Biochem. J. (1981) **195**, 627–637
Printed in Great Britain

The organization of formate dehydrogenase in the cytoplasmic membrane of *Escherichia coli*

Alexander GRAHAM and David H. BOXER
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

(*Received 17 December 1980/Accepted 4 February 1981*)

The arrangement of the proton-translocating formate dehydrogenase of the anaerobic respiratory chain of *Escherichia coli* within the cytoplasmic membrane was examined by direct covalent modification with non-membrane-permeant reagents. Three methods were employed, lactoperoxidase-catalysed radioiodination, labelling with diazotized [^{125}I]di-iodosulphanilic acid and labelling with diazobenzene[^{35}S]sulphonate. All three procedures yield results consistent with the view that the two larger subunits of the enzyme, M_r 110000 and 32000, both occupy transmembranous locations within the membrane. In each case the modification of the Ca^{2+} or Mg^{2+}-activated F_1-ATPase was monitored, and all reagents employed correctly located this enzyme at the cytoplasmic face of the membrane. A procedure involving agglutination with specific antibodies is described which appears to fractionate membrane vesicles of mixed orientation into two populations, one with the same membrane orientation as that of spheroplasts and the other of opposite orientation.

Formate dehydrogenase (EC 1.2.2.1) catalyses the proton-translocating oxidation of formate coupled to the reduction of quinone in the anaerobic respiratory chain of *Escherichia coli* (Garland *et al.*, 1975; Gutowski, 1976). The proton-translocating activity of this enzyme has been established by direct measurement of the stoichiometries of translocated protons per molecule of formate oxidized for the oxidation of formate by a variety of electron acceptors (Jones, 1980). The enzyme has been purified and partially characterized (Enoch & Lester, 1975). It is an iron–sulphur molybdenum-containing enzyme that is found in association with a cytochrome *b*. It comprises three types of polypeptide chain, α (M_r 110000), β (M_r 32000) and γ (M_r 20000). The haem is thought to be associated with the γ-subunit (Enoch & Lester, 1975). That the enzyme catalyses proton translocation implies that it is arranged in a transmembranous manner in the cytoplasmic membrane. In this paper we explore the organization of the enzyme within the membrane by direct covalent modification with non-membrane-permeant reagents. We conclude that both the α- and β-subunits of the enzyme span the cytoplasmic membrane.

Abbreviations used: [^{125}I]DDISA, diazotized [^{125}I]-di-iodosulphanilic acid; [^{35}S]DABS, diazobenzene[^{35}S]-sulphonate; F_1-ATPase, Ca^{2+}- or Mg^{2+}-activated soluble ATPase; SDS, sodium dodecyl sulphate.

Materials and methods

Growth of Escherichia coli

Escherichia coli (strain EMG29) was grown anaerobically in a medium based on that of Cohen & Rickenberg (1956) containing glucose (0.2%, w/v), casamino acids (0.1%, w/v), KNO_3 (1%, w/v), $(NH_4)_6Mo_7O_{24}$ (1 μM), K_2SeO_3 (1 μM), $MgCl_2$ (1 mM) and proline, histidine and tryptophan (10 μg/ml each). Growth was at 37°C in 500ml tightly stoppered bottles for anaerobic conditions.

For growth of bacteria on [^{35}S]sulphate, *E. coli* (strain EMG29) was grown in the medium described above except that casamino acids and $(NH_4)_2SO_4$ were omitted and were replaced by NH_4Cl (0.4%, w/v) and K_2[^{35}S]SO_4 (100 μM; sp. radioactivity 10Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.). Pre-cultures were grown aerobically in a nutrient broth, then washed three times, by centrifugation, in 50mM-sodium phosphate buffer, pH6.8, to remove residual sulphate before inoculation into the above medium (Graham, 1979).

Preparation of spheroplasts and membrane vesicles

Bacteria (grown on non-radioactive material or on [^{35}S]sulphate) were sedimented from cultures by centrifugation at 7000**g** for 15min at 4°C in the 6 × 750ml rotor of an MSE Mistral 6L centrifuge.

0306-3275/81/060627-11$01.50/1 © 1981 The Biochemical Society

Spheroplasts were prepared from bacteria by a lysozyme/EDTA method (Osborn *et al.*, 1972) and conversion into spheroplasts was monitored by phase-contrast microscopy and osmotic sensitivity. Sedimented bacteria were resuspended in 0.75 M-sucrose/20 mM-sodium phosphate, pH 7.5, to obtain an A_{600} of 10. Lysozyme (2 mg/ml) was added to a final concentraion of 100 μg/ml and the mixture was incubated on ice for 5 min. The suspension was then slowly diluted with 0.5 vol. of ice-cold 1.5 mM-EDTA, pH 7.5, over a period of 5 min. Spheroplasts were used immediately.

Membrane vesicles were prepared from spheroplasts by ultrasonic disruption, with an MSE 150W ultrasonicator [9.5 mm ($\frac{3}{8}$in) diam. probe] for five periods of 20 s (with 20 s intervals), followed by centrifugation at 7000 *g* for 15 min, all at 4°C. The supernatant so obtained was centrifuged at 250000 *g* for 1 h and the pellet (membrane vesicles) was resuspended in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5. The membrane vesicles were stored at −20°C before use, except for the [^{35}S]sulphate-labelled material, which was used immediately.

Preparation of antisera

Formate dehydrogenase was purified from *E. coli* essentially as described by Enoch & Lester (1975). Purified enzyme was assayed by using phenazine methosulphate-mediated reduction of the artificial electron acceptor dichlorophenol-indophenol, with formate as electron donor (Enoch & Lester, 1975). Rabbits were immunized with purified formate dehydrogenase (specific activity 23 μmol of dichlorophenol-indophenol reduced/min per mg of protein), which had only M_r-110000, -32000, and -22000 bands on protein-stained polyacrylamide gels, and contained 1.5 nmol of spectroscopically-determined cytochrome *b*/mg of protein.

Purified F_1-ATPase from *Escherichia coli* was a gift from Dr. G. Vogel (University of Tubingen, Federal Republic of Germany), and this was used to prepare antibodies.

Nitrate reductase was purified essentially as described by Enoch & Lester (1975) and had only M_r-150000 and -59000 polypeptides on protein-stained polyacrylamide gels and contained no spectroscopically detectable cytochrome *b*.

Antigens were injected subcutaneously into separate rabbits which had each been bled previously to obtain pre-immune control serum. Formate dehydrogenase (0.84 mg) and F_1-ATPase (0.2 mg) were injected on days 1 and 7 with an equal volume of Freund's Complete Adjuvant (Difco, Detroit, MI, U.S.A.), and on days 14 and 21 with an equal volume of water. Serum samples were clarified by centrifugation at 10000 *g* for 30 min and stored frozen at −20°C.

Anti-(formate dehydrogenase) serum [and anti-(nitrate reductase) serum] produced two precipitin arcs when analysed by crossed immunoelectrophoresis against a Triton X-100 extract of *E. coli* (Smyth *et al.*, 1978). Each antiserum produced one very strong arc and one weak arc. Since both these enzymes co-purify until the last step in the purification procedure (Enoch & Lester, 1975), it seemed likely that the weak arc present in each antiserum was to the other protein.

Anti-(formate dehydrogenase) serum was made specific by using immunoadsorption with a nitrate reductase preparation (which contained no detectable formate dehydrogenase). Anti-(nitrate reductase) serum was made specific by using immunoadsorption with a formate dehydrogenase preparation (which contained no detectable nitrate reductase). Immunoprecipitation was performed as described below, and the antisera were tested, by crossed immunoelectrophoresis against a Triton X-100 extract of membrane vesicles, each revealing only one precipitin arc.

Lactoperoxidase-catalysed radioiodination

Isolated formate dehydrogenase (50 μg) was incubated at room temperature with lactoperoxidase (5 μg/ml; Sigma), 0.4 nmol of $Na^{125}I$ (sp. radioactivity 6.25 Ci/mmol; The Radiochemical Centre) and 0.1 μmol of H_2O_2 for 5 min (in a final volume of 250 μl of 20 mM-Tris/HCl, pH 7.4). The reaction was quenched by addition of an equal volume of non-radioactive KI (2 μmol), and the formate dehydrogenase was recovered by immunoprecipitation.

Formate dehydrogenase (50 μg) was also labelled in the presence of 0.1% (w/v) SDS, by using lactoperoxidase (5 μg/ml), 0.4 nmol of $Na^{125}I$ (sp. radioactivity 6.25 Ci/mmol) and 0.5 μmol of H_2O_2 (in a final volume of 200 μl of 20 mM-Tris/HCl, pH 7.4). After 30 min at room temperature the proteins were precipitated with 10% (w/v) trichloroacetic acid, resuspended in 50 mM-Tris/HCl, pH 7.5, and were examined by SDS/polyacrylamide-gel electrophoresis.

Spheroplasts (4 mg of protein) and membrane vesicles (0.5 mg protein) were suspended together at room temperature to a final volume of 0.75 ml in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5, containing 50 μg of lactoperoxidase and 10 nmol of $Na^{125}I$ (sp. radioactivity 25 Ci/mmol). Five additions, each of 1 μmol of H_2O_2, were made at 3 min intervals. Spheroplasts and membrane vesicles from four parallel iodinations were separated by centrifugation at 2500 *g* for 15 min. The pellet (spheroplasts) were resuspended in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA/2 mM-KI, pH 7.5, and was washed three times by centrifugation at 5000 *g* for 15 min, the supernatant

being discarded each time. Membrane vesicles were prepared from the washed, labelled spheroplasts by ultrasonic disruption as described above. This vesicle preparation contained cytoplasmic membranes labelled on their periplasmic surface.

The supernatant fraction from the 2500 *g* centrifugation, containing the labelled membrane vesicles, was re-centrifuged at 5000 *g* for 15 min and the pellet was discarded. Membrane vesicles were then washed three times by suspension in a medium containing 50 mM-Tris/HCl, 1 mM-$MgCl_2$, 5 mM-benzamidine/HCl (Aldrich Chemical Co., Gillingham, Dorset, U.K.), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one hydrochloride ('TLCK'; Sigma; 0.1 mg/ml) and 2 mM-KI, pH 7.5, and resedimentation at 250000 *g* for 1 h. The washed, labelled vesicle preparations (from spheroplasts and membrane vesicles) were each made 2% (w/v) in Triton X-100 and stirred for 1 h at 4°C. The mixture was centrifuged at 100000 *g* for 1 h to remove Triton X-100-insoluble material and the detergent extracts were used for immunoprecipitation.

Labelling with diazotized [^{125}I]di-iodosulphanilic acid

[^{125}I]DDISA was synthesized from sulphanilic acid and Na^{125}I by the procedure used by Helmkamp & Sears (1970). The reagent (sp. radioactivity approx. 100 Ci/mol) was prepared in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5, and was used immediately after synthesis, although according to the authors above it is stable for months on freezing.

Isolated formate dehydrogenase (30 μg) in 50 mM-sodium phosphate, pH 7.5, was mixed with [^{125}I]-DDISA (final concn. 1 mM) in 100 μl final volume for 10 min at 4°C, then made 50 mM-Tris/HCl, pH 7.0. The enzyme was recovered by immunoprecipitation with anti-(formate dehydrogenase) serum.

Spheroplasts (30 mg of protein) and membrane vesicles (15 mg of protein in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5) were mixed. [^{125}I]DDISA was added to 1 mM final concentration (in a 10 ml volume). After 30 min on ice, labelled spheroplasts and membrane vesicles were separated by differential centrifugation as described for ^{125}I labelling, and each was washed three times in 0.5 M-sucrose/20 mM-Tris/HCl, pH 7.5. Membrane vesicles were prepared from the washed spheroplasts by ultrasonication as described above.

The labelled vesicle preparations (from spheroplasts and from membrane vesicles) were suspended separately to the same protein concentration, dispersed with Triton X-100 (final concn. 2%, w/v) and centrifuged at 100000 *g* for 1 h. Equal volumes of the extracts were used for immunoprecipitation by using antibodies specific for formate dehydrogenase, F_1-ATPase or nitrate reductase.

Labelling with diazobenzene[^{35}S]sulphonate

[^{35}S]DABS was synthesized from [^{35}S]sulphanilic acid (2.4 Ci/mmol; The Radiochemical Centre) as described by Tinberg *et al.* (1974). Non-radioactive sulphanilic acid was added to give a specific radioactivity of 4 Ci/mol. [^{35}S]DABS was prepared in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5, and was used immediately.

Formate dehydrogenase (50 μg) in 50 mM-sodium phosphate, pH 7.5, was mixed with [^{35}S]DABS (final concn. 2 mM) to a final volume of 100 μl for 10 min at 4°C, then made 50 mM in Tris/HCl, pH 7.0. The enzyme was recovered by immunoprecipitation with anti-(formate dehydrogenase) serum.

[^{35}S]DABS-labelling conditions for spheroplasts and membrane vesicles were identical with those for [^{125}I]DDISA, except that spheroplasts (15 mg) and membrane vesicles (8 mg) were used and [^{35}S]-DABS was added to a 2 mM final concentration. After 15 min at 4°C, the spheroplasts and membrane vesicles were separated and treated as for the [^{125}I]DDISA-labelling experiment.

Immunological techniques

Immunoprecipitation was performed by addition of sufficient of the specified antiserum to precipitate all the antigen present in a Triton X-100-solubilized extract, as determined by titration and enzyme assay. The mixtures were left for 1 h at 25°C, followed by 16 h at 4°C. The immunoprecipitates were collected and washed as described by Werner (1974), finally resuspended with 100 μl of 50 mM-Tris/HCl, pH 7.2, and ultrasonicated for 5 s with the micro-probe [3.2 mm ($\frac{1}{8}$in) diam.] of an MSE ultrasonicator. The immunoprecipitates were then disaggregated as described by Laemmli (1970).

'Rocket' immunoelectrophoresis of Triton X-100-solubilized membrane vesicles was performed essentially as described by Smyth *et al.* (1978). Barbital/HCl buffer ($I = 0.04$, pH 8.6) containing 1% (w/v) Triton X-100 was used throughout and was incorporated into all gels. Electrophoresis of 5 μl samples was performed in 1% (w/v) agarose gels, containing antiserum, on 50 mm × 50 mm glass plates. Wells (2 mm diameter) were cut along an origin 10 mm from one edge of the plate and samples were subjected to electrophoresis at 2 V/cm for 12–18 h in a water-cooled chamber. After electrophoresis the agarose plates were washed three times in 0.1 M-NaCl for 30 min, air-dried and stained in 0.1% (w/v) Coomassie Brilliant Blue. Polypeptide analysis of the precipitation arcs was performed by SDS/polyacrylamide-gel electrophoresis (Norrild *et al.*, 1977). The individual 'rockets' were cut out of dried, stained plates and were homogenized in 0.15 ml of

2% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/61mM-Tris/HCl/0.001% (w/v) Bromophenol Blue, pH6.8. After boiling for 5min, the molten agarose was applied directly to the polyacrylamide gels.

Polyacrylamide-gel electrophoresis and determination of radioactivity in gel slices

Electrophoresis in the presence of SDS was performed in 10% (w/v)-acrylamide separating gels (except where indicated) with 3% (w/v)-acrylamide stacking gels (Laemmli, 1970). Molecular weights were determined by using separating gels of 5% and 10% (w/v) acrylamide with the following marker polypeptides: fatty acid synthase (250000); glycogen-debranching enzyme (167000); phosphorylase (100000); bovine serum albumin (68000); catalase (60000); ovalbumin (43000); lactate dehydrogenase (36000); carbonic anhydrase (29500); and cytochrome *c* (12500).

After staining for protein, polyacrylamide gels were sliced uniformly into 1mm slices with a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey, U.K.). For ^{35}S determinations, each slice was incubated with 0.5ml of NCS (Amersham/Searle Corp., High Wycombe, Bucks., U.K.)/water (9:1, v/v) at 50°C for 2h, cooled, and 4.5ml of scintillation fluid added [6.6g of 2,5-diphenyloxazole and 82mg of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre of toluene]. Samples were counted for radioactivity in a Beckman LS 7000 liquid-scintillation spectrometer.

The distribution of ^{125}I was determined by counting radioactivity of samples directly in a model 300 Packard Auto-Gamma Spectrometer.

For double counting of ^{35}S and ^{125}I radioactivity, the ^{125}I was measured directly and then slices were transferred to scintillation vials and counted for ^{35}S as described above.

Assays

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V; BDH) as a standard. Nitrate reductase was assayed as described by Showe & DeMoss (1968).

Results

Characterization of anti-(formate dehydrogenase) serum

Anti-(formate dehydrogenase) serum could be used to immunoprecipitate formate dehydrogenase from detergent-solubilized *E. coli* preparations. Fig. 1(*a*) shows a titration of anti-(formate dehydrogenase) serum with Triton X-100-dispersed membrane vesicles. Formate dehydrogenase activity (measured as formate:dichlorophenol-indophenol oxidoreductase) was precipitated by the antiserum, but not by the pre-immune serum. Neither serum inhibited the formate dehydrogenase activity.

Analysis of the antiserum by crossed immunoelectrophoresis with Triton X-100-solubilized membrane vesicles as antigen produced a single protein-stained precipitin arc, which could also be

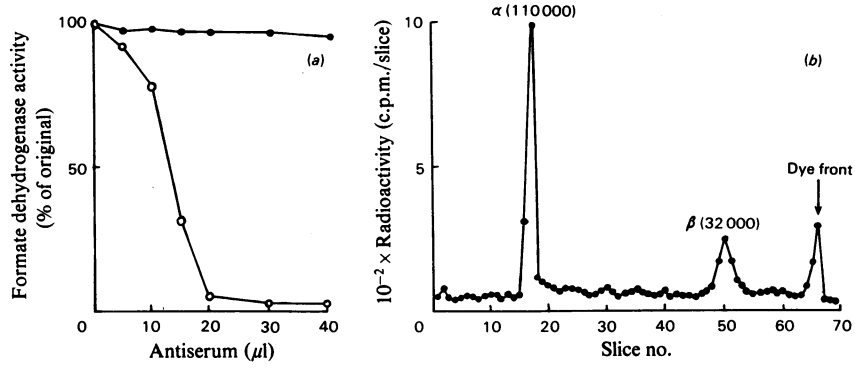

Fig. 1. *Immunoprecipitation of formate dehydrogenase from Triton X-100-solubilized membrane vesicles and polypeptide composition of the enzyme*

Triton X-100-solubilized extracts of membrane vesicles (each from 220μg of membrane protein) were used for immunoprecipitation with the specified amount of serum, as described in the Materials and methods section. Fig. 1(*a*) shows the formate:dichlorophenol-indophenol oxidoreductase activity in the supernatants of (●) control serum after centrifugation [or anti-(formate dehydrogenase) serum before centrifugation] and (○) anti-(formate dehydrogenase) serum after centrifugation. Fig. 1(*b*) shows the polypeptide composition of the formate dehydrogenase obtained from 'rocket' precipitin lines obtained by electrophoresis of Triton X-100-solubilized extracts from [^{35}S]sulphate-labelled cultures of *E. coli* against anti-(formate dehydrogenase) serum. The individual precipitin arcs were removed, the proteins extracted and analysed by SDS/polyacrylamide-gel electrophoresis. The anode is at the right. Subunits are shown with their M_r values.

stained for formate dehydrogenase activity before staining for protein (results not shown).

The subunit composition of the immunoprecipitated material was revealed by SDS/polyacrylamide-gel-electrophoretic analysis. Collected precipitin arcs from 'rocket' immunoelectrophoresis plates, obtained by using anti-(formate dehydrogenase) serum and Triton X-100-solubilized membrane vesicles prepared from [^{35}S]sulphate-grown bacteria, were analysed (Fig. 1*b*). The precipitate contained two radioactive bands of apparent M_r 110000 and 32000 (termed α and β respectively). The immunoprecipitate contained no component of M_r approx. 20000. The enzyme isolated by Enoch & Lester (1975) and by us, using their procedure, contained a cytochrome b_{556} component, and such preparations contained a protein-stained band of M_r approx. 20000, in addition to the α- and β-polypeptides.

Direct immunoprecipitation of the enzyme from Triton X-100-solubilized membranes is complicated by the fact that a contaminant is obtained in the immunoprecipiate with an electrophoretic mobility close to that of the β-subunit. This is also found when the extract is incubated for long periods without antisera. Hence we assign it as a contaminant and for accurate analyses use collected precipitin arcs. Nevertheless material obtained by direct immunoprecipitation does not contain a polypeptide of M_r 20000. Spectroscopic analysis of such preparations revealed that less than 1% of the total cytochrome *b* was precipitated by the antiserum. We conclude therefore that formate dehydrogenase recovered by immunoprecipitation does not contain cytochrome *b* under the conditions used in our experiments. It is still possible that it could be very loosely bound to the enzyme, since all our procedures involve extensive washing of immunoprecipitates. Our results indicate that neither the α- nor β-subunit of the enzyme contains haem, in accordance with the original suggestion of Enoch & Lester (1975) that the haem is associated with the M_r-20000 polypeptide.

Lactoperoxidase-catalysed radioiodination of membrane-bound formate dehydrogenase

The orientation of formate dehydrogenase within the cytoplasmic membrane was investigated by using lactoperoxidase-catalysed [^{125}I]iodination. The modification of spheroplasts was taken to be equivalent to labelling at the periplasmic surface of the membrane, and that of membrane vesicles, prepared by ultrasonic treatment (which possess predominantly inside-out orientation with respect to the intact cell), to labelling at the cytoplasmic surface of the membrane. Spheroplasts and membrane vesicles were iodinated as a mixture in a single tube. If the iodination were done separately, it would be difficult to ensure that the modifications were performed under equivalent conditions, since spheroplasts exhibit a 10-fold higher specific catalase activity than do membrane vesicles. Much greater incorporation of iodine into membrane vesicles than into spheroplasts would be obtained, since the degree of modification is a function of both the concentration of available sites and the concentration of H_2O_2, a substrate for lactoperoxidase. Thus by modifying the mixture and recovering the spheroplasts and vesicles separately by differential centrifugation afterwards, the extent of iodination of the enzyme in the two preparations could be compared directly. Since *E. coli* cells grown on [^{35}S]sulphate were used, the ^{125}I/^{35}S ratio was taken as an index of the extent of iodination of recovered proteins.

The incorporation of ^{125}I into subunits of formate dehydrogenase from modified spheroplasts and membrane vesicles is shown in Figs. 2(*a*) and 2(*b*); the α-subunit is significantly iodinated in both

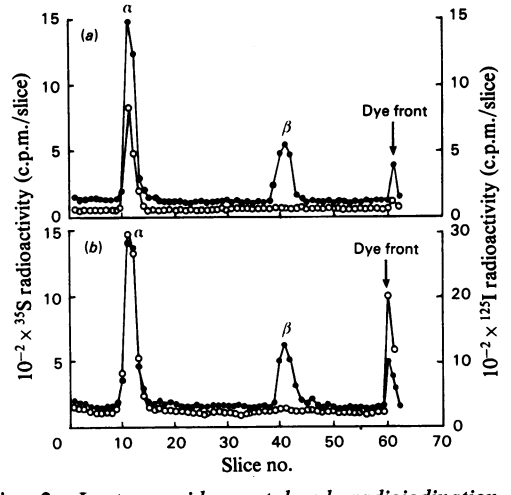

Fig. 2. *Lactoperoxidase-catalysed radioiodination of membrane-bound formate dehydrogenase*

Labelling was performed as described in the Materials and methods section. *E. coli* was grown on [^{35}S]sulphate to label protein. Spheroplasts and membrane vesicles from the ^{35}S-labelled bacteria were mixed and iodinated. After separation, both preparations were solubilized with Triton X-100 and the solubilized extracts were analysed by 'rocket' immunoelectrophoresis against anti-(formate dehydrogenase) serum. Polypeptide analysis of the individual precipitin arcs recovered from 'rocket' immunoelectrophoresis was performed by SDS/polyacrylamide-gel electrophoresis. The anode is at the right. The distribution of ^{125}I (○) and ^{35}S (●) in the subunits of formate dehydrogenase is shown for labelled spheroplasts (*a*) and labelled membrane vesicles (*b*).

spheroplasts and membrane vesicles. F_1-ATPase, an enzyme which is located exclusively at the cytoplasmic face of the membrane, was also recovered by immunoprecipitation from the same iodinated preparations. Figs. 3(*a*) and 3(*b*) show that the larger subunits of this enzyme (I and II) are iodinated only in the membrane vesicles, which is consistent with the known location of F_1-ATPase and confirms that the iodination has been confined to the membrane surfaces exposed to the bulk phase. It can be concluded therefore that the α-subunit of formate dehydrogenase is located, at least in part, at the periplasmic surface of the membrane. The β-subunit of the enzyme is not iodinated in either spheroplasts or membrane vesicles; its location therefore is not resolved by this technique.

The extent of iodination for the enzymes in spheroplasts and membrane vesicles was quantified. The α-subunit of formate dehydrogenase is iodinated in membrane vesicles ($^{125}I/^{35}S = 2.1$) to about four times the extent of the modification in spheroplasts ($^{125}I/^{35}S = 0.52$). Since our experimental conditions permit direct comparison of the $^{125}I/^{35}S$ ratios, it appears that more sites are available for iodination in membrane vesicles than in spheroplasts. Assuming that the structure of the membrane in the vesicles is unaltered from that in spheroplasts, except in respect of orientation, the results indicate that the α-subunit is also present at the cytoplasmic face of the membrane and is thus transmembranous. The ratios calculated for subunits I and II of F_1-ATPase show that an extent of iodination comparable with that of the α-subunit is achieved in membrane vesicles. The low $^{125}I/^{35}S$ ratio for F_1-ATPase from labelled spheroplasts (0.02), comparable with those obtained for the non-modified β-subunit of formate dehydrogenase (0.01 for spheroplasts, 0.02 for membrane vesicles), can be taken as a background value for the modification of membrane proteins which are not exposed at the periplasmic surface. Subunit III of F_1-ATPase does not appear to be radioiodinated in either preparation. No information on the lower-molecular-weight subunits of F_1-ATPase could be obtained from the gels used, since these subunits migrate with the dye front.

Nitrate reductase A- and B-subunits (M_r 150000 and 59000 respectively) were recovered, from the same labelled preparations as F_1-ATPase and formate dehydrogenase, by immunoprecipitation with antiserum specific for nitrate reductase. The ratios clearly show ($^{125}I/^{35}S = 0.04$ and 2.2 for spheroplasts and membrane vesicles respectively) that the A-subunit is located exclusively at the cytoplasmic surface, in agreement with Boxer & Clegg (1975). However, the B-subunit of nitrate reductase was not iodinated at either surface ($^{125}I/^{35}S < 0.03$ for both preparations), which is contrary to the interpretation of those authors. This error was due to the high background radioactivity found by those authors in the gels, in the region of the β-subunit.

The labelling patterns (Figs. 2 and 3) show that there are large peaks of ^{125}I radioactivity at the dye front. These can be partially decreased by lipid extraction by the method of Mersal *et al.* (1976). There have been several reports of iodinated lipid or glycolipid migrating at the dye front in SDS/polyacrylamide gels of detergent extracts from radioiodinated membranes (Butters & Hughes, 1975; Zimmermann & Chapman, 1977).

The results obtained from experiments with spheroplasts and membrane vesicles labelled separately are in complete agreement with those described above.

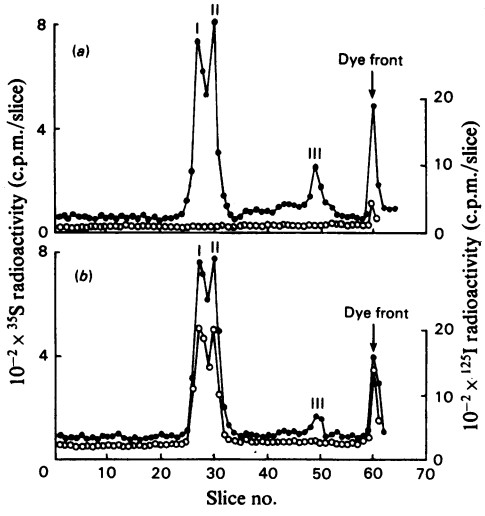

Fig. 3. *Lactoperoxidase-catalysed radioiodination of membrane-bound F_1-ATPase*

The modification of F_1-ATPase was investigated by using the same procedures and from the same labelled fractions as those described in Fig. 2, except that 'rocket' precipitin arcs, produced by using anti-(F_1-ATPase) serum, were used for polypeptide analysis. The distribution of ^{125}I (○) and ^{35}S (●) in the subunits of F_1-ATPase is shown for labelled spheroplasts (*a*) and labelled membrane vesicles (*b*).

Heterogeneity of orientation of membrane vesicles

It has been reported that vesicles from *E. coli* prepared by ultrasonic disruption are largely inverted with respect to whole cells (Hare *et al.*, 1974; Futai, 1974). We have estimated the heterogeneity of the membrane vesicles used in our experiments by a method similar to that used by Hare *et al.* (1974). This is important in our study, since the α-subunit of

formate dehydrogenase is labelled more strongly in membrane vesicles than in spheroplasts, which we interpret as modification at both the cytoplasmic and periplasmic surfaces of the membrane.

Antisera to both nitrate reductase (Graham & Boxer, 1978) and F_1-ATPase (Graham, 1979) have been shown, by immunofluorescence studies, to bind to membrane vesicles, but not to spheroplasts. This knowledge was exploited to fractionate membrane vesicles into populations of defined and opposite orientation. Membrane vesicles were challenged with anti-(nitrate reductase) or anti-(F_1-ATPase) serum, as shown in Fig. 4, and, by using the nitrate reductase activity as a marker for the membranes, it can be seen that about 75% of the vesicles could be agglutinated.

The agglutinated vesicles bind to the antisera, and therefore have at least part of their cytoplasmic surface exposed. The non-agglutinated vesicles do not have cytoplasmic surface determinants exposed and therefore are of the same orientation as spheroplasts. About 75% of the original vesicles are of inside-out orientation with respect to spheroplasts.

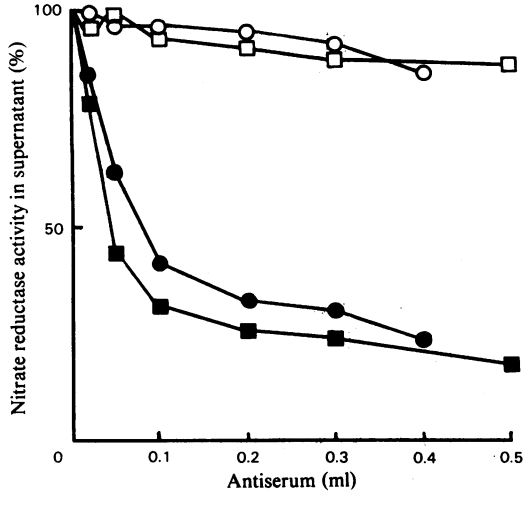

Fig. 4. *Agglutination of membrane vesicles with anti-(nitrate reductase) and anti-(F_1-ATPase) sera*
Membrane vesicles (1.26mg of protein/ml) were incubated separately with the specified amount of control, anti-(nitrate reductase) or anti-(F_1-ATPase) serum in a final volume of 0.5ml of 50mM-Tris/HCl, pH7.5 for 1h at 25°C, then for 12h at 4°C. The agglutinated material was removed by centrifugation (480*g* for 5min). The supernatant fractions (non-agglutinated vesicles) were assayed for nitrate reductase activity. ○, Anti-(nitrate reductase) serum before centrifugation; ●, anti-(nitrate reductase) serum after centrifugation; ■, anti-(F_1-ATPase) serum after centrifugation; □, control serum after centrifugation.

Table 1 shows the $^{125}I/^{35}S$ ratios for the α-subunit of formate dehydrogenase and the A-subunit of nitrate reductase which were calculated for a surface-radioiodinated membrane-vesicle preparation before and after agglutination. The $^{125}I/^{35}S$ ratios for the α-subunit of formate dehydrogenase and the A-subunit of nitrate reductase in the 'total vesicle' population were similar. The ratio for the α-subunit of formate dehydrogenase recovered from the agglutinated vesicles was higher than that for the 'total' fraction. Nitrate reductase could not be recovered from the agglutinated membrane vesicles, probably because the antibodies bound to the nitrate reductase, forming an insoluble reticulum.

That the A-subunit of nitrate reductase is not iodinated in non-agglutinated vesicles is consistent with these vesicles being of right-side-out orientation. The α-subunit of formate dehydrogenase is labelled in these vesicles, so confirming its presence at the periplasmic surface. Therefore the α-subunit of formate dehydrogenase is transmembranous. From the proportion of inside-out to right-side-out vesicles (approx. 70:30) and the $^{125}I/^{35}S$ ratios for the α-subunit of formate dehydrogenase, it can be calculated that the 'total' population should have a $^{125}I/^{35}S$ ratio of 0.93. This correlates well with the observed value of 0.95. The β- and B-subunits of the enzymes were not labelled with lactoperoxidase, in agreement with the results of the previous section.

Table 1. *Relative radioiodination of formate dehydrogenase and nitrate reductase in subfractionated vesicles*
Radioiodinated ^{35}S-labelled membrane vesicles were subfractionated by anti-(nitrate reductase) serum as described for Fig 4. The agglutinated vesicles were obtained by low-speed centrifugation (480*g* for 5min), and non-agglutinated vesicles were recovered by ultracentrifugation (100000*g* for 1h). Each vesicle population was solubilized with Triton X-100 (final concn. 2%, v/v) and analysed by 'rocket' immunoelectrophoresis by using anti-(formate dehydrogenase) or anti-(nitrate reductase) serum. Polypeptide analysis of the individual precipitin arcs by SDS/polyacrylamide-gel electrophoresis and radioactivity counting were performed as described in the Materials and methods section.

	$^{125}I/^{35}S$ ratio	
	Formate dehydrogenase α-subunit	Nitrate reductase A-subunit
'Total' vesicle population	0.95	1.1
Agglutinated membrane vesicles	1.2	—
Non-agglutinated membrane vesicles	0.3	0

Lactoperoxidase-catalysed radioiodination of isolated formate dehydrogenase

Since the membrane-bound formate dehydrogenase is not labelled in the β-subunit by lactoperoxidase-catalysed radioiodination, we investigated the labelling of isolated enzyme. Only the α-subunit (M_r 110000) of the isolated enzyme is ^{125}I-labelled by this method (results not shown). Polypeptides that are hidden or contain no available residues suitable for modification will not be labelled by this method, and this must be the reason for the lack of labelling in the other polypeptides.

Iodination of the isolated enzyme in the presence of 0.1% (w/v) SDS labels both the α- and β-subunits of the enzyme (results not shown). The amino acid residues of the β-subunit capable of being iodinated by lactoperoxidase are therefore inaccessible to the bulk phase, in the membrane-bound and isolated enzyme, so that no conclusion about the location of this subunit within the membrane can be made from the preceding experiments.

Labelling of isolated formate dehydrogenase with [^{125}I]DDISA and [^{35}S]DABS

Lactoperoxidase-catalysed radioiodination of membrane-bound formate dehydrogenase failed to locate the β-subunit on either surface of the cytoplasmic membrane. We investigated the reactivity of two other reagents, [^{125}I]DDISA and [^{35}S]DABS, towards isolated formate dehydrogenase, both of which have been used to study the topography of membrane proteins in other systems (George *et al.*, 1976; Ludwig *et al.*, 1979). Treatment of isolated formate dehydrogenase with [^{125}I]-DDISA resulted in both α- and β-subunits being modified (results not shown). The ratio of radioactivity found in each subunit was approximately equal to the molar ratio of the subunits. [^{35}S]DABS labelling of isolated formate dehydrogenase resulted in labelling of both subunits in a similar fashion (results not shown). Both subunits are accessible to each reagent in the isolated enzyme, and therefore they appear to be suitable probes for the study of the topography of both subunits of the membrane-bound enzyme.

Labelling of membrane-bound formate dehydrogenase with [^{125}I]DDISA

Under the labelling conditions normally used for modification with [^{125}I]DDISA (low reagent concentration), only a small fraction (assumed to be representative) of all potentially active sites on the membrane surfaces is labelled. Spheroplast and membrane vesicles were mixed before exposure to [^{125}I]DDISA and subsequently separated, to ensure that the incorporations of the reagent at the periplasmic and cytoplasmic surfaces of the membrane were directly comparable (Boxer & Clegg, 1975). The incorporation of label at the cytoplasmic face of the membrane was monitored by the measurement of the extent of modification of F_1-ATPase in spheroplasts and membrane vesicles. From the gel-electrophoretic analysis of the immunoprecipitate, it is evident that subunits I and II of F_1-ATPase are labelled with [^{125}I]DDISA only in membrane vesicles (Fig. 5*a*).

Formate dehydrogenase, obtained from the same labelled preparations as F_1-ATPase above, was labelled in both α- and β-subunits in spheroplasts and membrane vesicles (Fig. 5*b*). Since the same amounts of membrane protein from spheroplasts and membrane vesicles were used for the solubilization and subsequent immunoprecipitation, then

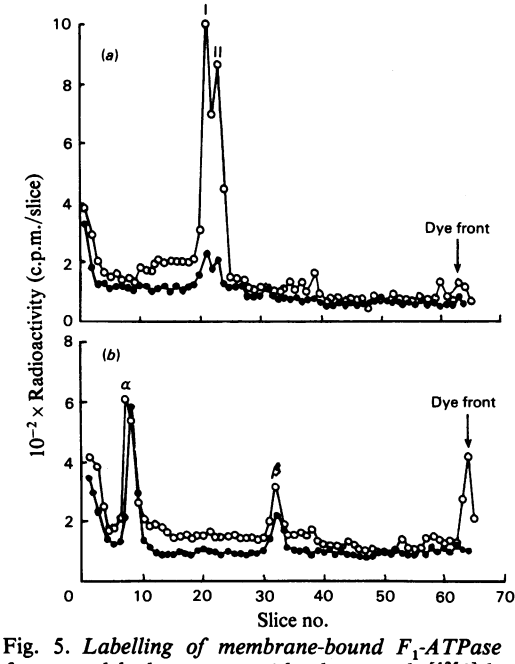

Fig. 5. *Labelling of membrane-bound F_1-ATPase and formate dehydrogenase with diazotized [^{125}I]di-iodosulphanilic acid*

A spheroplast and membrane-vesicle mixture was labelled with diazotized [^{125}I]di-iodosulphanilic acid, then each was separated as described in the Materials and methods section. Membrane vesicles were prepared from the spheroplasts, and both vesicle preparations (at the same protein concentrations) were dispersed separately with Triton X-100. F_1-ATPase (*a*) and formate dehydrogenase (*b*) were immunoprecipitated with antibodies specific for F_1-ATPase and formate dehydrogenase respectively. Immunoprecipitated material from spheroplasts (●) and from membrane vesicles (○) was analysed by SDS/polyacrylamide-gel electrophoresis [12% (w/v) polyacrylamide separating gels]. An arrow indicates the position of the dye front in each gel, and the anode is at the right.

the relative amounts of label incorporated at each surface are directly comparable, from the gels in Fig. 5(*b*). The results suggest that both polypeptides are transmembranous. Nitrate reductase recovered from the same experiment had the A- and B-subunits labelled only in membrane vesicles, in agreement with our earlier work (Graham & Boxer, 1980). Experiments performed with spheroplasts and membrane vesicles labelled with [^{125}I]DDISA independently produced similar results to those found for the mixture (results not shown).

We have also performed agglutination experiments with [^{125}I]DDISA-labelled vesicles similar to those performed with iodinated vesicles described above. Inside-out membrane vesicles were agglutinated by using specific anti-(nitrate reductase) serum. Non-agglutinated membrane vesicles were recovered by ultracentrifugation, and both vesicles were detergent-solubilized at equivalent protein concentrations.

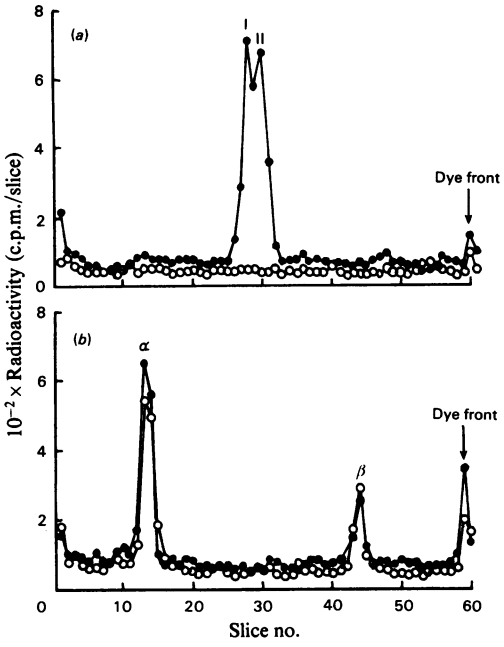

Fig. 6. *F_1-ATPase and formate dehydrogenase recovered from agglutinated and non-agglutinated [^{125}I]DDISA-labelled membrane vesicles*

[^{125}I]DDISA-labelled membrane vesicles were subfractionated by using anti-(nitrate reductase) serum, as described in the legend for Fig. 5. Agglutinated and non-agglutinated membrane vesicles were both resuspended to 3 mg of protein/ml and solubilized with Triton X-100. F_1-ATPase (*a*) and formate dehydrogenase (*b*) were both recovered from detergent extracts by using the respective antiserum. ●, Enzymes recovered from agglutinated membrane vesicles; ○, enzymes recovered from non-agglutinated membrane vesicles.

F_1-ATPase was recovered from agglutinated and non-agglutinated vesicles in the expected amounts (as shown by protein-stained gels), and subunits I and II were only labelled in the agglutinated membrane vesicles (Fig. 6). F_1-ATPase must be accessible to the bulk phase in the agglutinated membrane vesicles, and therefore the vesicles possess an inside-out orientation.

The α- and β-subunits of formate dehydrogenase are labelled to the same extent in agglutinated and non-agglutinated vesicles. Therefore both subunits are transmembranous.

Labelling of membrane-bound formate dehydrogenase with [^{35}S]DABS

[^{35}S]DABS is a non-membrane-penetrating reagent which has been used extensively in the study of the topography of membrane proteins. Although [^{35}S]DABS probably reacts with the same types of amino acid side chains as [^{125}I]DDISA, the iodine present in [^{125}I]DDISA make this reagent more lipophilic.

Subunits I and II of F_1-ATPase were labelled with [^{35}S]DABS only in membrane vesicles (results not shown). This is in accordance with the location of this enzyme at the cytoplasmic face of the membrane and supports the use of the reagent as a vectorial probe for membrane-protein location in this system.

The α- and β-subunits of formate dehydrogenase are labelled to the same extent in spheroplasts and membrane vesicles (results not shown), which agrees with the conclusions drawn from the experiments using the more lipophilic [^{125}I]DDISA and lactoperoxidase.

Discussion

The present paper establishes that formate dehydrogenase of *E. coli* occupies a transmembranous position in the cytoplasmic membrane of the organism. Both α- and β-subunits span the membrane. The use of F_1-ATPase as a marker enzyme for the cytoplasmic surface of the membrane facilitated the interpretation of our results. Distinct labelling patterns were obtained for this enzyme with spheroplasts and membrane vesicles with all reagents employed, thus demonstrating the intactness of the spheroplasts and the insignificant membrane permeation of these reagents. Unfortunately no protein has been identified at present as being located exclusively at the periplasmic surface of the membrane. Such a component would serve as a valuable control for the accessibility of this surface to the labelling agents. Although the location of F_1-ATPase in *E. coli* is well established (Downie

et al., 1979), its topography investigated by direct chemical modification has not been previously reported.

The method of labelling mixtures of spheroplasts and membrane vesicles for the establishment of the location of a membrane component proved most useful. Without the knowledge that the extent of modification is similar in both preparations of opposite orientation, it is difficult to prove the transmembranous nature of membrane components by direct coupling unless peptide 'mapping' is also undertaken. This would not be the case if saturation labelling was achieved, but this in itself is undesirable, since the membrane structure is then likely to be perturbed. Where a component is exclusively located at one or other face of the membrane, such complications of interpretation do not arise.

The lack of labelling of the β-subunit in the membrane by lactoperoxidase-catalysed iodination can be clearly ascribed to the absence of modifiable residues at the subunit's surface rather than to a buried location. The lack of iodination of this subunit in the isolated enzyme, its iodination in the presence of SDS, and its modification with [^{35}S]-DABS and [^{125}I]DDISA in both the solubilized and membrane-bound conditions, support this view.

Support for the transmembranous nature of both subunits of this enzyme was also obtained from the experiments using surface labelling of membrane vesicles and their subsequent immunological fractionation. Hare *et al.* (1974) originally reported that vesicles of heterogeneous orientation could be fractionated into populations of defined orientation by agglutination with antibodies specific for F_1-ATPase. We have confirmed and extended their findings by showing that the surface-labelling patterns of the subfractionated vesicles are distinct. Furthermore, modifying the unfractionated mixture and then separating it ensures that the surfaces of the two vesicle populations are labelled to the same extent. There does not appear to be a significant proportion of 'scrambled' vesicles in which the relative orientation of the enzymes studied is altered. Any such vesicles would be agglutinated in our experiments. It is difficult to rule out the unlikely possibility that formate dehydrogenase itself is independently 'scrambled' in the membrane vesicles.

We have been unable to obtain information about the location of the cytochrome *b* component that is present in isolated preparations of formate dehydrogenase. Although the cytochrome *b* was present in the enzyme used originally to immunize the rabbits, we have been unable to demonstrate that either spectroscopically detectable cytochrome or a polypeptide of M_r approx. 20000 is present in recovered immunoprecipitates. Two groups have reported that a cytochrome is physically associated with the enzyme (Enoch & Lester, 1975; Scott & De-Moss, 1976). It appears from our results that any such association must be weak.

Comparison of formate dehydrogenase with the proton-translocating respiratory complexes from mitochondria indicates that it is structurally much less complicated. Progress on the elucidation of the membrane disposition of these complexes has been hampered by the complexity of their polypeptide composition, although all three have now been shown to be transmembranous (Eytan *et al.*, 1975; Mendel-Hartvig & Nelson, 1978; Smith & Ragan, 1980). Why the mitochondrial complexes contain so many polypeptides is not known, but clearly, since formate dehydrogenase comprises only two or three, such complexity cannot be absolutely necessary for the catalysis of respiratory-driven protein translocation. Formate dehydrogenase is therefore an attractive candidate for the investigation of the mechanism of respiratory proton translocation.

We thank Dr. G. Vogel for a sample of F_1-ATPase. A. G. was in receipt of an S.R.C. Research Studentship. The work was supported by the S.R.C. (grant no. GR/B 28880) and The Royal Society. We thank Mr. A. D. Tucker for skilled technical assistance.

References

Boxer, D. H. & Clegg, R. A. (1975) *FEBS Lett.* **60**, 54–57

Butters, T. D. & Hughes, R. C. (1975) *Biochem. J.* **150**, 59–69

Cohen, G. N. & Rickenberg, H. W. (1956) *Ann. Inst. Pasteur, Paris* **91**, 693–702

Downie, J. A, Gibson, F. & Cox, G. B. (1979) *Annu. Rev. Biochem.* **48**, 103–132

Enoch, H. G. & Lester, R. L. (1975) *J. Biol. Chem.* **250**, 6693–6705

Eytan, G. D., Carroll, R. C., Schatz, G. & Racker, E. (1975) *J. Biol. Chem.* **250**, 8598–8608

Futai, M. (1974) *J. Membr. Biol.* **15**, 15–28

Garland, P. B., Downie, J. A. & Haddock, B. A. (1975) *Biochem. J.* **152**, 547–559

George, J. N., Potterf, R. D., Lewis, P. C. & Sears, D. A. (1976) *J. Lab. Clin. Med.* **88**, 232–246

Graham, A. (1979) Ph.D. Thesis, University of Dundee

Graham, A. & Boxer, D. H. (1978) *Biochem. Soc. Trans.* **6**, 1210–1211

Graham, A. & Boxer, D. H. (1980) *FEBS Lett.* **113**, 15–20

Gutowski, S. J. (1976) Ph.D. Thesis, Australian National University, Canberra

Hare, J. F., Olden, K. & Kennedy, E. P. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4843–4846

Helmkamp, R. W. & Sears, D. A. (1970) *Int. J. Appl. Radiat. Isot.* **21**, 683–685

Jones, R. W. (1980) *FEMS Microbiol. Lett.* **8**, 167–171

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Ludwig, B., Downer, N. W. & Capaldi, R. A. (1979) *Biochemistry* **18**, 1401–1407

Mendel-Hartvig, I. & Nelson, B. D. (1978) *FEBS Lett.* **92**, 36–40

Mersal, M., Benenson, A. & Doljanski, F. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1166–1171

Norrild, B., Bjerrum, O. J. & Vestergaard, B. F. (1977) *Anal. Biochem.* **81**, 432–441

Osborn, M. J., Gander, J. E. & Parisi, E. (1972) *J. Biol. Chem.* **247**, 3973–3986

Scott, R. H. & DeMoss, J. A. (1976) *J. Bacteriol.* **126**, 478–486

Showe, M. K. & DeMoss, J. A. (1968) *J. Bacteriol.* **95**, 1305–1313

Smith, S. & Ragan, C. I. (1980) *Biochem. J.* **185**, 315–326

Smyth, C. J., Siegel, J., Salton, M. R. J. & Owen, P. (1978) *J. Bacteriol.* **133**, 306–319

Tinberg, H. M., Melnick, R. L., Maguire, J. & Packer, L. (1974) *Biochim. Biophys. Acta* **345**, 118–128

Werner, S. (1974) *Eur. J. Biochem.* **43**, 39–48

Zimmermann, B. & Chapman, M. L. (1977) *J. Immunol. Methods* **15**, 183–192