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THE EFFECT OF LOW CONCENTRATIONS OF GLUTARALDEHYDE ON *MICROCOCCUS LYSODEIKTICUS* MEMBRANES: CHANGES IN THE RELEASE OF MEMBRANE-ASSOCIATED ENZYMES AND MEMBRANE STRUCTURE

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SUMMARY

The manner in which enzymes are associated with the plasma membrane of *Micrococcus lysodeikticus* can be modified in certain instances by the action of an aldehyde cross-linking agent. Enzymes such as ATPase (EC 3.6.1.3), NADH dehydrogenase (EC 1.6.99.3) and polynucleotide phosphorylase (EC 2.7.7.8) can normally be selectively released from the membrane by controlled washing with buffers. This release phenomenon is prevented when membranes are first treated with 0.5 % (v/v) glutaraldehyde. Under these conditions the enzymes become more strongly attached to the membrane while retaining activity. In the case of NADH dehydrogenase it can be shown that the total activity is unchanged after its association with the membrane has been strengthened by this treatment. The inhibition of enzyme release is supported by the results of polyacrylamide gel electrophoresis and by the fact that glutaraldehyde treated membranes constitute a larger fraction of total cell protein than untreated cell membranes. Electron microscopy reveals that the selective release of enzymes from untreated membranes is associated with a progressive reduction in granular substructure on the membrane surface. The smooth surfaced residue which remains, contains cytochromes and succinate dehydrogenase and is resistant to further release by washing. Membranes treated with glutaraldehyde retain the granular substructure despite the washing procedures.

INTRODUCTION

A large number of enzymes have been shown to be associated in some way with cellular membranes. The widely different techniques required to "solubilize" a number of these enzymes are believed to reflect differences in the strength and manner of their binding to the membrane. Previous reports from this laboratory have described a

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate.

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Ca^{2+}-dependent ATPase (EC 3.6.1.3) associated with the membrane of *Micrococcus lysodeikticus* which can be selectively released by two well defined steps[1-3]. Preliminary experiments indicated that NADH dehydrogenase (EC 1.6.99.3) activity could also be detached from the membrane complex under controlled conditions. In view of this "solubilization" of membrane components it was of interest to determine if the nature of the binding of these detachable proteins could be modified in the membrane prior to isolation, to the extent that their selective release would be prevented, without imparing their enzymatic function. Fixed in this way, the membrane might hopefully be isolated in a form more closely resembling the complex structural and functional system wihch is believed to characterize its *in vivo* activity. Such a membrane would be of use in immunological, biochemical and structural studies directed towards locating the functional site of membrane components.

MATERIALS AND METHODS

ATP (disodium salt) and ADP (sodium salt) were purchased from Pabst Laboratories Biochemicals.

Disodium succinate, phenazine methosulphate and NADH were obtained from the California Corporation for Biochemical Research. 2,6-Dichlorophenolindophenol was obtained from the Sigma Chemical Co. All other reagents were commercial products.

Membrane preparation

Micrococcus lysodeikticus (NCTC 2665) was grown and harvested as previously described[1,2,4]. Protoplasts were prepared and burst as described by MUÑOZ *et al.*[1] with the exceptions that 0.005 M $MgCl_2$ was added to the bursting mixture and deoxyribonuclease was omitted at this stage. Glutaraldehyde treatment of membranes was carried out by including various concentrations (0.1 % to 0.5 % (v/v)) of redistilled glutaraldehyde in the bursting buffer. This concentration of glutaraldehyde was based on the total volume of the bursting buffer *plus* the volume of the added protoplasts. The protoplasts were added as a thick suspension to the buffer with slow stirring. After bursting had occurred, the viscosity of the suspension was reduced by the addition of deoxyribonuclease (20 μg/ml). The resulting lysate was immediately centrifuged (30 min, 30000 $\times$ *g*), the cytoplasm and membrane separated and the pellets washed with various buffers adjusted to pH 7.5. The first 4 membrane washes were carried out on the same day and the pellets stored at 4° overnight. The remaining washes were completed on the second day. Membranes were recovered after each wash by centrifugation at 30000 $\times$ *g* for 30 min.

Analytical methods

Protein content was determined by the method of LOWRY *et al.*[5]. RNA + DNA content was determined by the orcinol reaction[6] with appropriate controls as described by SALTON AND FREER[4]. Control and treated membranes were examined after centrifugation for 2 h in an SW25 rotor at 24000 rev./min on discontinuous sucrose gradients as described by SALTON[6]. Sonicated membranes and membrane washes were also examined in double diffusion tests against antiserum to freshly prepared membrane[6]. Polyacrylamide disc gel electrophoresis of the membrane washes was carried out as described by SALTON[6].

Enzyme assays

ATPase activity was measured by the liberation of inorganic phosphorous (P_i) from ATP as described by MUÑOZ *et al.*[1,2]. One unit of ATPase activity is defined as the amount of enzyme able to liberate 1 μmole of P_i per 10 min under the assay conditions.

Polynucleotide phosphorylase (EC 2.7.7.8) activity was also measured by P_i liberation as described by MUÑOZ *et al.*[2]. One unit of activity in this case is defined as the amount of enzyme liberating 1 μmole P_i per h.

NADH dehydrogenase activity was followed spectrophotometrically at 25° and 600 nm in a Cary model 15 spectrophotometer by a modification of the method of ELLS[7]. The test system contained 0.02 ml of 2.5 mM 2,6-dichlorophenolindophenol (DCIP), 0.1 ml of $8 \cdot 10^{-5}$ M NADH, 0.05 ml of 0.01 M KCN and from 0.01 ml to 0.1 ml of sample containing the enzyme. The volume was made up to 1.0 ml in the cuvette (1-cm light path) by the addition of 0.05 M Tris buffer (pH 7.5) and the reaction initiated by the addition of NADH. One unit of enzyme activity is defined as the amount of enzyme dehydrogenating 1 μmole of NaDH per min under the assay conditions.

Succinate dehydrogenase (EC 1.3.99.1) activity was assayed in a test system based on that of ELLS[7]. The decrease in absorbance at 600 nm and 25° was followed in an assay system containing 0.2 ml of 0.02 M disodium succinate, 0.02 ml of 2.5 mM DCIP, 0.05 ml of 0.01 M KCN, 150 μg of phenazine methosulphate (PMS) and from 0.05 ml to 0.1 ml of sample containing the enzyme. The volume was made up to 1.0 ml by the addition of 0.05 M Tris buffer (pH 7.5). The reaction was initiated by the addition of PMS in the form of a freshly prepared solution containing 3 mg/ml, which was stored in the dark during all assays. In all assays of membrane preparations the fractions were activated by preincubation for 20 min at 25° in 40 mM succinate. No additional activation was obtained by the use of phosphate buffer. One unit of succinate dehydrogenase activity is the amount of enzyme dehydrogenating 1 μmole of succinate per min under the assay conditions. Correction was made for any nonenzymic reduction of DCIP.

Electron microscopy

Membranes were examined after negative staining with ammonium molybdate essentially as described by MUÑOZ *et al.*[1].

RESULTS

The effect of different treatments on the partition of protein and enzyme activities in a series of experiments is shown in Table I. The results indicate a wide variation in the relative amounts of cytoplasm and pellet material in the treated and untreated protoplasts. Membranes isolated from untreated protoplasts comprised 65 % of the total cellular protein, whereas when the protoplasts were exposed to 0.5 % glutaraldehyde, 80 % of the protoplast protein was isolated in the membrane pellet. Although in the course of washing both treated and untreated membranes lost protein into the supernatants, the final amount of protein in the treated and untreated membranes differed. Washed glutaraldehyde treated membranes as seen in Table I represented 12.7 % of the total cell protein, whereas untreated washed membranes comprised only 8.8 % of this total. Chemical analyses of these membrane fractions revealed that there

TABLE I

PARTITION OF PROTEIN, ATPASE, NADH DEHYDROGENASE AND POLYNUCLEOTIDE PHOSPHORYLASE IN FRACTIONS OBTAINED FROM UNTREATED PROTOPLASTS AND PROTOPLASTS TREATED WITH GLUTARALDEHYDE

Expt. I: Untreated protoplasts. Expts. II and III: Protoplasts treated with 0.2 % and 0.5 % of glutaraldehyde, respectively. Washes 1–6 comprise the supernatant obtained after the membranes had been suspended in the appropriate washing buffer and subsequently sedimented by centrifugation as described in MATERIALS AND METHODS. For washes 1 to 4 the membrane pellet was resuspended in 125 ml of Tris–EDTA. For washes 5 and 6, the membranes were resuspended in twice this volume of 3 mM Tris. The figures for total activity for each of the three enzymes represent the sum of the activities recovered in each of the washing steps together with the activities in the residual membrane and cytoplasmic fraction.

	Cytoplasm	*Pellet membrane*	*30 mM Tris + 1 mM EDTA*				*3 mM Tris*		*Residual membrane*	*Total Activity*
			1st wash	*2nd wash*	*3rd wash*	*4th wash*	*5th wash*	*6th wash*		
Protein (mg)										
Expt. I	353	647	256	80	53	64	53	53	88	
Expt. II	301	699	198	94	69	83	87	87	81	
Expt. III	196	804	81	81	65	93	193	164	127	
ATPase (units)										
Expt. I	63	+++*	18	18	77	113	462	315	−*	1066
Expt. II	0		0	36	84	134	334	268	++*	857
Expt. III	0		6	8	18	36	59	59	++*	186
NADH dehydrogenase (units)										
Expt. I	38		55	25	50	206	440	441	80	1335
Expt. II	10		30	59	30	118	470	378	96	1191
Expt. III	4		2	5	7	16	47	67	1200	1348
Polynucleotide phosphorylase (units)										
Expt. I	157		204	155	176	149	252			1093
Expt. II	90		204	191	187	191	252			1115
Expt. III	36		29	48	57	79	158			407

* Indicates (+ → +++) relative amount present or (−) absence of trypsin activated ATPase; exact units not given because of complexity of trypsin activation kinetics.

was a slight increase in the amount of nucleic acids associated with the glutaraldehyde treated membranes. Membranes treated with 0.5 % glutaraldehyde contained 3 % nucleic acid compared with 2.5 % for the untreated membranes.

The measured activity of NADH dehydrogenase activity in the various fractions shown in Table I confirms the findings of MITCHELL[8], GELMAN[9] and others, that this enzyme is located in the membrane fraction. Approx. 97 % of the total NADH dehydrogenase is detectable in the membrane pellet obtained from untreated protoplasts. Of this membrane associated activity, 94 % is released under these defined washing conditions leaving only 6 % of the original membrane associated activity in the residual pellet. This situation is reversed in membranes obtained from protoplasts treated with 0.5 % glutaraldehyde. In this instance less than 1 % of the total NADH dehydrogenase activity is measurable in the cytoplasm and only 10 % of the total activity is released upon washing. However almost 90 % of the NADH dehydrogenase activity remains in the residual membrane pellet. This distribution of NADH dehydrogenase activity is summarised in Table II. The total measurable NADH dehydrogenase activities in Experiments I and III are seen to be approximately the same in-

dicating that the glutaraldehyde is in fact strengthening the association between enzyme and membrane without inhibiting it selectively in either the free or bound state.

TABLE II

PARTITION OF NADH DEHYDROGENASE ACTIVITY IN FRACTIONS FROM UNTREATED PROTOPLASTS AND PROTOPLASTS TREATED WITH GLUTARALDEHYDE

Treatment	% *Total NADH dehydrogenase activity*		
	Cytoplasm	*Washings*	*Residual membrane*
None (Expt. I)	3	91	6
0.5 % glutaraldehyde (Expt. III)	0.3	10	89

Table I also shows that 6 % of the total number of ATPase units from untreated protoplasts were detectable in the cytoplasmic fraction. No ATPase activity was found in the cytoplasm from protoplasts treated with either 0.2 % or 0.5 % glutaraldehyde. The total number of ATPase units in the washes from each experiment decreases markedly, suggesting that this enzyme may also be bound more strongly to the membrane complex after glutaraldehyde treatment. In support of this possibility, the selective release of ATPase in Experiment I is partially inhibited in Expt. II and completely absent in Expt. III. The relative amount of ATPase remaining in the membrane residues after this washing procedure can be estimated by means of trypsin activation as described by MUÑOZ *et al.*[3]. While it is not possible to obtain quantitative totals of this residual ATPase activity owing to the complexity of the kinetics of trypsin activation, experiments indicate that a substantial amount of trypsin activatable ATPase remains associated with the 0.5 % glutaraldehyde treated membranes after washing. Because of this difficulty in quantitating residual ATPase activity, it is not possible to determine to what extent the ATPase is inhibited by these concentrations of glutaraldehyde as is the case with NADH dehydrogenase.

As shown in Table I there is a substantial reduction in the amount of polynucleotide phosphorylase detectable in the cytoplasm of protoplasts treated with 0.5 % glutaraldehyde compared to that of untreated protoplasts. This reduction was also marked in washes 1 through 5, although the actual amount released did increase significantly with each wash. Once again, because of the difficulty of measuring residual polynucleotide phosphorylase activity in the washed membrane it is not possible to distinguish clearly between an inhibition of the release process and an inhibition of activity. Although there was a substantial reduction in the total polynucleotide phosphorylase activity released in Expts. II and III compared to Expt. I, the fraction of the total activity detectable in the cytoplasm in each case was not correspondingly reduced.

The results of the polyacrylamide disc gel electrophoresis seen in Figs. 1A and 1B support to some extent the suggestion that the glutaraldehyde is preventing the release of membrane protein upon washing. The characteristic banding patterns obtained from the washes of untreated membranes are similar to those already reported by MUÑOZ *et al.*[2] and include the prominent band identified as ATPase by these workers. These bands are seen to be absent from the corresponding washes of membranes

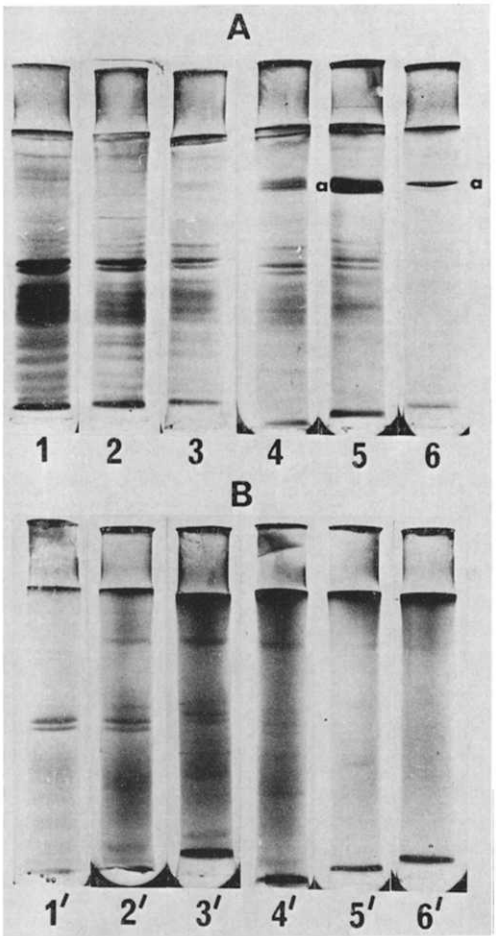
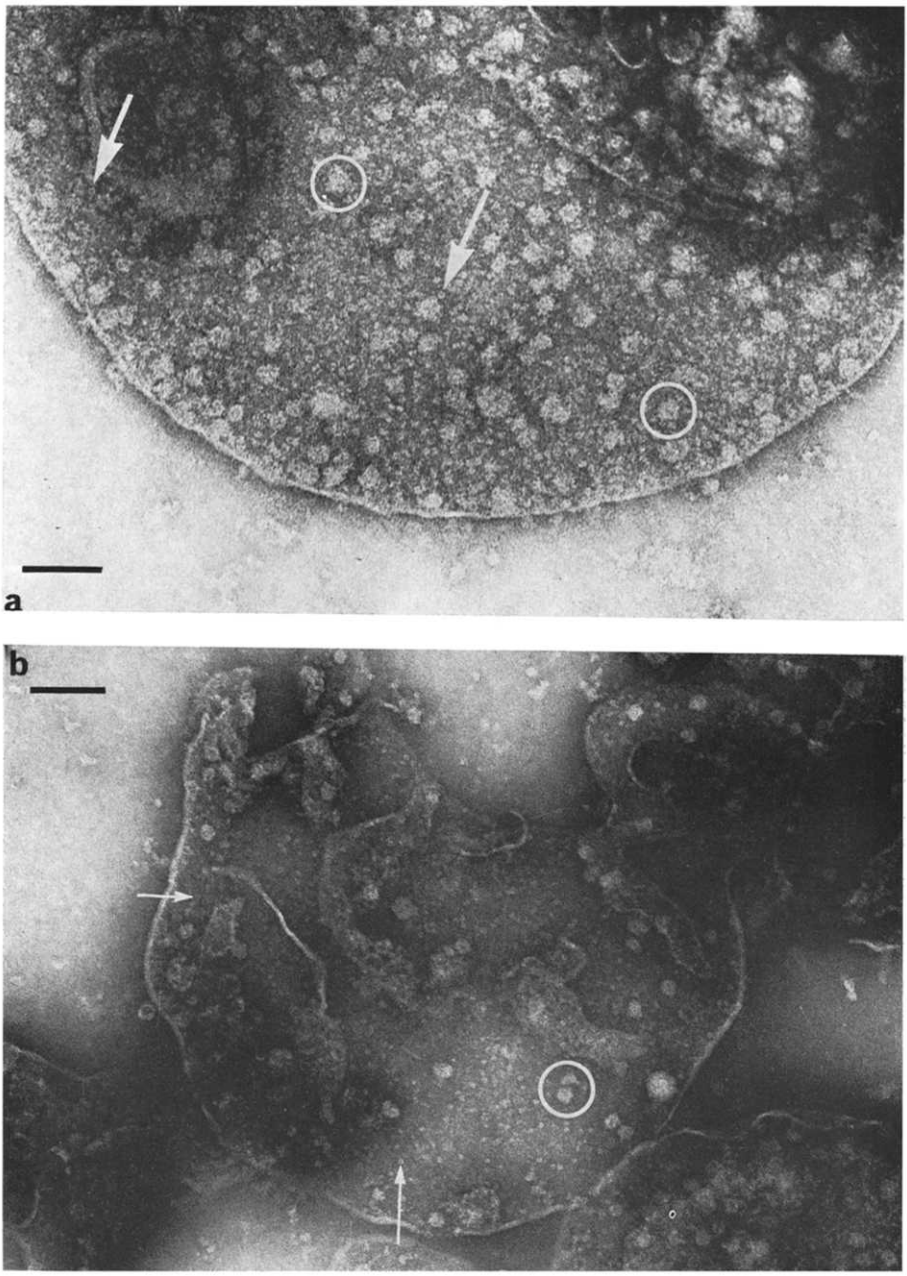


Fig. 1. Disc electrophoresis of supernatants from membrane washes. Staining was with aniline black; migration was towards the anode (bottom of the gels). (A) Gels (1 through 6) showing electrophoresis of the six consecutive washes of untreated membrane (Expt. I). (B) Gels (1′ through 6′) showing electrophoresis of the six consecutive washes of membrane treated with 0.5% glutaraldehyde (Expt. III). ATPase activity is located in bands labelled a.

TABLE III

ACTIVITY OF SUCCINATE DEHYDROGENASE IN MEMBRANE PREPARATIONS

Membrane preparation	*Succinate dehydrogenase activity*	
	Units (nmoles/min)	*Specific activity (nmoles/min per mg portein)*
Untreated residue (Expt. I)	3200	340
0.2% glutaraldehyde residue (Expt. II)	3000	370
0.5% glutaraldehyde residue (Expt. III)	3400	270
Freshly isolated pellet membrane	3600	180



Figs. 2a and 2b. Electron micrographs of untreated *M. lysodeikticus* membranes prior to washing (Expt. 1). Preparations are negatively stained with ammonium molybdate. Marker in all instances corresponds to 0.1 μm.

treated with 0.5 % glutaraldehyde. There is, however, an indication of an increase in the amount of material remaining at the interface between the stacking and separating gels in the washes from glutaraldehyde treated membranes.

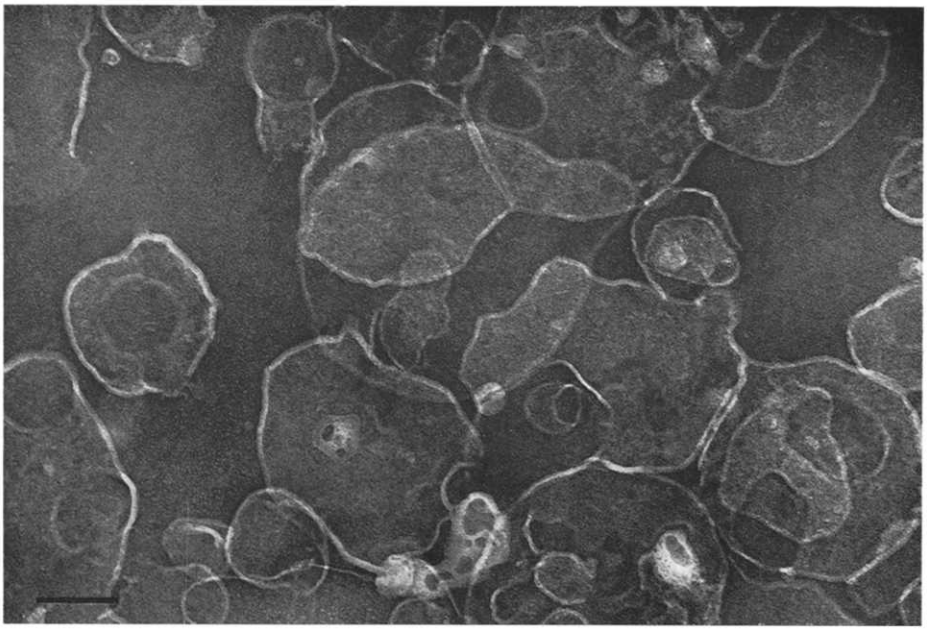


Fig. 3. Electron micrograph of untreated *M. lysodeikticus* membrane after the six consecutive washes of Expt. I.

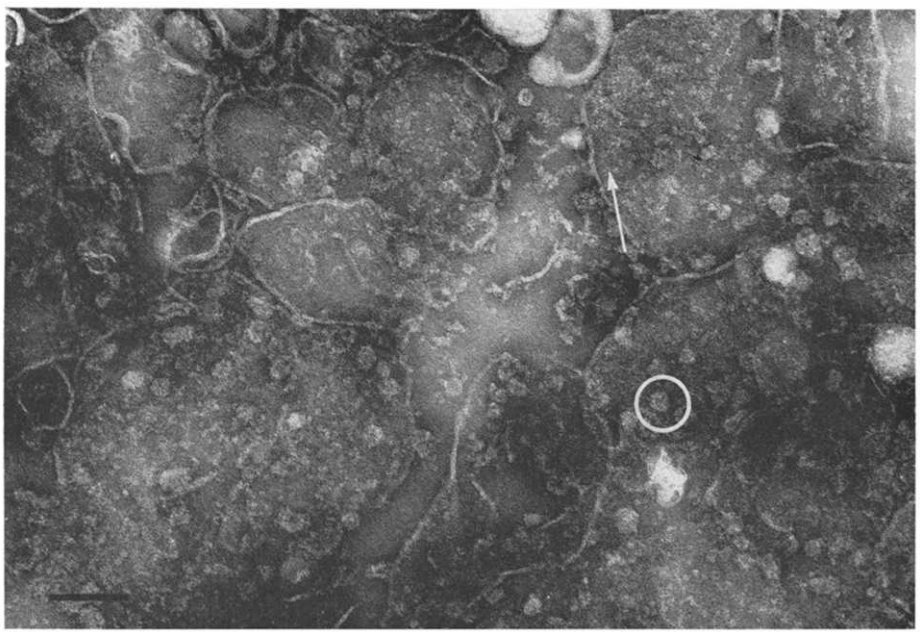


Fig. 4. Electron micrograph of 0.5 % glutaraldehyde treated *M. lysodeikticus* membranes after the six consecutive washes of Expt. III.

Table III shows the activity of succinate dehydrogenase in the various membrane residues after washing, together with the activity in a sample of untreated pellet membrane washed six times with 0.05 M Tris buffer (pH 7.5). The results indicate that while there is little change in the total succinate dehydrogenase activity after glutaraldehyde treatment, there is a doubling in the specific activity of the enzyme in the untreated membrane residue in Experiment I compared to that in pellet membrane washed six times in 0.05 M Tris. Since the protein content of the untreated membrane residue comprises only 12 % of the freshly isolated pellet membrane protein, these results suggest that the increase in specific activity of succinate dehydrogenase is associated with the removal by washing of other membrane proteins such as ATPase and NADH dehydrogenase *etc.* This suggestion is further supported by the finding that there is a correspondingly smaller increase in specific activity of succinate dehydrogenase in the 0.5 % glutaraldehyde treated membrane residue, which contains approx. 44 % more portein than the untreated residue. No significant succinate dehydrogenase activity was detectable in the cytoplasm or any of the washes from Expts. I, II or III.

The observed loss of membrane protein under these conditions of washing is accompanied by well defined changes in the appearance of the various membrane fractions in the electron microscope. Figs. 2a and 2b illustrate the appearance of the freshly isolated membrane pellet prior to any washing. The membrane surface is seen to be almost completely covered with granule-like structures of varying size. Some of these (arrows) appear to consist of a hollow stained core surrounded by a number of subunits, whilst others resemble closely the structures previously reported for the ATPase of *M. lysodeikticus*[1]. Other larger structures (circles) appear in some instances to be composed of aggregates of smaller subunits oriented in different directions. When the untreated membranes are washed under the conditions of Experiment I the difference in the appearance of the membranes is very marked as shown in Fig. 3. There is almost a complete dissappearance of granule-like substructure from the membrane surface. The membrane fragments are generally smaller in size and less electron dense than the starting membrane with a degree of transparency which permits one fragment to be seen through a second superimposed fragment. The difference between these untreated residues and the residues from membranes treated with 0.5 % glutaraldehyde in Expt. III is equally striking. As illustrated in Fig. 4, glutaraldehyde treated membranes closely resemble freshly isolated membranes in the degree and variety of surface structure which they possess.

DISCUSSION

The value of glutaraldehyde treatment in preserving *in vivo* enzymatic activity is well known.[10–12] In their work with mitochondria, UTSUMI AND PACKER[13] and DEAMER *et al.*[14] have recently shown that glutaraldehyde functions to preserve enzymatic activities and also to trap conformational states. *In vitro*, glutaraldehyde has been shown to cross-link crystals of carboxypeptidase A to give a product with considerable enzymic activity[15,16]. With regard to the site of action of glutaraldehyde, HABEEB AND HIRAMOTO[17] have recently shown that with proteins *in vitro*, glutaraldehyde reacts predominantly with the epsilon amino group of lysine to form mainly intermolecular cross linkages. Some reaction also occurs with tyrosine, histidine and

sulphydril residues. Proteins treated in this way were still capable of reacting with antibodies to the native proteins. These results together with those reported in this study suggest that the controlled use of glutaraldehyde may indeed be useful in the preparation of membranes which retain their *in vivo* complement of enzymic and antigenic activity in a three dimensional structure closely resembling the native configuration.

MIZUSHIMA[18] has recently reported the extraction of a significant amount of NADH dehydrogenase from the membrane of *Bacillus megaterium* by mild treatment with alkali. With this method, 70 % of the total NADH dehydrogenase activity in the membrane could be extracted. The activity remaining in the membrane after this treatment was much more sensitive to acidification than the extractable activity suggesting that this enzyme might occur in the membrane in two different forms. Our results for *M. lysodeikticus* show that 94 % of the total NADH dehydrogenase activity in the membrane can be extracted by these relatively mild washing treatments. The procedure may therefore prove to be a more effective method of solubilising this enzyme. The yield of NADH dehydrogenase after this treatment suggests that only one form of this enzyme may exist in *M. lysodeikticus*. Since 90 % of the total NADH dehydrogenase activity is not extracted from glutaraldehyde treated membranes by this procedure, it would seem that while the cross-linking action of glutaraldehyde has no effect on the activity of the enzyme, it does result in a marked strengthening of its binding to the membrane. From what is now known of the chemistry of glutaraldehyde action[17] this may enable us to speculate on the nature of chemical groups at the surface of both membrane and enzyme.

These results also confirm the low level of cytoplasmic ATPase in untreated protoplasts reported by MUÑOZ *et al.*[2]. However no ATPase was detectable in the cytoplasm of protoplasts treated with glutaraldehyde. This was the case even with 0.2 % glutaraldehyde treated membranes which were still capable of releasing substantial amounts of ATPase upon washing. These findings might indicate that *in vivo* all ATPase is associated with the membrane and that the small amount which is detectable in the cytoplasm in the absence of glutaraldehyde is the inevitable result of the mechanical dislocation during protoplasting. Alternatively, only that fraction of ATPase which exists in the free state *in vivo* may be susceptible to inhibition as a result of glutaraldehyde treatment. The remaining enzyme although not inhibited, is of course bound to the membrane upon exposure to the aldehyde. These alternatives are at present being studied.

Washed membranes from 0.5 % glutaraldehyde treated protoplasts constitute 12.7 % of total cell protein compared to 8.8 % for the membranes from untreated protoplasts. This increase can be partly explained by the additional binding of NADH dehydrogenase, ATPase or polynucleotide phosphorylase, although once again the problem is complicated by the difficulty of assaying the last two of these enzymes when they are associated with the membrane.

Analyses fail to indicate any substantial increase in nucleic acid associated with glutaraldehyde treated membranes. Part of this increase in membrane protein may reflect the binding of other enzymes which are normally only weakly associated with the membrane and which are usually dislodged during protoplasting. A more extensive survey of the enzymic activity of glutaraldehyde treated membranes is at present being carried out to investigate this possibility.

It is clear from the electron micrographs that the changes in enzymic capacity of the membranes are reflected in their appearance. As enzymes are selectively and progressively removed by washing, the granular sub-structure on the membrane surface gradually disappears. The smooth-surfaced residue which remains is resistant to further washing and would seem to differ in some way from the 'detachable' proteins which were originally attached to it. One such difference which these studies reveal is that the total succinate dehydrogenase activity of the membrane is associated with this residual fraction. A similar location of succinate dehydrogenase activity was found in a residual fraction obtained after deoxycholate treatment of intact membrane from *M. lysodeikticus*[19]. The electron micrographs demonstrate a marked similarity between the deoxycholate treated residue and that obtained in this work by controlled washing with buffers. Both preparations are clearly electron transparent with no surface particles. In addition, the cytochromes of *M. lysodeikticus* are almost exclusively localised in the deoxycholate residue[19] and in the residue from the washing treatment (unpublished observation). The evidence therefore suggests that certain components of the electron transport chain in this organism are located in a relatively rigid fraction which is resistant to various procedures designed to remove 'detachable' proteins. The fact that this fraction can be obtained from the membrane by the relatively mild washing treatments described in this work, lends support to the idea that it represents an important structural and functional component of the native membrane.

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