorophenolindophenol (DCIP), phenazine methosulfate (PMS), and formate; and the influence of temperature and pH on the membrane-bound enzyme, solubilized enzyme and enzyme resolved by chromatography on agarose gel columns (see below for details) were assayed. Comparative data of apparent K_m values for phenazine methosulfate and formate, optimum pH and optimum temperature are shown in Table II. It may be observed that optimum pH, optimum temperature, and K_m for formate were not affected appreciably by solubilization and chromatography of the enzyme. On the other hand, K_m for PMS increased after solubilization and resolution of the enzyme by chromatography. Optimum concentration of PMS was 0.3–0.4 mM, higher concentrations resulted in inhibition of the enzyme (Fig. 3). Optimum concentration of DCIP was also between 0.3 and 0.4 mM. Higher concentrations inhibited the enzymatic activity (Fig. 4). When the results obtained from analyzing the effect of formate concentration were plotted as the double reciprocals, biphasic kinetics which suggest activation by the substrate were

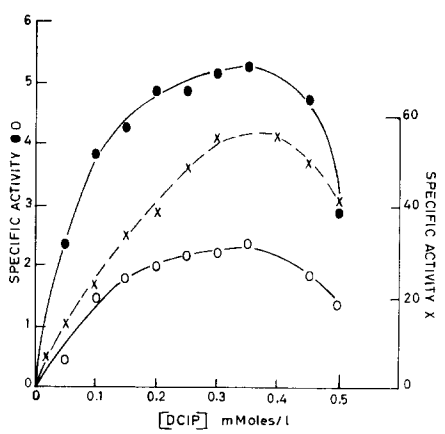


Fig. 3. Effect of PMS concentration on the activity of formate dehydrogenase N. Enzymic activity was measured with three different preparations. ×—×, whole membranes; ○—○, solubilized membranes; ●—●, enzymic peak recovered after chromatography in an agarose-gel column.

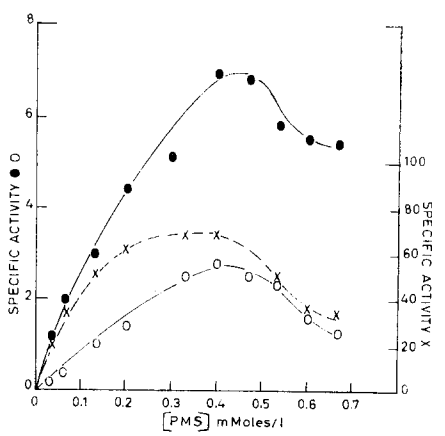


Fig. 4. Effect of DCIP concentration on the activity of formate dehydrogenase N. Enzymic activity was measured with three different preparations. ×—×, whole membranes; ○—○, solubilized membranes; ●—●, enzymic peak recovered after chromatography in an agarose-gel column.

observed both with the cell membrane-bound enzyme and the enzyme recovered from the agarose-gel column (Fig. 5).

Kinetic properties of formate dehydrogenase H

Kinetic analysis of the enzyme were performed using frozen–thawed cells and enzyme solubilized with BRIJ-36T. It was not possible to make experiments with cell membranes since the enzyme was inactivated completely in less than 2 h after obtaining the membranes. All attempts made to preserve enzymatic activity were unsuccesful. When freshly obtained membranes were solubilized with BRIJ-36T, the enzyme was stabilized and kept its activity at 0 °C for several days if treated with a thiol compound such as dithioerythritol and selenite. As mentioned above chromatographic separation of the enzyme on the agarose gel column brought about its almost complete inactivation. The activity was very low and the enzyme had a very long lag phase (about 2 h) before some activity, could be observed. Several attempts were made to recover enzymatic activity. A higher concentration of dithioerythritol was included in the buffer, reduced glutathione was also tried; formate was added to the elution buffer; active material was pooled, and tested, mixed with each one of the remaining fractions. All fractions eluting after the active material were pooled, concentrated and added to the active fractions. Lipids were extracted from *E. coli* and added to the active fractions. All these tratments failed to increase the activity of the enzyme. Results obtained with frozen–thawed cells and solubilized membranes are shown in Table III. It may be seen that solubilization of the enzyme did not affect optimum temperature and optimum pH, but affinity for the substrate and for the electron acceptor decreased somewhat after solubilization. It was observed that high concentrations of benzyl viologen did not inhibit enzymatic activity, in contrast to the effect of PMS on formate dehydrogenase N. There were no biphasic kinetics when the effect of formate concentration on enzymatic activity was tested (Fig. 6).

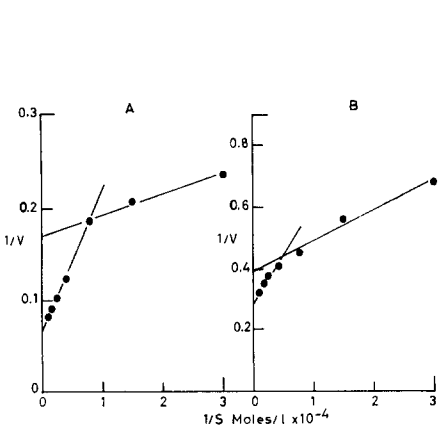
TABLE III

KINETIC PROPERTIES OF CELL MEMBRANE-BOUND AND SOLUBILIZED FORMATE DEHYDROGENASE H

Parameter	*Stage of the enzyme*	
	Frozen–thawed cell	*Solubilized*
K_m for benzyl viologen	$2.9 \cdot 10^{-4}$ M	$5.6 \cdot 10^{-4}$ M
K_m for formate	$1.5 \cdot 10^{-3}$ M	$2.5 \cdot 10^{-3}$ M
Optimum temperature	38 °C	38 °C
Optimum pH	6.4	6.4

DISCUSSION

Contrary to reports existing in the literature[1,2] we found that formate dehydrogenase H is a cell membrane-bound enzyme, the same as formate dehydrogenase N. The differences may be due to the fact that we used a milder method for the isolation of the membranes which insures that small non-sedimenting fragments do not remain in the supernatant after centrifugation. Solubilization of the two enzymes was achieved by treatment with the nonionic detergent BRIJ-36T. Other non-ionic deter-



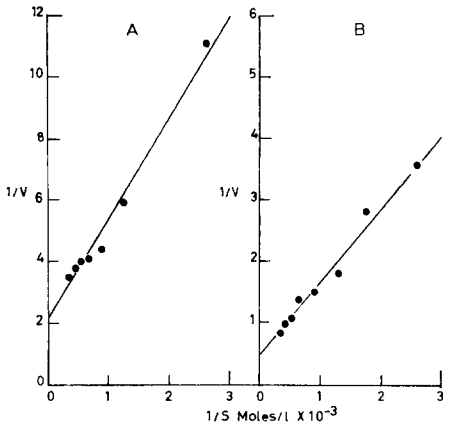


Fig. 5. Formate dehydrogenase N activity. Dependence of reaction velocity (v) on formate concentrations (S). Velocity was determined under standard reaction conditions and is expressed as nmoles of formate oxidized per 1 mg of protein in 1 min of incubation. (A) whole membranes, (B) enzyme solubilized and purified by agarose-gel chromatography.

Fig. 6. Formate dehydrogenase H activity. Dependence of reaction velocity (v) on formate concentration (S). Velocity was determined under standard reaction conditions and is expressed as change in absorbance at 550 nm per minute per mg of protein. (A) frozen–thawed cells, (B) solubilized membranes.

gents such as BRIJ-35, Triton X-100 and Tween-80; anionic detergents as sodium dodecyl sulfate and the cationic detergent cetyltrimethylammonium bromide; and chaotropic salts were useless in the solubilization of the enzymes[20]. It has been suggested[13] that the positively charged group of cationic detergents interact in the membrane with polyphosphatidic acids, lysis being a secondary process following the disturbance of the permeability of the membrane. On the other hand, anionic detergents react with the protein components of the membranes. Sodium dodecyl sulfate for example inhibits activity of membrane enzymes when it is added in excess[14]. This effect is due to progressive denaturation of the proteins with concomitant disruption of the hydrophobic interactions between membrane lipids and proteins[15]. Comparing the effect of several detergents Gitler *et al.*[16] reported that Triton X-100, cetyltrimethylammonium bromide and sodium dodecyl sulfate solubilized membranes from erythrocytes. Triton X-100 did not inhibit any of the tested enzymes, cetyltrimethylammonium bromide inhibited NAD nucleosidase and acetylcholinesterase whereas acid phosphatase was not affected; and concentrations of sodium dodecyl sulfate which solubilized the membrane inhibited all these enzymes. According to the behavior in agarose-gel columns it may be assumed that both formate dehydrogenases form micelles with detergent radii corresponding to that of a globular protein having a molecular weight between 5 and $15 \cdot 10^6$. The presence of such large micelles suggests that the protein molecules which form them are aggregates of several polypeptide subunits.

According to this, BRIJ-36T possibly breaks only hydrophobic bonds between the lipids and proteins of the cell membrane. According to their kinetic behavior and their separation in agarose-gel columns the two formate dehydrogenases appear to be quite different enzymes. K_m for formate, optimum temperature, optimum pH and

molecular weight differ greatly. It is interesting to mention that formate dehydrogenase H had a lower optimum pH than formate dehydrogenase N; whereas Peck and Gest[1] reported exactly the opposite. The difference may be due to the fact that different strains were used. It has been suggested[4,5] that both formate dehydrogenases might have a common genetic element, perhaps the structural gene. According to the results reported here, it would be necessary to assume that the polypeptide product of the gene is bound to other protein moieties not released by BRIJ-36T which give different characteristics to both enzymes. Only an analysis of the monomer components of each enzyme will throw some light on this matter.

There are few reports which compare the kinetic behavior of particulate *versus* solubilized membrane enzymes. Gawron *et al.*[17] observed a different kinetic behavior between the soluble and particulate succinate dehydrogenase. These authors attribute the differences observed to possible interactions with other electron carriers. Cerletti *et al.*[18] have discussed the different properties of membrane-bound and soluble succinate dehydrogenase and have stressed the importance of a hydrophobic medium on the behavior of the enzyme. According to our data, solubilization of formate dehydrogenases did not greatly affect the general properties of both enzymes. These results suggest that the microenvironment of the membrane-bound enzyme is not very different from that found in the detergent suspension.

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REFERENCES

1 H. D. Peck and H. Gest, *J. Bacteriol.*, 73 (1957) 706.
2 C. T. Gray, J. W. T. Wimpenny, D. E. Hughes and M. R. Mossman, *Biochim. Biophys. Acta*, 117 (1966) 22.
3 J. Ruíz-Herrera, M. K. Showe and J. A. De Moss, *J. Bacteriol.*, 97 (1969) 1291.
4 J. Ruíz-Herrera and J. A. De Moss, *J. Bacteriol.*, 99 (1969) 720.
5 R. L. Lester and J. A. De Moss, *J. Bacteriol.*, 105 (1971) 1006.
6 P. S. Sypherd and N. Strauss, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 400.
7 D. W. Morris and J. A. De Moss, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 262.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
9 C. W. Fiske and Y. SubbaRow, *J. Biol. Chem.*, 8 (1929) 629.
10 E. Itagaki, T. Fujita and R. Sato, *Biochem. Biophys. Res. Commun.*, 5 (1961) 30.
11 A. W. Linnane and C. W. Wrigley, *Biochim. Biophys. Acta*, 77 (1963) 408.
12 J. P. Williams, J. T. Davidson and H. D. Peck, *Bacteriol. Proc.*, (1964) 110.
13 A. Gilby and A. Few, *Nature*, 181 (1958) 55.
14 V. T. Marchesi and G. E. Palade, *J. Cell Biol.*, 35 (1967) 385.
15 D. F. H. Wallach, in *Membrane Proteins*, Little Brown Co., Boston, 1969, p. 3.
16 C. Gitler, G. Martinez-Cedillo, D. Martinez-Rojas and G. Chavez-Diaz, *National Cancer Institute Monographs*, 27 (1967) 153.
17 O. Gawron, K. P. Mahajan, M. Limetti, G. Kananen and A. J. Glaid, *Biochemistry*, 5 (1966) 411.
18 P. Cerletti, S. Giovenco, G. Testolin and I. Binotti, in L. Bolis and B. A. Pethica, *Membrane Models and the formation of Biological Membranes*, North Holland, Amsterdam, 1968, p. 166.
19 J. Ruíz-Herrara and A. Alvarez, *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, in the press.
20 J. Ruíz-Herrara and I. Figueroa, *Rev. Latinoam. Microbiol.*, 14 (1972) 99.