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Immunochemical Properties of NAD$^+$-Linked Glycerol Dehydrogenases from *Escherichia coli* and *Klebsiella pneumoniae*

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An NAD$^+$-linked glycerol dehydrogenase hyperproduced by a mutant of *Escherichia coli* K-12 was found to be immunochemically homologous to a minor glycerol dehydrogenase of unknown physiological function in *Klebsiella pneumoniae* 1033, but not to the glycerol dehydrogenase of the *dha* system responsible for anaerobic dissimilation of glycerol or to the 2,3-butanediol dehydrogenase of *K. pneumoniae*.

Glycerol dissimilation by *Escherichia coli* K-12 is initiated by an ATP-dependent kinase of the *glp* system. The product, *sn*-glycerol 3-phosphate, is converted to dihydroxyacetone phosphate at the eventual expense of an exogenous hydrogen acceptor (6). *Klebsiella pneumoniae* 1033 has a similar system, but in addition there is a *dha* system that enables the cell to grow fermentatively on glycerol without the intervention of an exogenous hydrogen acceptor. An NAD$^+$-linked dehydrogenase initiates the utilization of glycerol as a carbon source by the *dha* system (4–9, 13). A mutant *E. coli* K-12 (strain 424) was isolated in which the ATP-dependent kinase was replaced by an NAD$^+$-linked dehydrogenase as the first enzyme for aerobic growth on glycerol. This enzyme was found also to be present in wild-type *E. coli*, but only at a level of about 0.1% of that found in strain 424 (15, 17).

The present work was undertaken to probe for possible structural homology between the *K. pneumoniae* dehydrogenase and the mutant *E. coli* dehydrogenase by an immunochemical approach. On account of overlapping substrate specificity, two other dehydrogenases were also included in some of the studies: a minor *K. pneumoniae* glycerol dehydrogenase (GDH II) with electrophoretic mobility different from that of the inducible catabolic enzyme but similar to that of the *E. coli* enzyme, and *K. pneumoniae* 2,3-butanediol dehydrogenase.

MATERIALS AND METHODS

Bacteria. *E. coli* strain 424 was derived from a K-12 line (15). *K. pneumoniae* 2002 (6) is an arginine and guanine double auxotroph of *K. pneumoniae* 1033 (9).

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Unless otherwise specified, cells were grown at 37°C in a phosphate-buffered medium (16).

Chemicals. Sepharose 6B, Sepharose 4B, and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Alkaline phosphatase (type VII), ethanolamine, goat anti-rabbit immunoglobulin G (IgG), NAD$^+$ (grade III), protamine sulfate (salmon), and Tween 20 were purchased from Sigma Chemical Co., St. Louis, Mo. Alkaline phosphatase conjugated to goat anti-rabbit IgG was obtained from Bionetics Laboratory Products, Kensington, Md. All other reagents used were previously described (J. C.-T. Tang, E. St. Martin, and E. C. C. Lin, submitted for publication).

Enzyme assays. Activities of various NAD$^+$-linked dehydrogenases were assayed at 30°C in the presence of 100 mM substrate, 0.6 mM NAD$^+$, 30 mM ammonium sulfate, and 100 mM potassium carbonate (pH 9.0) as previously described (12). Units of activity are expressed in nanomoles of NADH formed per minute.

Purification of enzymes. Glycerol dehydrogenase from *E. coli* K-12 (strain 424) and glycerol dehydrogenase from *K. pneumoniae* 2002 were prepared and purified to electrophoretic homogeneity by procedures already described (14, 17). GDH II and butanediol dehydrogenase were partially purified from *K. pneumoniae* grown anaerobically at 30°C to early stationary phase in a medium containing 0.4% DL-1,2-propanediol, 0.4% L-arabinose, 0.03% casein acid hydrolysate, 40 μg of arginine per ml, and 40 μg of guanine per ml. Extracts of sonically disrupted cells were treated with 2.4 mg of protamine sulfate per ml, and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant fraction, and the proteins precipitated between 30 and 60% saturation were collected and suspended in 50 mM phosphate buffer, pH 7.0. The two dehydrogenases were separated from each other by gel filtration through Sepharose 6B equilibrated with 50 mM phosphate buffer containing 50 mM DL-1,2-propanediol. The partially purified GDH II showed a specific activity of 2×10^3 U/mg of protein with DL-1,2-propanediol as substrate, and the partially purified butanediol dehydrogenase showed a specific activity of 1.9×10^4 U/mg of protein.

Purification of IgG. Specific antisera against the glycerol dehydrogenase of *E. coli* K-12 (strain 424) (17) and against the glycerol dehydrogenase of *K. pneumoniae* 1033 (14) were obtained from rabbits immunized with the appropriate enzyme proteins purified to electrophoretic homogeneity. One volume of saturated ammonium sulfate was added to two volumes of antiserum at room temperature. After 1 h of stirring, the precipitate was collected by centrifugation and dissolved in 0.01 M phosphate–0.015 M NaCl, pH 7.5. The material was then dialyzed against two changes of 100 volumes of the same buffer overnight at 4°C and was batch adsorbed with DEAE-cellulose to remove non-IgG components.

For immobilization on a Sepharose gel, a portion of the IgG against the *E. coli* enzyme was further purified by adsorption to glycerol dehydrogenase–Sepharose 4B (2 mg/ml). After 30 min, the gel was washed with 0.9% NaCl until no protein was detectable (optical density at 280 nm [OD_{280}]) in the supernatant fluid. The IgG was then eluted with 0.1 M acetate (pH 2.8) at 20°C. The eluate was dialyzed against 50 mM phosphate buffer (pH 7.0) and was concentrated by pressure dialysis.

Immobilization of *E. coli* glycerol dehydrogenase and IgG against the enzyme by coupling to a Sepharose gel. Cyanogen bromide-activated Sepharose 4B swollen for 15 min with 1 mM HCl was washed with the same solution on a sintered glass filter (1 g of gel per 200 ml of 1 mM HCl). This was followed by a brief wash with 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl. Glycerol dehydrogenase from *E. coli* K-12 (strain 424) was then added to the washed Sepharose (0.1 mg of enzyme per ml of gel) and was incubated in the pH 8.3 buffer for 2.5 h at 20°C as recommended by Pharmacia Fine Chemicals. The mixture was centrifuged, and the pellet was washed with 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl until no protein was detectable (OD_{280}) in the supernatant fluid. After the material was incubated with 1 M ethanolamine (pH 8) for 1.5 h to block excess reactive groups on the gel, the Sepharose-coupled protein was subjected to three cycles of alternating washing with 1 M NaCl buffered with 0.1 M acetate at pH 4 and with 1 M NaCl buffered with 0.1 M borate at pH 8. The pelleted material was fully suspended in 50 mM phosphate (pH 7.0) with 0.02% sodium azide for storage at 4°C. A 1-ml portion of the suspension dehydrogenated 0.3 μmol of glycerol per min per ml at 20°C. The same procedure was used for coupling IgG against *E. coli* glycerol dehydrogenase (10 mg of IgG purified by affinity chromatography per ml of gel).

Test for enzyme adsorption by IgG coupled to Sepharose. A 100-μl sample containing 0.6 U of a glycerol dehydrogenase or 2,3-butanediol dehydrogenase in 50 mM phosphate buffer (pH 7.0) was incubated with 0.5 ml of Sepharose 4B-coupled IgG against *E. coli* glycerol dehydrogenase (1.5-fold dilution of the stock suspension; see above) for 10 min at 20°C in a test tube. After centrifugation for 1 min at 4°C, 100 μl of the supernatant fraction was removed for enzyme assay.

Analysis of antigens and antibodies by enzyme-linked immunosorbent assay. Various NAD^+-linked dehydrogenases were tested for immunochemical cross-reactivity with IgG against *E. coli* glycerol dehydrogenase essentially by the procedure of Engvall et al. (2). The particular conditions used were described elsewhere (17a).

IgG against *E. coli* glycerol dehydrogenase and IgG against the *K. pneumoniae* dehydrogenase of the *dha* system were tested against the *E. coli* enzyme essentially by the method of Engvall and Perlmann (3). A 1-ml portion of 0.1 M sodium carbonate buffer (pH 9.8) containing 2 μg of *E. coli* glycerol dehydrogenase and 0.02% sodium azide was added to each of a series of polystyrene tubes and was incubated for 3 h at 30°C. The tubes were stored at 4°C with the antigen solution. Before use, the antigen-coated tubes were washed three times with 0.9% NaCl containing 0.05% Tween 20. Rabbit IgG against the *E. coli* enzyme or rabbit IgG against the *K. pneumoniae* enzyme (0 to 100 ng) was added in 0.5 ml of PBS-Tween (15 mM sodium phosphate buffer (pH 7.2) containing 0.02% sodium azide, 0.05% Tween 20, 0.9% NaCl) to a washed tube and incubated at room temperature for 6 h. After three washings with PBS-Tween, 0.5 ml of PBS-Tween containing alkaline phosphatase-conjugated goat anti-rabbit IgG (0.20 OD_{400} unit per min) was added. The tubes were incubated overnight (15 h) at 20°C and then were washed three times with PBS-Tween. The amount of goat IgG retained was determined by measuring the activity of alkaline phosphatase with *p*-nitrophenylphosphate as a substrate.

Immunoprecipitation of glycerol dehydrogenases. A 200-μl sample containing 0.5 U of glycerol dehydrogenase in 50 mM phosphate buffer at pH 7.0 was incubated with 50 μl of specific rabbit IgG for 14 h at 4°C. Then, 10 μl of goat antiserum specific for rabbit IgG was added, and further incubation was carried out for 3 h at 20°C. After clarification by centrifugation, the supernatant fraction was assayed for enzyme activity.

RESULTS

Substrate specificity of four NAD^+-linked dehydrogenases. Among the tested compounds that acted as substrates for the dehydrogenase of *E. coli* K-12 (strain 424), only glycerol, 1,2-propanediol (fermentation product of L-fucose [1]), and 2,3-butanediol are known as metabolites of enteric bacteria. These compounds also exhibited high activity with *K. pneumoniae* dehydrogenase of the *dha* system. This dehydrogenase, however, is not the only one in *K. pneumoniae* that can act on the above-mentioned substrates. When an extract of cells grown anaerobically on L-arabinose and casein hydrolysate was subjected to polyacrylamide gel electrophoresis and stained for NAD^+-linked dehydrogenase activity (17), a minor band active towards all three substrates was identified. Its mobility was similar to that of the *E. coli* dehydrogenase but lower than that of the dehydrogenase of the *dha* system. This band was found to contain both GDH II and 2,3-butanediol dehydrogenase, as was found previously in a different strain of *K. pneumoniae* (5).

Table 1 compares the substrate specificities of the *E. coli* dehydrogenase, the *K. pneumoniae* dehydrogenase of the *dha* system, GDH II, and butanediol dehydrogenase. The first three dehydrogenases acted on 1,2-propanediol and 2,3-

TABLE 1. Substrate specificity of four NAD^+-linked dehydrogenases[a]

Enzyme	Relative activity on[b]:		
	Glycerol	DL-1,2-propanediol	DL-2,3-butanediol
E. coli glycerol dehydrogenase	100	97	66
K. pneumoniae glycerol dehydrogenase	100	66	72
K. pneumoniae GDH II	100	160	71
K. pneumoniae butanediol dehydrogenase	0	4	100

[a] Preparation and assay of enzyme activity are described in the text.
[b] Full activity (100) represents 25 U.

butanediol as well as on glycerol. In contrast, butanediol dehydrogenase acted only on its specific substrate.

Immunochemical analysis of the four enzymes by the Ouchterlony double diffusion technique. Since only the *E. coli* dehydrogenase and the *K. pneumoniae* dehydrogenase of the *dha* system were available in highly purified form, antibodies raised against these two antigens were used as structural probes. IgG against the *E. coli* enzyme formed a line of immunoprecipitation with 1 μg of the immunogen, but not with the *K. pneumoniae* enzyme of the *dha* system (Fig. 1A). Figure 1B shows the mirror image result, with IgG raised against the *K. pneumoniae* enzyme. IgG against the *E. coli* enzyme gave no visible precipitation with 50 μg of partially purified butanediol dehydrogenase from *K. pneumoniae*, but it clearly gave visible precipitation with 50 μg of the partially purified GDH II from *K. pneumoniae* (Fig. 1C). Moreover, GDH II is immunochemically identical with the *E. coli* glycerol dehydrogenase since a continuous band without a spur was formed in the agar between the central well charged with the specific IgG and the adjacent wells charged, respectively, with the pure *E. coli* enzyme and GDH II. A mixture of 25 μg of each of the two *K. pneumoniae* enzymes gave a visible line of precipitation, although this is not clearly revealed by photography. The *E. coli* enzyme thus appears to be an evolutionary relative of GDH II of *K. pneumoniae* but has no detectable resemblance to the *dha* enzyme by immunochemical criteria. This conclusion is supported by more quantitative tests.

Enzyme adsorption tests of the four enzymes by immobilized IgG. The cross-reactivities of the four dehydrogenases with IgG against *E. coli* glycerol dehydrogenase coupled to Sepharose 4B are compared in Table 2. Binding of the *E. coli* enzyme and *K. pneumoniae* GDH II was complete. There was no significant binding of *K. pneumoniae* dehydrogenase of the *dha* system or of butanediol dehydrogenase.

Comparison of antigenic properties of the four enzymes by enzyme-linked immunosorbent assay. Binding tests of the *E. coli* and *pneumoniae* enzymes to IgG against *E. coli* glycerol dehydrogenase coated on the walls of plastic tubes again revealed structural similarity between the *E. coli* glycerol dehydrogenase and *K. pneumoniae* GDH II (Table 3). Assuming that the two enzymes have the same molecular weight, it can be estimated that the crude extract of *K. pneu-*

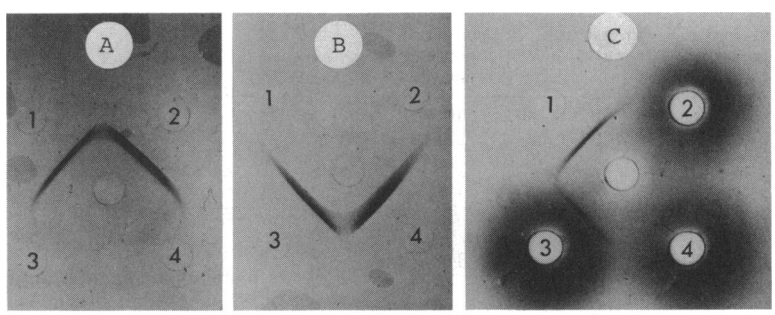

FIG. 1. Analysis of dehydrogenases by double immunodiffusion (11). Central wells contained 20 μg of IgG, and surrounding wells contained either a pure glycerol dehydrogenase or partially purified GDH II or butanediol dehydrogenase. Antibodies and enzymes were tested in three configurations. (A) Central well, IgG against *E. coli* glycerol dehydrogenase; wells 1 and 2, *E. coli* dehydrogenase (1 μg); wells 3 and 4, *K. pneumoniae* glycerol dehydrogenase of the *dha* system (1 μg). (B) Central well, IgG against *K. pneumoniae* glycerol dehydrogenase of the *dha* system; wells 1 and 2, *E. coli* glycerol dehydrogenase (1 μg); wells 3 and 4, *K. pneumoniae* glycerol dehydrogenase of the *dha* system (1 μg). (C) Central well, IgG against *E. coli* glycerol dehydrogenase; well 1, *E. coli* glycerol dehydrogenase (1 μg); well 2, *K. pneumoniae* butanediol dehydrogenase (50 μg); well 3, *K. pneumoniae* GDH II (50 μg); well 4, *K. pneumoniae* GDH II (25 μg) plus butanediol dehydrogenase (25 μg). The protein was stained by Coomassie brilliant blue.

TABLE 2. Enzyme adsorption by Sepharose-IgG against *E. coli* glycerol dehydrogenase

Enzyme	% Activity remaining in supernatant fraction after incubation with:	
	Sepharose 4B[a]	IgG-Sepharose 4B
E. coli glycerol dehydrogenase	100	0
K. pneumoniae glycerol dehydrogenase	100	95
K. pneumoniae GDH II	100	0
K. pneumoniae butanediol dehydrogenase	100	93

[a] Recovery of enzyme activity in the supernatant fraction exposed to uncoupled Sepharose 4B was normalized as 100%. The initial activities in units per 100 μl were 12 (line 1), 10.5 (line 2), 13 (line 3), and 7.0 (line 4). Further details are given in the text.

moniae contained about 2 μg of GDH II per g of total soluble protein.

Quantitative analysis of the two major purified dehydrogenases by immunoprecipitation. When 0.5 U of *E. coli* glycerol dehydrogenase was titrated against IgG against this enzyme, >90% of the enzyme was bound with the addition of 50 μg of IgG. Under the same conditions, up to 100 μg of IgG against *K. pneumoniae* glycerol dehydrogenase of the *dha* system had no effect (Fig. 2A). Opposite results were obtained when *K. pneumoniae* glycerol dehydrogenase of the *dha* system was used as the antigen (Fig. 2B).

Quantitative analysis of the two major purified dehydrogenases by enzyme-linked immunosorbent assay. Binding of alkaline phosphatase-conjugated glycerol dehydrogenase of *E. coli* to a plastic surface coated with rabbit IgG against this dehydrogenase was 50% inhibited by incubation with 25 ng of unlabeled *E. coli* enzyme (Fig. 3A). In contrast, no inhibition by incubation with the *K. pneumoniae* enzyme was evident even at 250 ng. Binding of alkaline phosphatase-conjugated goat anti-rabbit IgG to a plastic surface coated with *E. coli* glycerol dehydrogenase was enhanced as a result of incubation with rabbit IgG against the *E. coli* enzyme, but not with rabbit IgG against the *K. pneumoniae* enzyme of the *dha* system (Fig. 3B).

TABLE 3. Enzyme-linked immunosorbent assay of NAD^+-linked dehydrogenases

Competing enzyme protein added	Protein (ng)	OD_{400}	% Alkaline phosphatase retained
None	0	0.140	100
E. coli glycerol dehydrogenase[a]	100	0.043	31
K. pneumoniae dehydrogenase[a]	100	0.140	100
K. pneumoniae GDH II[a]	5,000	0.100	75
K. pneumoniae butanediol dehydrogenase[a]	5,000	0.150	110
E. coli glycerol dehydrogenase[b]	100	0.000	0

[a] Walls of tubes were coated with IgG against *E. coli* glycerol dehydrogenase. Alkaline phosphatase-labeled *E. coli* glycerol dehydrogenase was then incubated with a competing enzyme in the tube, and the phosphatase activity was assayed after washing.

[b] The wall of the tube was coated instead with IgG against the *K. pneumoniae* dehydrogenase of the *dha* system.

DISCUSSION

The glycerol dehydrogenase of *E. coli*, whose true function remains to be discovered, exists in two catalytically active forms: a major form of about 310,000 daltons and a minor form of about 81,000 daltons. They appear to be octomers and dimers, respectively, of a common subunit of about 39,000 daltons. The enzyme activity is strongly stimulated by NH_4^+, K^+, and Rb^+, but

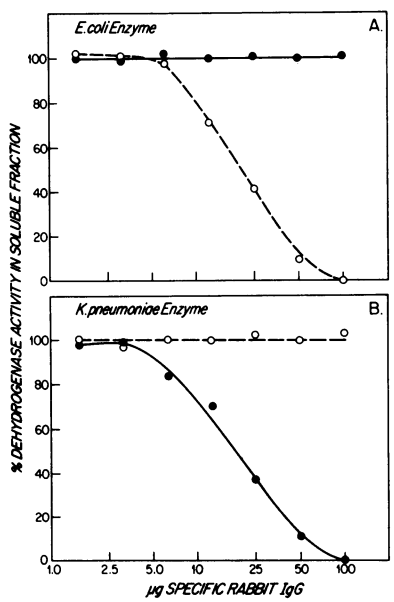

FIG. 2. Immunoprecipitation of glycerol dehydrogenases by specific antibodies. (A) *E. coli* glycerol dehydrogenase was incubated with various amounts of IgG against the same enzyme (○) or IgG against *K. pneumoniae* glycerol dehydrogenase of the *dha* system (●). Goat antiserum specific for rabbit IgG was then added to the sample, and the immunoglobulin complexes were removed by centrifugation. The enzyme activity remaining in the supernatant fraction was assayed. (B) *K. pneumoniae* glycerol dehydrogenase of the *dha* system was analyzed in a similar manner. For details, see the text.

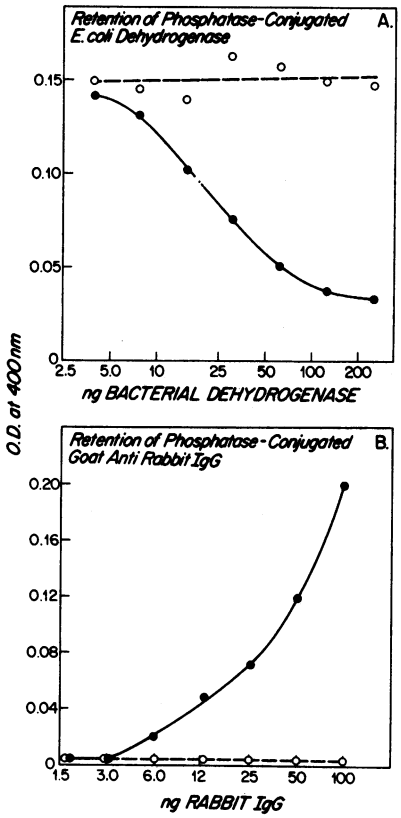

FIG. 3. Enzyme-linked immunosorbent analysis of glycerol dehydrogenases. (A) Tubes coated with rabbit IgG against *E. coli* glycerol dehydrogenase were incubated with various amounts of the *E. coli* enzyme (●) or the *K. paneumoniae* enzyme of the *dha* system (○) along with alkaline phosphatase-conjugated glycerol dehydrogenase. After washing, the retention of alkaline phosphatase activity was determined. Units are in OD_{400} change per hour. (B) Tubes coated with *E. coli* glycerol dehydrogenase were incubated with various amounts of rabbit IgG against the *E. coli* enzyme (●) or rabbit IgG against the *K. pneumoniae* enzyme of the *dha* system (○). After rinsing, the amount of rabbit IgG retained was estimated by the binding of alkaline phosphatase-conjugated goat anti-rabbit IgG. For details, see text.

not at all by Li^+. In the presence of 100 mM NH_4^+, the apparent K_m for glycerol is 1.4 mM, and that for dihydroxyacetone is 1 mM. The enzyme is highly sensitive to inhibition by divalent cations, e.g., Cu^{2+}. On the other hand, it is also susceptible to inhibition by chelating agents, 8-hydroxyguinoline being the most powerful (17).

The glycerol dehydrogenase belonging to the *dha* system of *K. pneumoniae* was also found to exist in two catalytic forms: a major form of about 160,000 daltons and a minor form of about 79,000 daltons. They appear to be tetramers and dimers, respectively, of a common subunit of about 40,000 daltons (14). The enzyme activity is strongly stimulated by NH_4^+, K^+, and Rb^+, but not at all by Li^+ (8). In the presence of 100 mM NH_4^+, the apparent K_m for glycerol is about 2 mM, and that for dihydroxyacetone is about 0.1 mM (10). The enzyme is highly sensitive to inhibition by divalent cations, e.g., Zn^{2+}. On the other hand, it is also susceptible to inhibition by chelating agents, 8-hydroxyquinoline being the most powerful (8).

The multiple resemblance in physical and catalytic properties between the two enzymes led us to think that their genes might have evolved from a common ancestor before the speciation of the two enteric organisms and that some homology in protein structure might be revealed immunochemically. However, no such evidence came forth. Instead, we unexpectedly found that the GDH II in *K. pneumoniae* does show immunochemical homology with the *E. coli* enzyme. These two enzymes are likely to play the same physiological role. If the genes of all three enzymes have the same origin, this would be an example of more rapid evolution in protein surface structure under the influence of functional specialization than under the influence of taxonomic divergence.

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LITERATURE CITED

1. **Cocks, G. T., J. Aguilar, and E. C. C. Lin.** 1974. Evolution of L-1,2-propanediol catabolism in *Escherichia coli* by recruitment of enzymes for L-fucose and L-lactate metabolism. J. Bacteriol. **118**:83–88.
2. **Engvall, E., K. Jonsson, and P. Perlmann.** 1971. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim. Biophys. Acta **251**:427–434.
3. **Engvall, E., and P. Perlmann.** 1971. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129–135.
4. **Forage, R. G., and M. A. Foster.** 1979. Resolution of the coenzyme B_{12}-dependent dehydratase of *Klebsiella* sp. and *Citrobacter freundii*. Biochim. Biophys. Acta **569**:249–258.
5. **Forage, R. G., and M. A. Foster.** 1982. Glycerol fermentation in *Klebsiella pneumoniae*: functions of the coenzyme B_{12}-dependent glycerol and diol dehydratases. J. Bacteriol. **149**:413–419.
6. **Lin, E. C. C.** 1976. Glycerol dissimilation of its regulation in bacteria. Annu. Rev. Microbiol. **30**:535–578.
7. **Lin, E. C. C., A. P. Levin, and B. Magasanik.** 1960. The effect of aerobic metabolism on the inducible glycerol dehydrogenase of *Aerobacter aerogenes*. J. Biol. Chem. **235**:1824–1829.

8. **Lin, E. C. C., and B. Magasanik.** 1960. The activation of glycerol dehydrogenase from *Aerobacter aerogenes* by monovalent cations. J. Biol. Chem. **235**:1820–1823.
9. **Magasanik, B., M. S. Brooke, and D. Karibian.** 1953. Metabolic pathways of glycerol dissimilation. A comparative study of two strains of *Aerobacter aerogenes*. J. Bacteriol. **66**:611–619.
10. **McGregor, W. G., J. Phillips, and C. H. Suelter.** 1974. Purification and kinetic characterization of a monovalent cation-activated glycerol dehydrogenase from *Aerobacter aerogenes*. J. Biol. Chem. **249**:3132–3139.
11. **Ouchterlony, O.** 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26**:507–515.
12. **Ruch, F. E., J. Lengeler, and E. C. C. Lin.** 1974. Regulation of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. **119**:50–56.
13. **Ruch, F. E., and E. C. C. Lin.** 1975. Independent constitutive expression of the aerobic and anaerobic pathways of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. **124**:348–352.
14. **Ruch, F. E., Jr., E. C. C. Lin, J. D. Kowit, C.-T. Tang, and A. L. Goldberg.** 1980. In vivo inactivation of glycerol dehydrogenase in *Klebsiella aerogenes*: properties of active and inactivated proteins. J. Bacteriol. **141**:1077–1085.
15. **St. Martin, E. J., W. B. Freedberg, and E. C. C. Lin.** 1977. Kinase replacement by a dehydrogenase for *Escherichia coli* glycerol utilization. J. Bacteriol. **131**:1026–1028.
16. **Tanaka, S., S. A. Lerner, and E. C. C. Lin.** 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. **93**:642–648.
17. **Tang, C.-T., F. E. Ruch, Jr., and E. C. C. Lin.** 1979. Purification and properties of a nicotinamide adenine dinucleotide-linked dehydrogenase that serves an *Escherichia coli* mutant for glycerol catabolism. J. Bacteriol. **140**:182–187.

17a. **Tang, J. C.-T., E. J. St. Martin, and E. C. C. Lin.** 1982. Derepression of an NAD-linked dehydrogenase that serves an *Escherichia coli* mutant for growth on glycerol. J. Bacteriol. **152**:1001–1007.