IMMUNOCHEMICAL ANALYSIS OF THE MEMBRANE-BOUND HYDROGENASE OF *ESCHERICHIA COLI*

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1. Introduction

The membrane-bound hydrogenase [EC 1.12.-.-] of *Escherichia coli* is involved in the energy-conserving oxidation of hydrogen [1–4] via fumarate reductase [EC 1.3.99.1] and also in the formate hydrogenlyase pathway which converts formate to CO_2 and H_2 [5]. *E. coli* hydrogenase from aerobically grown cells has been isolated and characterised [6] and the enzyme from anaerobically grown cells has been partially characterised [7]. Antibodies specific for *Bacillus subtilis* membrane-bound succinate dehydrogenase [EC 1.3.99.1] have been raised from activity-stained precipitin arcs located after analysis of crude fractions by crossed immunoelectrophoresis using antisera raised to detergent-solubilised membranes [8]. We have used a similar approach to prepare antibodies specific for *E. coli* hydrogenase and its use has enabled the subunit MW of the enzyme from anaerobically grown cells to be determined. We also report the isolation and immunological characterisation of two new *E. coli* mutants which specifically lack hydrogenase activity.

2. Materials and methods

2.1. *Cell growth and preparation of detergent-solubilised membranes*

E. coli (strain A1002) was grown anaerobically at 37°C for 12–16 h in 5 l of mineral salts medium [9] supplemented with glucose (0.5% w/v), bacteriological peptone (0.5% w/v), 1 mM $MgCl_2$, L-isoleucine, L-valine and L-methionine (20 μg/ml of each), 1 μM $(NH_4)_6Mo_7O_{24}$ and 1 μM K_2SeO_3 adjusted to pH 6.4. The cultures were harvested [10], washed once with 50 mM Tris–HCl pH 7.5, and broken in the same buffer, but containing the protease inhibitor 5 mM benzamidine–HCl in the presence of DNase and RNase, using a French pressure cell (30 000 lb in^{-2}). Unbroken cells were removed by centrifugation at 12 000 × *g* for 15 min and the membrane fraction sedimented by further centrifugation of the supernatant at 250 000 × *g* for 1 h. The membranes were washed once in the above buffer and resuspended in the same buffer to a protein concentration of 15–20 mg/ml. Triton X-100-dispersed membranes were prepared by the addition of Triton X-100 to the membrane suspension to a final concentration of 4% (w/v) and, after incubation at 4°C for 1 h, centrifuging at 250 000 × *g* for 1 h. The supernatant was immediately stored as 20 μl beads in liquid nitrogen until required.

Cell labelled with [^{35}S]sulphur were obtained as previously [11] except that the growth medium also contained Na fumarate (5.8 g/l) and casein hydrolysate (0.1 g/l). Cells were harvested, membrane vesicles prepared and treated with Triton X-100 as described above. For analytical purposes mutant *E. coli* strains were grown anaerobically overnight in 100 ml cultures at 37°C in a minimal salts medium [9] adjusted to pH 6.5, containing glucose (0.4%, w/v), casein hydrolysate (0.1%, w/v) and L-methionine (10 μg/ml). The cells were harvested, washed once with growth medium lacking added carbon source and suspended in 20 mM barbitone–HCl pH 8.6 to $A_{600} \triangleq 10$ (~1–2 ml). The cells were broken by ultrasonic treatment and unbroken bacteria were removed by centrifugation as above. The supernatant was used directly for enzymic analysis. For immunological analysis,

Table 1
Strains of *Escherichia coli* K12 used in this study

Strain	Characteristics	Source
A1002	F⁻ *ato fadR*^{C} *ilv lacI metE rha rpsL*	H. U. Schairer
EMG-29	F⁻ *pro trp his lac rpsL*	D. Old
P4X	Hfr *metB*1	E. Wollman
FD10	Hfr *metB*1 *hyd*	This study
FD12	Hfr *metB*1 *hyd*	This study
520	F⁻ *cysC purF argF thi malA mtl xyl rpsL* (T_6^R)	M. Novel (E. MacFall) Pasteur Institute (Paris)
KL165	Hfr *thi recA*1 *rif*	
5201	F⁻ As 520 but *recA*1	Derived by mating a *thy*⁻ derivative of 520 (isolated as trimethoprim resistant [23]) with KL165 and selecting for a *thy*⁺ *recA*⁻ transconjugant.

Triton X-100 was added to a final concentration of 4% (w/v) and, after placing at 4°C for 1 h, was centrifuged at 250 000 × *g* for 1 h. The supernatant was either analysed directly or stored in liquid nitrogen. The *E. coli* strains used in this study are listed in table 1.

2.2. *Antibody production*

Membrane vesicles (10 mg protein/ml) were prepared as described above, washed three times and used to immunise a rabbit. On days 1 and 4 the rabbit was injected s.c. above the scapula with an emulsion of 200 μl membrane vesicles and 200 μl Freund's complete adjuvant (Difco Labs., Detroit, MI, USA). On days 10, 17 and 24, the rabbit was injected with 200 μl membrane vesicles in the absence of adjuvant. From day 28 the rabbit was bled once weekly (20 ml blood) and the serum obtained was termed anti-membrane vesicle serum.

Hydrogenase-specific antiserum was raised against excised hydrogenase-activity-stained precipitin arcs located on crossed immunoelectrophoresis plates of Triton X-100-solubilised membranes (*E. coli* strain A1002) analysed with anti-"membrane vesicle"-serum. The plates were also stained for protein with Coomassie blue and the hydrogenase-containing arcs from 3 air-dried plates were removed with a scalpel, taking care not to remove parts of other precipitin arcs. The arcs were homogenised with 400 μl 50 mM Tris–HCl, pH 7.5 and 400 μl Freund's complete adjuvant, and injected s.c. into a rabbit on day 1. This process was repeated on days 7, 14 and 21 except that on the last two occasions the adjuvant was omitted. The rabbit was bled twice each week from day 28.

The immunoglobulin fraction from sera was purified as described by Harboe and Ingild [12]. Antisera and immunoglobulins were stored at −20°C.

Absorption of the anti-hydrogenase-serum was performed on 3 ml of immunoglobulin fraction (from 15 ml of serum) with 1.1 ml of Triton X-100-solubilised membranes from strain FD10 prepared as described above. The absorption was allowed to proceed overnight at 4°C and the supernatant obtained after removal of the immunoprecipitate by centrifugation (10 000 × *g* × 4 min), was specific for hydrogenase by the criterion of crossed immunoelectrophoretic analysis.

2.3. *"Rocket" and crossed immunoelectrophoresis*

Crossed immunoelectrophoresis of Triton X-100-solubilised membrane vesicles was performed essentially as described in [13]. Barbital–HCl buffer (μ = 0.04, pH 8.6) containing 1% (w/v) Triton X-100 was used throughout. Electrophoresis of 5 μl samples was performed in 1% agarose gels on 5 × 5 cm glass plates in a water-cooled chamber. "Rocket" immunoelectrophoresis was performed in the same manner as the second dimension for the crossed immunoelectrophoresis except that the 2 mm diameter wells were cut in the positions shown in fig.3. Precipitin arcs were detected by staining with Coomassie blue. Hydrogenase activity was specifically located by immersing the washed plate in 50 mM phosphate buffer, pH 6.8 containing 1 mM benzyl viologen,

1 mM methyl viologen and 1 mM 2,3,5-triphenyltetrazolium chloride. This solution was sparged constantly with a mixture of 95% H_2 and 5% CO_2 until the red-coloured hydrogenase-specific precipitin arc appeared. The plates were stained for protein as described above.

2.4. *Polyacrylamide gel electrophoresis and assay procedures*

Electrophoresis under non-dissociating conditions was performed at pH 8.9 in 7.3% (w/v) polyacrylamide gels as in [14]. Activity staining of gels for hydrogenase was performed as described above.

Electrophoresis in the presence of dodecyl sulphate was performed in 10% (w/v) polyacrylamide gels [15]. Determinations of MW were made using the following marker polypeptides: glycogen debranching enzyme, 167 000; phosphorylase b, 100 000; bovine serum albumin, 68 000; catalase, 60 000; heavy chain immunoglobulin, 50 000; ovalbumin, 43 000.

The distribution of [^{35}S]radioactivity in 1 mm slices of polyacrylamide gels was determined by liquid scintillation counting [16]. The distribution of ^{125}I radioactivity in polyacrylamide gels was measured directly in 1 mm gel slices using a gamma spectrometer.

Formate-dependent reduction of 2,6-dichlorophenolindophenol (mediated by *N*-methylphenazonium methosulphate) and of benzyl viologen [10] and the hydrogen-dependent reduction of benzyl viologen [17] were assayed as described previously. Protein was assayed by the method of Lowry et al. [18] using bovine serum albumin as standard.

3. Results and discussion

3.1. *Production of antiserum specific for* E. coli *hydrogenase*

Analysis of Triton X-100-solubilised membrane from anaerobically grown wild type *E. coli* (strain A1002) by crossed immunoelectrophoresis using anti-"membrane vesicle"-serum produced, as expected, a large number (~15) of protein-stained precipitin arcs. Activity staining of the immunoplate for hydrogenase activity, prior to staining for protein, gave a single, symmetrical, stained arc. Marking of the activity-stained arc allowed the hydrogenase containing arc to be identified as a major component in the protein-stained plate (fig.1a,b). Fig.1(a) does not

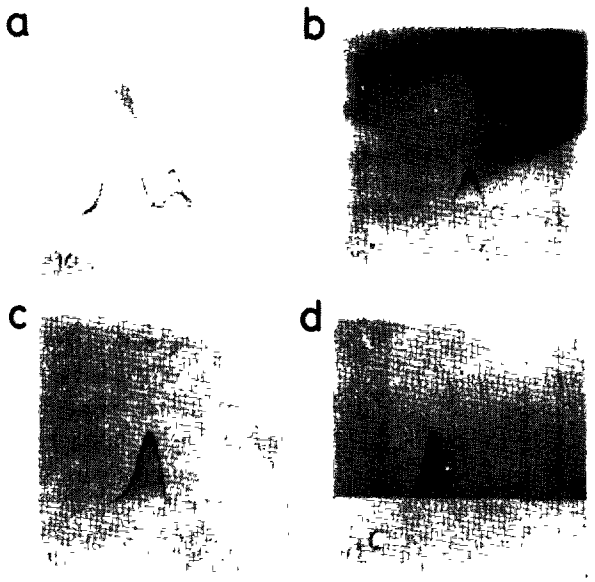

Fig.1. Crossed immunoelectrophoretic analysis of antiserum to membrane vesicles and to hydrogenase. Triton X-100-solubilised membranes (5 μl) of strain A1002 was electrophoresed in each case. (a) The second dimension employed 300 μl of anti-membrane vesicle-serum mixed with 2.35 ml of buffered agarose. Stained for protein; (b) as (a) but stained for hydrogenase activity; (c) the second dimension employed 250 μl of absorbed anti-hydrogenase immunoglobulin fraction mixed with 2.4 ml of buffered agarose. Stained for protein; (d) as (c) but stained for hydrogenase activity.

reveal all the precipitin arcs that could be detected by this procedure since the conditions for electrophoresis were such that good resolution of the hydrogenase-containing arc from the other protein-staining arcs was obtained. The absence of an activity-stained arc when N_2 is used in place of H_2 indicated that the activity was indeed due to hydrogenase. Almost identical results were obtained when Triton X-100-solubilised membrane vesicles from the other wild-type *E. coli* strains (EMG-2 and P4X) used in this study, grown under the same conditions, were examined.

Antiserum specific for hydrogenase was obtained by immunising a rabbit with excised hydrogenase activity-stained precipitin arcs from crossed immunoelectrophoresis plates as described in Section 2. The antiserum so obtained gave two precipitin arcs on crossed immunoelectrophoretic analysis of Triton X-100-solubilised membranes from wild type (strain A1002); a major arc which stained for hydrogenase activity, and a minor arc, lacking hydrogenase activ-

ity, produced by an electrophoretically very mobile component in the detergent extract. Antibodies to this non-active component were also found in the pre-immune serum from the immunised rabbit, suggesting that it was unrelated to hydrogenase. Triton X-100-solubilised membrane preparations from a hydrogenase-deficient mutant of *E. coli* (strain FD10) isolated in this study, completely lack an antigen that corresponds to wild-type hydrogenase (see Sections 3.3 and 3.4). Exploiting this knowledge the antibodies to the non-hydrogenase component were removed by immunoabsorption of the immunoglobulin fraction of the antiserum with a small amount of the solubilised membranes from the mutant (strain FD10). The hydrogenase-active precipitin arc formed in crossed immunoelectrophoretic analysis remained unaltered in size by the immunoabsorption procedure whereas the minor inactive arc was completely removed. Results of the analysis of the absorbed anti-hydrogenase preparation are shown in fig.1(c) and (d).

3.2. *Polypeptide analysis of hydrogenase*

The polypeptide composition of the enzyme catalysing the H_2-dependent reduction of benzyl viologen in wild type *E. coli* was established by dodecyl sulphate polyacrylamide gel analysis of immunoprecipitates recovered from ^{35}S-labelled solubilised membranes, using the hydrogenase-specific antibodies. The result of such an experiment is shown in fig.2. A single radioactive band was obtained of M_r = 58 000. An indistinguishable result was obtained when a Triton X-100-solubilised extract from a non-radioactive growth was ^{125}I-radioiodinated using Chloramine T and analysed in the same way. The nature of the radioactivity located at the dye front of the gels is unknown.

This result is in close agreeement with that of Adams and Hall [6] who reported that hydrogenase purified from areobically grown *E. coli* was composed of a single type of polypeptide of M_r = 56 000. Our results are inconsistent with the view [19] that there are multiple molecular forms of the enzyme in *E. coli*.

3.3. *Mutant isolation and genetic characterisation*

Two independent mutants deficient in hydrogenase activity (table 1) were identified using the general procedures and dye overlay technique used previously for the isolation of formate dehydrogenase-deficient mutants [20]. Such mutants differed from *fdhA* and *fdhB* mutants [21] in that, although they lacked

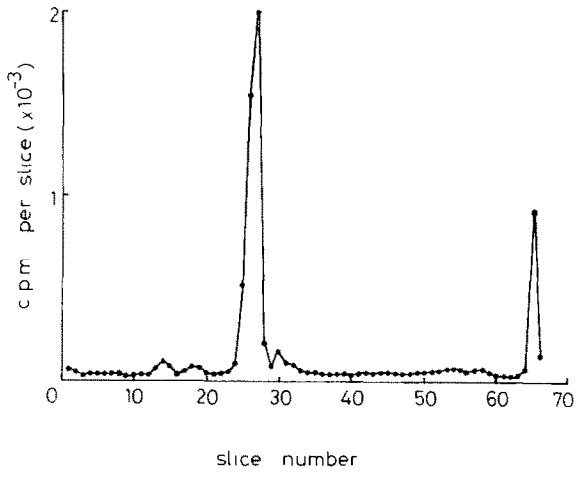

Fig.2. Polyacrylamide gel analysis of immunoprecipitated hydrogenase. Dodecyl sulphate polyacrylamide gel analysis of the total immunoprecipitate obtained when 340 μl of Triton X-100-solubilised membrane vesicles from a ^{35}S-labelled culture of wild type strain (EMG-2) was challenged with 400 μl of the absorbed antibodies to hydrogenase. Immunoprecipitation proceeded for 1 h at 20°C and overnight at 4°C. The immunoprecipitate was recovered and treated as described by Werner [24].

formate-dependent benzyl viologen reductase activity, they retained some formate-dependent, *N*-methylphenazonium methosulphate-mediated, 2,6-dichlorophenolindophenol reductase activity. Furthermore such mutants lacked hydrogen-dependent benzyl viologen reductase activity (table 2), unlike *fdhA* and *fdhB* mutants, and, thus, they phenotypically resemble the *hyd* mutants described earlier [22]. Previous work had suggested that the *hyd* allele was located at about 58 min on the recalibrated *E. coli* chromosome [22]. This observation was confirmed and extended by phage Plkc-mediated transduction with strain FD12 (*cysC*$^+$ *hyd*$^-$ *recA*$^+$) as donor and strain 5201 (*cysC*$^-$ *hyd*$^+$ *recA*$^-$) as recipient. Among 152 *cysC*$^+$ transductants analysed, 50% were *recA*$^+$ and 92% *hyd*$^-$ and the RecA and Hyd phenotypes were distributed as follows: Hyd$^-$ RecA$^+$, 71 (47%); Hyd$^-$ RecA$^-$, 69 (45%); Hyd$^+$ RecA$^+$, 5 (3%) and Hyd$^+$ RecA$^-$, 7 (5%). These data suggest that the *hyd* and *cysC* genes are closer than was previously reported [22]. Assuming that the product of quadruple cross-over events will be the least frequent, this analysis indicates the gene order *cysC-hyd-recA*. Essentially similar results obtained using strain FD10.

Table 2
Enzymatic activities of wild type and derived *hyd* mutants of *Escherichia coli*

Strain	Formate-dependent DCPIP[a] reductase activity (μmol DCPIP reduced/min/mg protein)	Formate-dependent BV[b] reductase activity (μmol BV^{2+} reduced/min/mg protein)	H_2-dependent BV reductase activity (μmol BV^{2+} reduced/min/mg protein)
P4X	0.032	0.010	0.034
FD10	0.033	<0.001	<0.001
FD12	0.029	<0.001	<0.001

[a]DCIPIP, 2,6-dichlorophenol-indophenol; [b]BV, benzyl viologen

For details see Section 2

3.4. *Immunological analysis of hydrogenase mutants*

Analysis of Triton X-100-solubilised membranes from strains FD10 and FD12 by crossed immunoelectrophoresis against anti-"membrane vesicle" serum gave precipitin patterns very similar to those found using the wild type strain (P4X and A1002) except that the precipitin arc corresponding to hydrogenase was absent. The absence of the hydrogenase antigen in the solubilised membranes of mutant strain FD10 was confirmed by adsorption of the anti-"membrane vesicle" serum with the solubilised mutant membranes. Crossed immunoelectrophoretic analysis of the anti-"membrane vesicle" serum absorbed with increasing amounts of the strain FD10 solubilised membranes, showed that the area of all the precipitin arcs, except that due to hydrogenase, increased. Since the area beneath an arc is inversely proportional to the amount of specific antibody present in the

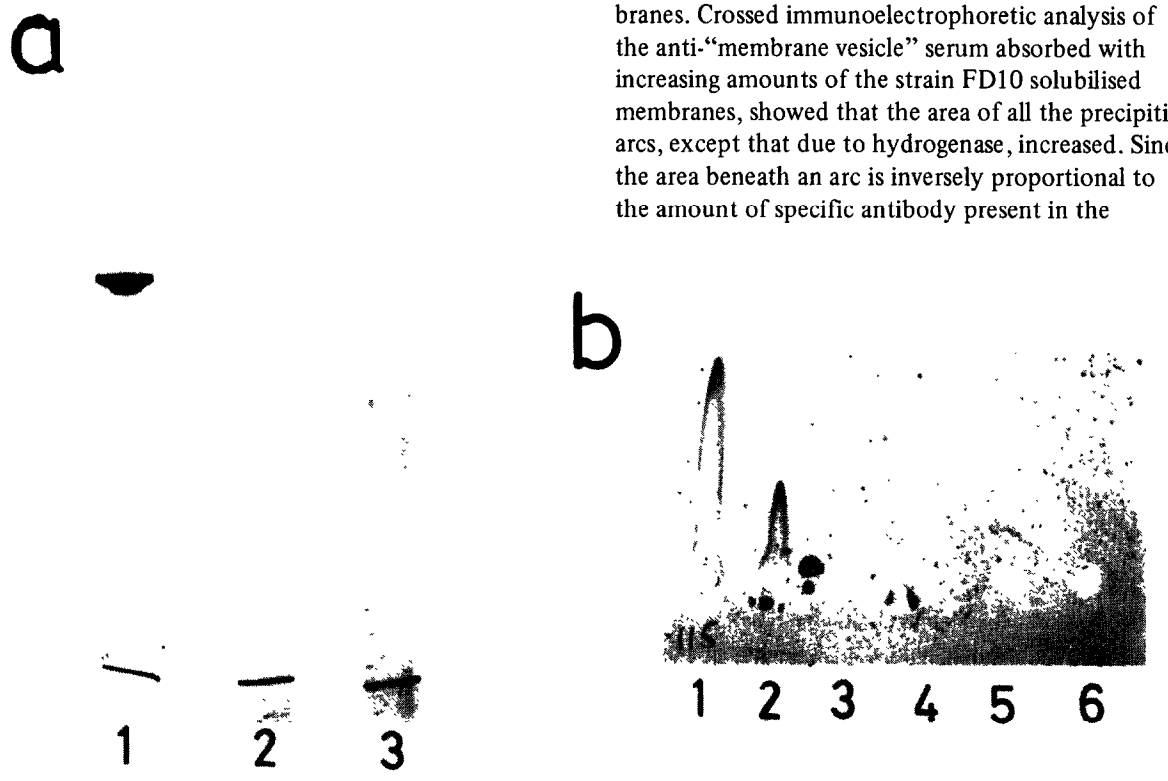

Fig.3. Analysis of whole cell lysates of hydrogenase-deficient mutants. (a) Non-dissociating polyacrylamide gel electrophoresis of Triton X-100-treated whole cell lysates. 300 μg of protein was applied to each gel. Stained for hydrogenase activity: (1) strain P4X (wild type); (2) strain FD10 (*hyd*); (3) strain FD12 (*hyd*). (b) The same Triton X-100-treated whole cell lysates as used in (a) examined by "rocket" immunoelectrophoresis with 200 μl of absorbed anti-hydrogenase immunoglobulin fraction incorporated into 3.0 ml of the agarose layer. (1) Strain P4X (wild type), 60 μg protein; (2) as (1) but 20 μg protein; (3) strain FD10 (*hyd*), 60 μg protein; (4) as (3) but 20 μg protein; (5) strain FD12 (*hyd*), 60 μg protein; (6) as (5) but 20 μg protein.

agarose layer [13], it can be concluded that the hydrogenase antigen is absent from the membranes of this mutant. In a similar manner the same conclusion was reached for strain FD12.

The possibility that defective hydrogenase accumulates in the cytoplasm as has been found for succinate dehydrogenase in some mutants of *B. subtilis* [8] was examined by analysis of Triton X-100 treated whole cell lysates. Initially whole cell lysates from strains P4X, FD10 and FD12 were analysed by non-dissociating polyacrylamide gel electrophoresis. Consistent with the data in table 2, only the lysate from the wild-type organism (strain P4X) gave a positive hydrogenase stain, a small amount at the gel origin and a single activity band having a relative mobility of 0.40 (fig.3). When the same lysates were analysed by "rocket" immunoelectrophoresis against antibodies specific for hydrogenase only the wild type (Strain P4X) gave a positive result (fig.3). The specific absence of detectable hydrogenase protein in strains FD10 and FD12 is consistent with their specific lack of hydrogenase activity. We have previously reported [21] a similar lack of nitrate reductase protein in many *chlC* mutants and of formate dehydrogenase protein in *fdhA* and *fdhB* mutants. Whether these findings indicate a bias in the selection procedures towards early nonsense mutations or that the defective enzymes are very rapidly degraded remains to be established. The availability of antiserum specific for hydrogenase provides the means for the detection and analysis of defective enzyme in further hydrogenase-deficient mutants as they become available.

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References

[1] Macy, J., Kulla, H. and Gottschalk, G. (1976) J. Bacteriol. 125, 423–428.
[2] Bernhard, Th. and Gottschalk, G. (1978) Arch. Microbiol. 116, 235–238.
[3] Jones, R. W. (1979) Biochem. Soc. Trans. 7, 1136–1137.
[4] Jones, R. W. (1980) in the press.
[5] Gray, C. T. and Gest, H. (1965) Science 148, 186–192.
[6] Adams, M. W. W. and Hall, D. O. (1979) Biochem. J. 183, 11–22.
[7] Bernhard, Th. and Gottschalk, G. (1978) in Hydrogenases: their Catalytic Activity, Structure and Function (Schlegel, H. G. and Schneider, K., eds.) pp. 199–208. Goltze, Göttingen.
[8] Rutberg, R., Hederstedt, L., Holmgren, E. and Rutberg, L. (1978) J. Bacteriol. 136, 304–311.
[9] Cohen, G. N. and Rickenberg, H. W. (1956) Ann. Inst. Pasteur (Paris) 91, 693–720.
[10] Jones, R. W. and Garland, P. B. (1977) Biochem. J. 164, 199–211.
[11] Giordano, G., Graham, A., Boxer, D. A., Haddock, B. A. and Azoulay, E. (1978) FEBS Lett. 95, 290–294.
[12] Harboe, N. and Ingild, A. (1973) in A Manual of Quantitative Immunoelectrophoresis (Axelson, N. H., Kroll, J. and Weeke, B., eds.) pp. 161–164. Universitetsforlaget, Oslo.
[13] Smyth, C. J., Sigel, J., Salton, M. R. J. and Owen, P. (1978) J. Bacteriol. 133, 306–319.
[14] Ornstein, L. and Davies, B. J. (1963) Disc Gel Electrophoresis, Distillation Products Industries, Rochester, NY.
[15] Laemmli, U. K. (1970) Nature 227, 680–685.
[16] Jenkins, H. E., Graham, A. and Haddock, B. A. (1979) FEMS Microbiol. Lett. 6, 169–173.
[17] Jones, R. W. (1979) Biochem. Soc. Trans. 7, 724–725.
[18] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[19] Yamamoto, I. and Ishimoto, M. (1978) J. Biochem. (Tokyo) 84, 673–679.
[20] Mandrand-Berthelot, M. A., Wee, M. Y. K. and Haddock, B. A. (1978) FEMS Microbiol. Lett. 4, 37–40.
[21] Graham, A., Jenkins, H. E., Smith, N. H., Mandrand-Berthelot, M. A., Haddock, B. A. and Boxer, D. H. (1980) FEMS Microbiol. Lett. 145–151.
[22] Pascal, M. C., Casse, F., Chippaux, M. and Lepelletier, M. (1975) Mol. Gen. Genet. 141, 173–179.
[23] Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 218–220, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[24] Werner, S. (1974) Eur. J. Biochem. 43, 39–48.