

# Characterization of *Escherichia coli* Mutant Incapable of Maintaining a Transmembrane Potential

METC\* ECF<sup>ts</sup> MUTATIONS\*

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A temperature-sensitive mutant of *Escherichia coli* defective in coupling energy to the active transport of amino acids and some sugars (Lieberman and Hong (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 4395-4399) is further characterized. The use of starved cells allows us to demonstrate that the mutant, in addition to being defective in active transport, is also unable to synthesize adenosine 5'-triphosphate under nonpermissive conditions. The mutant is thus blocked in energy coupling to both transport and oxidative phosphorylation.

The *ecf<sup>ts</sup>* mutation maps at 64 min on the revised *E. coli* chromosome map and is closely linked with the *metC* gene.

The transport defect is not due to a generalized membrane permeability, as determined by noncarrier-mediated diffusion of orthonitrophenyl- $\beta$ -D-galactoside and measurements of membrane permeability to protons. In the absence of an exogenous energy source the mutant rapidly loses its ability to transport due to a de-energization of the energized membrane state. Under these conditions the mutant is incapable of establishing a membrane potential, as determined by triphenylmethylphosphonium ion uptake. This de-energization is related to a reduced efficiency with which energy is coupled to transport. The addition of an exogenous energy source to the mutant energizes the membrane and stimulates transport, although energization remains inefficient. The addition of low concentrations of glucose (<1 mM) to the mutant brings about a transitory stimulation of transport, the rate and extent of which is dependent upon the glucose concentration. The glucose addition also establishes a membrane potential, the extent of which parallels the extent of transport activity.

This stimulation of transport activity by glucose is fully sensitive to cyanide, suggesting that membrane energization via the (Ca,Mg)-ATPase route is totally defective in the mutant at the nonpermissive temperature, whereas membrane energization via respiration is only partially defective.

Since both respiration and (Ca,Mg)-ATPase activity are normal at 42° the defect lies in the coupling of energy to active transport and oxidative phosphorylation.

Powerful genetic methods have been recently applied to the problem of the energy-coupling mechanism in *Escherichia coli* and *Salmonella typhimurium* (for review, see Refs. 1 and 2), and many mutants have been found, primarily in the (Ca,Mg)-ATPase.<sup>1</sup> The study of these mutants (*uncA*, *B*) has led to the general conclusion that the (Ca,Mg)-ATPase is an energy-transducing ATPase and may also serve an important role in maintaining the structural integrity of the membrane.

We have previously described a temperature-sensitive mutant of *E. coli* which is defective in the coupling of energy to transport, and which is distinct from the other energy-uncoupled mutants thus far isolated (3). This paper further characterizes the mutant demonstrating that at the nonpermissive temperature the mutant cannot efficiently energize its membrane, thus resulting in a loss of those functions (transport and oxidative phosphorylation) which require the energized membrane state. The defect of the mutant appears to be a novel one and may reside in an as yet unspecified step in the complex energy-transducing machinery.

## MATERIALS AND METHODS

### Media and Organisms

All strains used in this work are derived from *Escherichia coli* K-12 and are listed in Table I.

Media used were nutrient broth and minimal salts medium E (per liter: 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.0 g of citric acid·H<sub>2</sub>O, 10 g of K<sub>2</sub>HPO<sub>4</sub>, 3.5 g of NaNH<sub>4</sub>HPO<sub>4</sub>·4H<sub>2</sub>O) (6) supplemented with 0.5% carbon source and 40  $\mu$ M B1. When required for growth, amino acids were added to 0.4 mM. A carbon-free salts medium (N<sup>+</sup>C<sup>-</sup>) (per liter: 1.0 g of NH<sub>4</sub>Cl, 1.0 g of K<sub>2</sub>SO<sub>4</sub>, 13.5 g of K<sub>2</sub>HPO<sub>4</sub>, 4.7 g of KH<sub>2</sub>PO<sub>4</sub>, 0.1 g of MgSO<sub>4</sub>·7H<sub>2</sub>O) (7) was often employed as a buffer for the transport experiments.

### Genetic Techniques

Transductions, preparation of phage lysates, and isolation of mutants have been described previously (3).

<sup>1</sup> The abbreviations used are: (Ca,Mg)-ATPase, calcium- or magnesium-stimulated adenosine 5'-triphosphatase; N<sup>+</sup>C<sup>-</sup>, a carbon-free salts medium; TMG,  $\beta$ -methyl-D-thiogalactoside; IPTG, isopropyl- $\beta$ -D-thiogalactoside; TDG, thiodigalactoside; TPMP<sup>+</sup>, triphenylmethylphosphonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazide; Suc, ability to grow on succinate as sole carbon source.

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TABLE I  
Bacterial strains used

	Relevant genotype	Source
JSH1	HfrH: <i>thi</i>	Wild type, from R. F. Schleif, Brandeis University
JSH4	HfrH: <i>thi ecf<sup>ts</sup> metC*</i>	Ref. 3
JSH202	F <sup>-</sup> <i>:thi strA</i>	JSH1 made <i>strA</i> by P1 transduction and F <sup>-</sup> by sodium dodecyl sulfate treatment (4)
JSH203	F <sup>-</sup> <i>:thi strA thyA</i>	JSH202 resistant to trimethoprim
KL228	Hfr: <i>thi leu-6 gal-6</i> . . .	Obtained from B. Bachmann CGSC 4316. Hfr, transfer <i>metC</i> gene early
AT2699	F <sup>-</sup> <i>:argG-6 metC-69 his-1 thyA-3</i> . . .	Obtained from B. Bachmann CGSC 4524
MAL112	Hfr: <i>thi metC-69 his-1</i> . . .	KL228 × AT2699. Selection for <i>arg<sup>+</sup> thy<sup>+</sup></i> to insert Hfr in AT2699
JSH210	F <sup>-</sup> <i>:thi strA metC-69</i>	MAL112 × JSH203
JSH80	Hfr: <i>thi uncA</i>	Ref. 5
MAL300	F <sup>-</sup> <i>:thi ecf<sup>ts</sup> metC* strA</i>	JSH210 transduced to <i>metC<sup>+</sup></i> with P1 grown on JSH4
MAL303	F <sup>-</sup> <i>:ilv thi metC-69 strA</i>	Ref. 5
MAL304	F <sup>-</sup> <i>:thi ilv metC* ecf<sup>ts</sup> strA</i>	Ref. 5
MAL310	F <sup>-</sup> <i>:thi strA uncA metC</i>	Ref. 5
MAL312	F <sup>-</sup> <i>:thi strA uncA metC* ecf<sup>ts</sup></i>	Ref. 5
A586	F <sup>-</sup> <i>:tolC-3</i> . . .	Obtained from B. Bachmann CGSC 4925
MAL350	F <sup>-</sup> <i>:thi strA metC-69 thyA</i>	JSH210 resistant to trimethoprim
MAL351	F <sup>-</sup> <i>:thi strA metC* ecf<sup>ts</sup> thyA</i>	MAL350 transduced to <i>metC<sup>+</sup></i> with P1 grown on MAL300
KLF16/KL110	F <sup>-</sup> <i>:lysA<sup>+</sup> tyrA<sup>+</sup>/tyrA2</i> . . .	Obtained from B. Bachmann Episome F <sup>-</sup> 116. CGSC 4254

## Transport Assays

**Amino Acid and Sugar Uptake**—Transport in both whole cells and membrane vesicles was measured as described previously (3). The specific activities and final concentrations of the radioactive substrates used were as follows: L-[U-<sup>14</sup>C]proline (236 Ci/mol), 8.3  $\mu$ M; L-[U-<sup>14</sup>C]lysine (312 Ci/mol), 6.6  $\mu$ M; L-[U-<sup>14</sup>C]serine (156 Ci/mol), 12.9  $\mu$ M; L-[U-<sup>14</sup>C]tyrosine (460 Ci/mol), 4.4  $\mu$ M; L-[U-<sup>14</sup>C]glutamine (204 Ci/mol), 9.8  $\mu$ M; L-[U-<sup>14</sup>C]valine (232 Ci/mol), 8.9  $\mu$ M; methyl- $\alpha$ ,D-[<sup>14</sup>C]glucopyranoside (108 Ci/mol), 37  $\mu$ M;  $\beta$ -methyl-D-[<sup>14</sup>C]-thiogalactoside (TMG) (14.4 Ci/mol), 100  $\mu$ M.

**Orthonitrophenyl- $\beta$ -D-galactoside Transport**—The carrier- and noncarrier-mediated transport of orthonitrophenyl- $\beta$ -D-galactoside by the *lac* system was assayed as described by Rosen (8) utilizing a Zeiss spectrophotometer equipped with a constant temperature block. Cells were induced for the *lac* operon by growth in minimal salts media plus glycerol and 0.5 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG). Cells were harvested in midlog phase ( $2$  to  $4 \times 10^8$  cells/ml), washed once in N<sup>+</sup>C<sup>-</sup>, and resuspended in N<sup>+</sup>C<sup>-</sup> at a density of  $2.8 \times 10^9$  cells/ml. The assay was performed in 1.0 ml of N<sup>+</sup>C<sup>-</sup> containing 50  $\mu$ l of cells and 10  $\mu$ mol of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. To determine the noncarrier-mediated diffusion of orthonitrophenyl- $\beta$ -D-galactoside, 3  $\mu$ mol of thiodigalactoside (TDG) was added. After a 10-min incubation at the desired temperature, 2  $\mu$ mol of orthonitrophenyl- $\beta$ -D-galactoside were added and the increase in absorbance at 420 nm recorded. In order to determine the rate of orthonitrophenyl- $\beta$ -D-galactoside transport in the absence of a membrane barrier, cells were treated with toluene. These cells were prepared by adding 10  $\mu$ l of toluene to the assay mixture and blending on a Vortex mixer vigorously. This procedure disrupts the membrane.

**Triphenylmethylphosphonium ion (TPMP<sup>+</sup>) Uptake**—Uptake of [<sup>3</sup>H]TPMP<sup>+</sup> was measured in tris(hydroxymethyl)aminomethane/ethylenediaminetetraacetate (Tris/EDTA)-treated cells using a modification of the procedure described by Schuldner and Kaback (9). Glucose-grown cells (160 ml) were harvested in midexponential phase, washed twice with N<sup>+</sup>C<sup>-</sup>, and resuspended in 0.1 M Tris/HCl, pH 7.8, at a cell density of  $2.8$  to  $4.2 \times 10^9$  cells/ml. The cells were immediately transferred to an Erlenmeyer flask such that the flask volume was at least 10 times that of the sample volume and the

potassium salt of EDTA, adjusted to pH 7.0, was added to a final concentration of 10 mM. The cells (about 15 ml) were rapidly swirled by hand in a 30° water bath for 30 s, then diluted at least 4-fold with N<sup>+</sup>C<sup>-</sup>. The cells were then pelleted, washed once with N<sup>+</sup>C<sup>-</sup>, and resuspended in N<sup>+</sup>C<sup>-</sup> containing 100  $\mu$ g of chloramphenicol/ml. [<sup>3</sup>H]TPMP<sup>+</sup> (0.4 mM, 28.5 or 80 Ci/mol) uptake was assayed as described by Schuldner and Kaback (9). Cells could be stored in ice for up to 3.5 h with no loss in their initial level of TPMP<sup>+</sup> uptake.

## S-Adenosylmethionine Synthetase Assays

S-Adenosylmethionine (AdoMet) synthetase (EC 2.5.1.6) was assayed in sonicated cell extracts following the procedure of Holloway *et al.* (10).

## Starvation of Cells

**For ATP Synthesis Determination**—Cells were starved by a modification of the procedure developed by Berger (11). Cultures were grown on glucose minimal salts medium at 25°, harvested in midexponential phase, washed twice with Medium B (consisting of 1 mM MgCl<sub>2</sub>, 0.1 M potassium phosphate, pH 7.0, and 100  $\mu$ g of chloramphenicol/ml), and resuspended in Medium B with 5 mM 2,4-dinitrophenol (twice recrystallized from water) at a cell density of  $7 \times 10^9$  cells/ml. The cells were then starved by incubating in a 26° water bath for either 2.0 (mutant) or 4.5 (wild type) h. The starved cells were then washed three times with Medium B and resuspended in the same at  $5 \times 10^8$  cells/ml. The cells were stored on ice. One-half of the cell suspension was heated at 42° for 5 min and cooled on ice for at least 10 min prior to beginning the experiments, all of which were conducted at 26°.

**For Efficiency Determinations**—Glucose-grown cells (25°) were harvested in midexponential phase, washed twice with N<sup>+</sup>C<sup>-</sup>, and resuspended in Medium A (12) (per liter: 4.69 g of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 11.15 g of K<sub>2</sub>HPO<sub>4</sub>, 2.64 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.074 g of MgSO<sub>4</sub>, 10<sup>-6</sup> M FeSO<sub>4</sub>, 10<sup>-6</sup> M ZnCl<sub>2</sub>, and 10<sup>-5</sup> M CaCl<sub>2</sub>) to a cell density of  $2.1$  to  $4.2 \times 10^9$  cells/ml. Solid dinitrophenol was then added to 5 mM and the cells were incubated at 26° for 10 to 13 h. The cells were processed by washing three times with N<sup>+</sup>C<sup>-</sup> and resuspending in N<sup>+</sup>C<sup>-</sup> containing chloramphenicol (100  $\mu$ g/ml) at a density of  $2.1 \times 10^9$  cells/ml. The cells were stored on ice.

## Measurement of ATP Synthesis in Starved Cells

Starved cells (2.8 ml in a 50-ml Erlenmeyer flask) were placed in a 26° shaking water bath. ATP synthesis was initiated by adding 30  $\mu$ l of 2.0 M ascorbate (pH 7.0 with KOH) followed by 20  $\mu$ l of 15 mM phenazine methosulfate. At desired times samples (0.4 ml) were removed and placed in a boiling water bath for 10 min. Both ascorbate and phenazine methosulfate were prepared freshly before each experiment and were stored on ice in the dark.

External standards were obtained by taking samples (0.4 ml) of ATP solutions (0 to 6.0  $\mu$ M, in Medium B) and quenching similarly. Internal standards for ATP formation were obtained by assaying each sample in the presence of four different concentrations of ATP (0 to 20 mM). Both internal and external standards were necessary to account for any medium-induced quenching of the luciferin-luciferase assay. Further controls were run using either the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (5  $\mu$ M) or the respiratory inhibitor, KCN (3 mM). The procedure for analyzing the amount of ATP formed has been described previously (13).

## Oxygen Consumption

Rates of oxygen consumption were measured using a Clark electrode in conjunction with a YSI model 53 oxygen monitor. For both nonstarved cells and starved cells used for efficiency determinations the sample chamber contained 3.0 ml of cells (at a density of  $3.5 \times 10^8$  to  $1.4 \times 10^9$  cells/ml) in N<sup>+</sup>C<sup>-</sup> plus chloramphenicol (100  $\mu$ g/ml). The cells were preincubated for 5 min to reach a given temperature after which the probe was inserted and an endogenous rate determined for 5 more min. Glucose was then added and the stimulated rate followed for at least 7 more min.

With the starved cells which had been used to determine ATP synthesis, the following procedure was used. Medium B (2.4 ml) alone or with several different cell concentrations were preincubated for 2.5 min. Fifty microliters of 1.0 M ascorbate, pH 7.0, was added, followed immediately by 50  $\mu$ l of 5 mM phenazine methosulfate, and the rate of oxygen consumption monitored. The rate of oxygen consumption without cells was taken as background. By plotting the

rate of oxygen consumption *versus* cell concentration, a straight line was obtained, the slope of which was independent of oxygen concentration. The rate of oxygen consumption was taken as the slope.

#### (Ca,Mg)-ATPase Assays

Membrane particles were prepared by sonication as described by Fisher *et al.* (14) and the ATPase activity measured spectrophotometrically (Assay D) as described by Hanson and Kennedy (15).

#### Measurement of Membrane Proton Permeability

**Apparatus**—The apparatus used in these experiments is adapted from that described by Mitchell and Moyle (16). It consists of a water-jacketed Lucite cylinder with a screw-on lid. Three openings were present in the lid. One allowed the introduction of a Radiometer combination pH electrode (GK2321C) into the cylinder. A second was for the inflow of water-saturated nitrogen gas, and the third was for both nitrogen outflow and the introduction of various material to the cylinder. The pH electrode was connected to an Orion 801A digital ionalyzer.

**Experimental Conditions**—The method described is adapted from that of West and Mitchell (17). Cells grown in glucose at 25° were harvested in midexponential phase, washed twice with  $\text{N}^+\text{C}^-$ , and Tris/EDTA treated if desired. The cells were then washed twice with 150 mM KCl, 3.0 mM glycylglycine, pH 7.0, and resuspended in 100 mM KCl, 1.5 mM glycylglycine, pH 7.0, at a cell density of  $3.3 \times 10^9$  cells/ml, and stored on ice. Cells (3.8 ml) were placed in the apparatus described above along with 0.2 ml of 1.0 M KSCN (pH 6.8) (a permeant anion) and 0.025 ml of carbonic anhydrase (4 mg/ml) to equilibrate the  $\text{CO}_2$ /carbonic acid system (18). The mixture was stirred vigorously, through the use of a small magnetic stirring bar, for 40 min under a steady stream of water-saturated nitrogen. The temperature was maintained at 25°. At this point, 5  $\mu\text{l}$  of 100 mM KCl, 50 mM KOH, which had been previously aspirated for 10 min to remove dissolved oxygen and had had nitrogen bubbled through it for at least 30 min, was added. The pH was monitored for 12 min. A second measurement was then made on the same cells. CCCP (freshly made, in 95% ethanol) was added to 12.5  $\mu\text{M}$  and the incubation continued for 10 min. Base was then added as described above and the ensuing pH recorded for 7 min.

**Treatment of Data**—The experiment measures the neutralization of KOH added at time zero to a suspension of anaerobic bacteria. The observed pH is plotted as a function of time and the rate of decay was exponential for the first 5 min, followed by a slow linear decrease in pH. This linear decrease was taken as background drift. The log of the difference between the observed hydrogen ion concentration and the extrapolated background hydrogen ion concentration is plotted against time. This treatment yields a straight line from which  $t_{1/2}$  is obtained.  $t_{1/2}$  corresponds to the time required for a sufficient number of protons to diffuse through the cell membrane to neutralize one-half of the added base. Cells treated with CCCP were used as a control to determine membrane proton permeability for a freely permeable membrane.

#### Protein Determinations

Protein was determined by the method of Lowry *et al.* (19) using bovine serum albumin as the standard.

#### Materials

[ $^3\text{H}$ ]Triphenylmethylphosphonium bromide was the generous gift of Dr. H. R. Kaback (Roche Institute of Molecular Biology, Nutley, N. J.). For some experiments we used [*methyl- $^3\text{H}$* ]TPMP $^+\text{Br}^-$  which was synthesized in our laboratory. Details of this synthesis will be described elsewhere. All other labeled compounds were obtained from New England Nuclear. CCCP and carbonic anhydrase were from Sigma. 2,4-Dinitrophenol was from Eastman. Cellulose acetate filters (EHW02500) were from Millipore. Bacto-Difco agar was used for succinate plates; Moorhead agar for all other plates.

#### RESULTS

**Mapping of *ecf<sup>ts</sup>* Mutation**—We have previously reported (3) that the *ecf<sup>ts</sup>* mutation of JSH4 maps between 61 and 67 min on the revised *Escherichia coli* chromosome map (20) and have now found that it is closely linked to the *metC* gene. When strain JSH210 (*metC*) was transduced to methionine prototrophy with P1 grown on JSH4 at 28°, 72% of the transductants

(145/200) were found to exhibit the properties of JSH4, namely temperature-sensitive growth on nonfermentable substrates (succinate (Suc), malate, fumarate, D-lactate), reduced growth rate and yield on glucose, and defective transport at 42°.

One of these transductants, MAL300, was used as a donor in the transductions described below. When JSH210 is transduced to *MetC* $^+$ , 89% (454/510) of the transductants were found to receive the *ts* growth pattern of MAL300 (succinate was tested). It was observed, however, that when some of the Suc $^+$  (at 42°) transductants were streaked on nutrient broth plates at 42° colonies exhibiting the phenotypic growth properties (Suc $^{ts}$ ) of MAL300 were present. This implies that some of the transductants which were scored as Suc $^+$  at 42° are actually revertants of transductants which had received the Suc $^{ts}$  phenotype. Therefore, the linkage of *ecf<sup>ts</sup>* to *metC* is probably greater than 89%. When a *tolC* mutant (strain A586) was transduced to *tolC* $^+$  (selected for the ability to grow in the presence of 0.08% deoxycholate (21)), 3.8% (6/160) of the transductants were found to receive the Suc $^{ts}$  property of MAL300. In all cases tested the Suc $^{ts}$  phenotype correlated to a temperature-sensitive transport phenotype. The *ecf<sup>ts</sup>* gene is thus cotransducible with both the *metC* and *tolC* genes, with *ecf<sup>ts</sup>* very close to *metC*. This places the *ecf<sup>ts</sup>* mutation at about 64 min on the *E. coli* chromosome map (20). It is worth noting that there are no known carrier protein genes which map in this area. Strains MAL300 and JSH210 were chosen for the studies described in this paper.

**Genetic Properties of MAL300 and Its Revertants**—We have shown previously that some revertants of JSH4 to Suc $^+$  at 42° appear to result from intergenic suppressions which reside in the *metC* gene and abolish cystathionase (*metC*) activity (3). Revertants of MAL300 were also examined. About  $1.5 \times 10^{-5}$  of the bacteria in a fully grown nutrient broth culture of MAL300 revert to Suc $^+$ . These revertants were isolated by their ability to grow on succinate ( $\pm$ methionine) at 42°, and can be divided into two classes of equal size. Class I revertants retain cystathionase activity whereas Class II revertants are methionine auxotrophs lacking this activity. Both classes transport normally at 42°.

Our previous work suggested that two mutations are necessary for the Suc $^{ts}$  and transport $^{ts}$  defects in JSH4 to occur. We examined this possibility for MAL300 by a series of transductions, always using Class II revertants as the recipient and selecting for *Met* $^+$  at 25°. For this purpose 10 independent Class II (*metC*) revertants were selected. It was reasoned that if the Class II reversion is due to a second site mutation in the *metC* gene (an intergenic suppression) then the original *ecf<sup>ts</sup>* mutation should still be present in these revertants. To determine whether this is the case each of these Class II revertants was transduced to *MetC* $^+$  with P1 phage grown on either JSH1 (wild type), JSH4 (mutant), or MAL300 (isogenic mutant). When JSH1 is the donor we found that 100% of the transductants arising from each of the 10 Class II revertants are Suc $^+$  at 42° (at least 50 transductants were examined for each revertant). Furthermore, of the transductants tested all were transport $^+$  at 42°. This result indicates that the introduction of a *metC* $^+$  allele (wild type) into a Class II revertant results in a wild type phenotype.<sup>2</sup> The results are quite different when P1

<sup>2</sup> This result differs from that reported previously (3); when Class II revertants of JSH4 were transduced to *met* $^+$  with P1 phage grown on wild type (JSH1) all of the transductants were Suc $^+$ , transport $^+$  at 42°. We attribute this difference to the difference in genetic backgrounds between JSH4 and MAL300. JSH4 was isolated utilizing nitrosoguanidine mutagenesis and could contain mutations outside of the *metC* region. MAL300 is derived directly, by transduction,



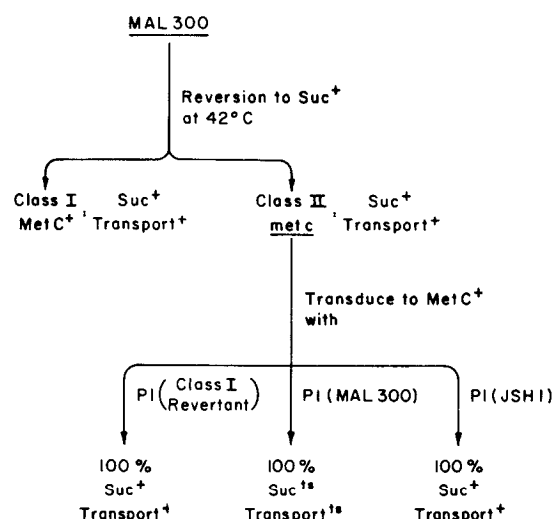


FIG. 1. Summary of the reversion pattern exhibited by MAL300. *P1(X)* refers to a phage lysate prepared on strain X, *Suc<sup>ts</sup>* to a temperature-sensitive growth phenotype on succinate.

grown on JSH4 (or MAL300) was used as the donor. In these cases 100% of all transductants (*MetC<sup>+</sup>*) arising from each of the 10 Class II revertants are *Suc<sup>+</sup>* and transport<sup>+</sup> at 42°. Since the linkage between *ecf<sup>ts</sup>* and *metC* is  $\geq 89\%$ , and the observed frequencies in these transductions is 100%, we tentatively conclude that the *metC* gene in MAL300, while retaining wild type levels of cystathionase activity, is different from the *metC* gene in JSH1 (wild type). If this interpretation is correct then 11% of the transductants arising from the transduction of Class II revertants by P1 grown on JSH1 should have an *ecf<sup>ts</sup> metC<sup>+</sup>* genotype (the other 89% should be *ecf<sup>+</sup> metC<sup>+</sup>*). Yet these 11% retained wild type phenotypic properties. Thus, a *metC<sup>+</sup>* gene can also suppress the *ecf<sup>ts</sup>* mutation (as can a *metC<sup>-</sup>* gene). It therefore appears as if two mutations are necessary for any phenotypic defects to be expressed: one in the *ecf<sup>ts</sup>* gene, and the other in the *metC* gene. We call the altered cystathionase in MAL300 *metC\**. This conclusion is strengthened when the result of the transduction of Class II revertants by P1 grown on Class I revertants is examined. In this case 100% of the transductants obtained acquired a wild type phenotype (*Suc<sup>+</sup>*, transport<sup>+</sup> 42°). This result indicates that the reversion event to form Class I revertants occurs within the *metC* gene, most probably *metC\** to *metC<sup>+</sup>*.

These transduction results are summarized in the scheme depicted in Fig. 1 and suggest that there are two mutations present in MAL300 (*metC\**, *ecf<sup>ts</sup>*) and that the presence of both mutations is necessary for any phenotypic defects to be observed.

Since the linkage between the *metC\** and *ecf<sup>ts</sup>* mutations is very close, alternative explanations may be considered. The first assumes that the mutations are in the *ecf* gene and that all reversions occur within this gene. If this model were correct, revertants of both classes would be intragenic revertants, *ecf<sup>ts</sup>* to *ecf<sup>+</sup>*, but with one difference. The reverted *ecf<sup>+</sup>* in Class II revertants of both classes would be intragenic revertants, *ecf<sup>ts</sup>*

from JSH210, which is a strain that had never been exposed to mutagens. Thus, all the defects associated with MAL300 and its revertants are due to genetic alterations within the *metC* area. We therefore believe the *Suc<sup>-</sup>* phenotype (at 42°) exhibited by the *met<sup>+</sup>* transductants in the cross of the Class II revertants of JSH4 with P1 grown on JSH1 is due to mutational alterations outside of the *metC* *ecf<sup>ts</sup>* region.

could occur in several ways. (a) The Class II reversion could alter the *ecf* gene product in such a way as to be inhibitory to or inactivate cystathionase. This model can only be tested when proteins from both mutant and wild type strains are isolated, purified, and their properties compared. (b) The Class II reversion abolishes the synthesis of cystathionase. For this to occur the *ecf* and *metC* genes would have to belong to the same operon such that the reversion event would create a nonsense mutation in the *ecf<sup>ts</sup>* gene that has a strong polar effect upon the translation of cystathionase. The possibility that the two genes are in the same operon is likely because an 89% co-transduction frequency implies a distance of only 2 to 3 genes as estimated according to the method of Wu (22). However, this model can be argued against by the following experiment. The introduction of any of five different suppressors (*supF*, *su<sub>3</sub><sup>+</sup>*; *supD*, *su<sub>1</sub><sup>+</sup>*; *supE*, *su<sub>2</sub><sup>+</sup>*; *sup-51*, *su<sub>6</sub><sup>+</sup>*; *supG*, *su<sub>5</sub><sup>+</sup>*) into a Class II revertant of an *ecf<sup>ts</sup> metC\** mutant did not restore growth in the absence of methionine, nor did it result in a *ts* growth pattern on succinate (data not shown). This indicates that the reversion event in Class II revertants is not suppressible by any of the above suppressors. In addition, antibody prepared against purified cystathionase cross-reacted with material in extracts of Class II revertants,<sup>3</sup> implying that the *metC* gene is transcribed and the mRNA translated in these revertants.

Another possible explanation is that the *ecf<sup>ts</sup>* and *metC\** mutations are really one mutation residing in the *metC* gene. This is a possibility since the observed 89% co-transduction frequency between the *ecf<sup>ts</sup>* and *metC* is a minimal figure. This model would require that the altered cystathionase is completely inhibitory to energy-coupling. Reversion to *metC* would eliminate such inhibition.

It is difficult at this time to distinguish between the one and two mutation hypotheses. All hypotheses require, however, interaction between cystathionase and the *ecf* gene product. For the purposes of the rest of this paper we shall use two mutations, *ecf<sup>ts</sup>* and *metC\**, and assume that both these mutations are necessary for any phenotypic defects to be observed.

**Dominance of *ecf<sup>ts</sup> metC\** Mutations**—The introduction of F'116, an episome which carries the *metC* region of the *E. coli* chromosome, into MAL351 (*metC\* ecf<sup>ts</sup> thyA*) and selection of *Thy<sup>+</sup>* merodiploids allows the dominance or recessiveness of the *metC\* ecf<sup>ts</sup>* alleles to be studied. When the selection was carried out at room temperature all the merodiploids obtained were found to exhibit the phenotypic properties of MAL300, namely, temperature-sensitive growth on succinate and *ts* transport properties. These results indicate a dominance of the *ecf<sup>ts</sup> metC\** mutations over their wild type alleles.

**Growth and Transport Properties of MAL300**—As stated above, MAL300 is unable to grow on nonfermentable substrates at 42°. MAL300 also exhibits a slower growth rate and lower growth yield on glucose or nutrient broth at the restrictive temperature than does JSH210. Fig. 2 shows typical growth utilizing glucose and succinate as the sole carbon sources in a temperature shift from 25 → 42°. In the absence of an added energy source the transport defect in MAL300 is easily observed after only a 2-min incubation at 42°. Typical data are presented in Fig. 3. The half-time of inactivation of transport at 42° is 1 min. Efflux of accumulated solute upon a temperature shift is also demonstrable in MAL300 (data not shown), indicating that the mutant loses the ability to retain solutes against a concentration gradient at the nonpermissive

<sup>3</sup> S. Kagan and J.-S. Hong, unpublished results.

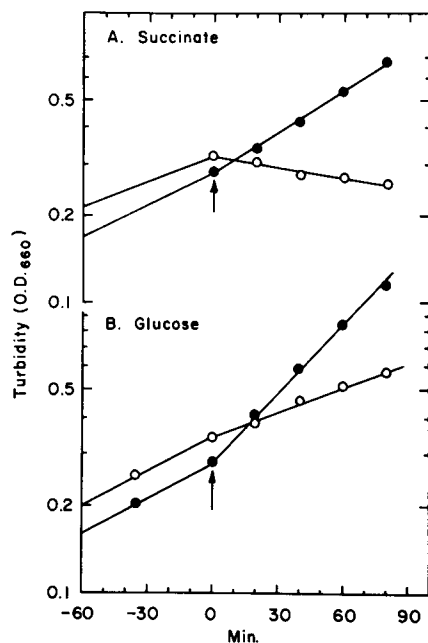


FIG. 2. Growth of MAL300 following temperature shift. Cells were growing for at least three generations at 25° before the temperature shift. Cells were grown in minimal salts medium E supplemented with either glucose or succinate as the sole carbon source. MAL300, ○—○; JSH210, ●—●. A, succinate as sole carbon source; B, glucose as sole carbon source.

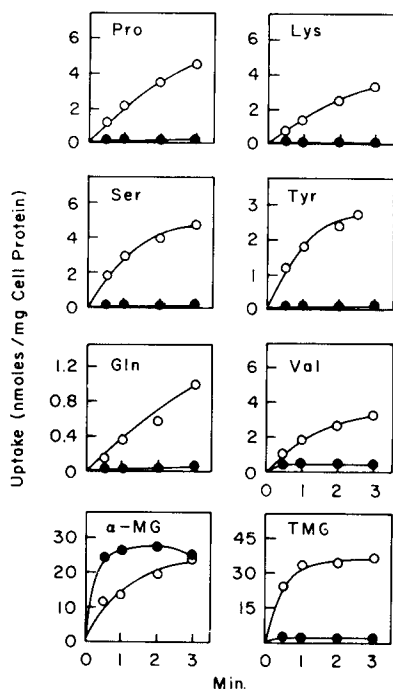


FIG. 3. Uptake of amino acids, TMG, and  $\alpha$ -methylglucose ( $\alpha$ -MG) by cells of MAL300. Cells were grown in minimal glucose medium at 25° and transport assayed as described under "Materials and Methods." Uptake measured at 25° (○—○); 42° (●—●).

temperature. The defect affects the transport of solutes belonging to both osmotic shock-sensitive and osmotic shock-resistant transport systems. Heat inactivation of transport is irreversible; prolonged cooling in ice (up to 24 h) after a 10-min incubation at 42° could not restore transport activity when assayed at 25°.

TABLE II

*Rates of orthonitrophenyl- $\beta$ -D-galactoside hydrolysis*

Cells were prepared and the assays carried out as described under "Materials and Methods."

Conditions	JSH210	MAL300
	nmol/min/mg cell protein	
24.5°	82	103
+TDG	17	20
+Toluene	1,000	1,070
42.5°	510	610
+TDG	180	220

*Absence of Generalized Membrane Permeability*—To ensure that the transport defect observed with the mutant is not due to a generalized membrane permeability we examined  $\alpha$ -methylglucoside transport. As shown in Fig. 3 the mutant transports  $\alpha$ -methylglucoside normally at both 25° and 42°. This indicates that the mutational lesion is specific, affecting only those transport systems that depend upon coupled energy.

To ensure that the transport defect is not due to an alteration of membrane carrier molecules themselves, we examined the functioning of the lactose carrier molecules (the M protein). It is well established that orthonitrophenyl- $\beta$ -D-galactoside, an artificial substrate of the *lac* system, is hydrolyzed slowly by intact cells, with the transport of orthonitrophenyl- $\beta$ -D-galactoside into the cell being the rate-determining step (23). It is therefore possible to measure the carrier-mediated diffusion of orthonitrophenyl- $\beta$ -D-galactoside into the cells, as well as the noncarrier-mediated diffusion (passive diffusion). The latter is accomplished by performing the assay in the presence of TDG, which is a competitive inhibitor of orthonitrophenyl- $\beta$ -D-galactoside for the M protein. The results of such measurements are summarized in Table II. It is evident that the rate of the noncarrier-mediated diffusion of orthonitrophenyl- $\beta$ -D-galactoside in MAL300 is similar to wild type at 42°. This shows that no gross membrane defects exist in the mutant at 42° which would allow a rapid flux of orthonitrophenyl- $\beta$ -D-galactoside across the membrane. To ensure that transport is indeed the rate-limiting step in these experiments, cells were treated with toluene. As shown in the table, toluenized cells hydrolyze orthonitrophenyl- $\beta$ -D-galactoside much more rapidly than nontoluenized cells owing to the destruction of the cell permeability barrier by toluene. It is also clear that the carrier-mediated diffusion of orthonitrophenyl- $\beta$ -D-galactoside is normal in the mutant at 42°. This result indicates that the M protein is not altered at the nonpermissive temperature, yet at that temperature, the active transport of TMG is totally defective (Fig. 3). We therefore conclude that the *metC\** *ecf<sup>ts</sup>* mutations do not inactivate the carrier proteins.

*ATPase Activity*—The (Ca,Mg)-ATPase activity in MAL300 was examined using membrane particles which had been prepared by sonication. As can be seen in Table III, the enzyme was capable of hydrolyzing ATP at both 25° and 42°, indicating that its activity was normal.

*Stimulation of Transport by Glucose and Other Energy Sources*—Since the transport defect in MAL300 is not due to an alteration of membrane permeability, inactive carrier molecules, a failure in the respiratory chain (3), or a deficiency in (Ca,Mg)-ATPase activity, we considered the possibility that the mutation affected the process of coupling energy to active transport. Support for this contention came from the study of the effect of exogenous energy sources upon transport in the

mutant. As shown in Fig. 4A transport is observed upon addition of 20 mM glucose to the cells (at the nonpermissive temperature). However, the initial rate of stimulation, as well as the steady state accumulation achieved, is less than that observed with the wild type (JSH210) in the absence of exogenous energy. Similarly, transport is also observed in the presence of succinate, D-lactate, or ascorbate-phenazine methosulfate (an artificial electron donor) (Fig. 4B) although the extent of stimulation is smaller than that observed with glucose.

When glucose is added at low concentrations (below 2 mM), transient uptake is observed (Fig. 4C). Within the first 30 s of glucose addition, the cells are capable of accumulating solutes, yet within the next 2 to 3 min the accumulated substrate has completely effluxed from the cells. The examination of the initial rates of transport as a function of time after glucose addition (Fig. 5) indicates clearly that during the efflux period the cells can no longer transport solute. This shows that when efflux occurs the cells have lost both the ability to actively transport and to retain accumulated solutes. If a second dose of glucose is added to the cells after the efflux period, a restimulation of transport occurs, as shown in Fig. 6. This experiment rules out the possibility that a long-lived glucose metabolite is interfering with the energy-coupling process.

There are two routes of membrane energization in *E. coli*. The first is derived from respiratory energy; the second from ATP hydrolysis catalyzed by the membrane-bound (Ca,Mg)-ATPase. We investigated each pathway in MAL300 by examining the glucose effect in the presence of cyanide, which

blocks the respiratory pathway. As is shown in Fig. 7, the addition of cyanide to the cells completely inhibits the glucose effect for proline uptake. Similar data (not shown) were obtained for serine. This indicates that the mutant cannot promote uptake of solute at the nonpermissive temperature via the ATPase pathway. All of the uptake, therefore, must be due to the respiratory pathway of membrane energization.

#### Reduced Ability in Maintaining Transmembrane Poten-

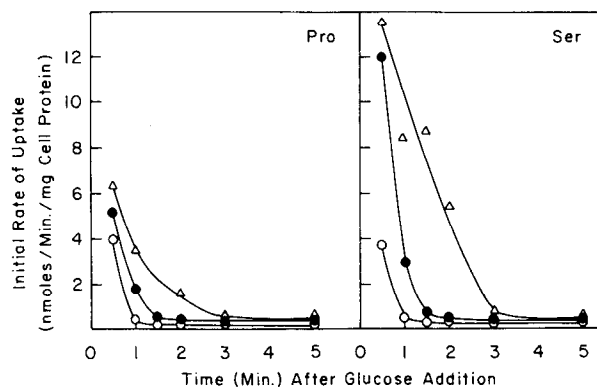


Fig. 5. Initial rate of amino acid uptake by MAL300 at 38.5°. Glucose-grown MAL300 cells were prepared for transport and preincubated for 5 min at 38.5°. Glucose was then added and at various times after glucose addition  $^{14}\text{C}$ -labeled amino acid was added and the uptake measured for 15 s. Symbols: +0.1 mM glucose ( $\circ$ — $\circ$ ); +0.2 mM glucose ( $\bullet$ — $\bullet$ ); +0.4 mM glucose ( $\Delta$ — $\Delta$ ).

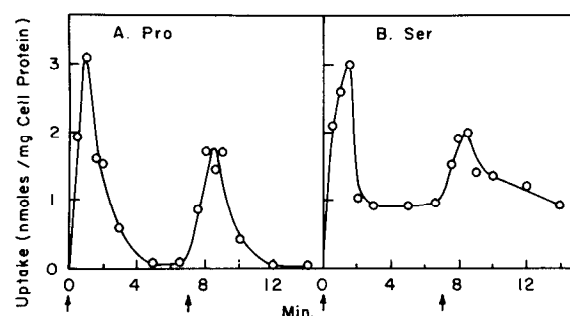


Fig. 6. Double glucose effect. Glucose-grown MAL300 was prepared for transport and preincubated for 2 min at 42°.  $^{14}\text{C}$ -labeled substrate was added and 30 s later, at  $t = 0$ , glucose was added at 0.4 mM. Glucose was added again at  $t = 7$ , as indicated by the arrow.

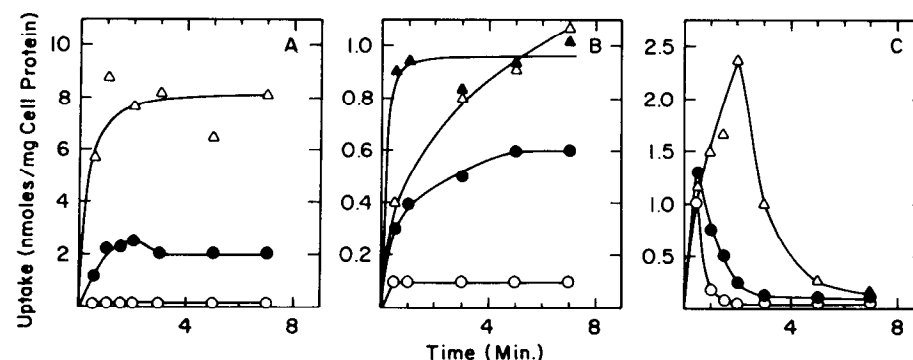


Fig. 4. Effects of exogenous energy sources upon transport in MAL300 under nonpermissive conditions. Cells were grown in either minimal glucose (A, C) or minimal succinate (B) medium at 25° and prepared for transport. To test the effect of exogenous energy sources [ $^{14}\text{C}$ ]proline was first added to cells followed 30 s later by the energy source. All uptake was measured at 42° after a 2-min preincubation. Uptake was measured as a function of time following energy addi-

tion. Symbols: A, JSH210 (wild type), no glucose ( $\Delta$ — $\Delta$ ); MAL300, no glucose ( $\circ$ — $\circ$ ); MAL300 + 20 mM glucose ( $\bullet$ — $\bullet$ ). B, MAL300, endogenous ( $\circ$ — $\circ$ ); +20 mM succinate ( $\bullet$ — $\bullet$ ); +20 mM D-lactate ( $\Delta$ — $\Delta$ ); +20 mM ascorbate, 0.2 mM phenazine methosulfate ( $\blacktriangle$ — $\blacktriangle$ ). C, MAL300 + 0.04 mM glucose ( $\circ$ — $\circ$ ); +0.12 mM glucose ( $\bullet$ — $\bullet$ ); +0.4 mM glucose ( $\Delta$ — $\Delta$ ).

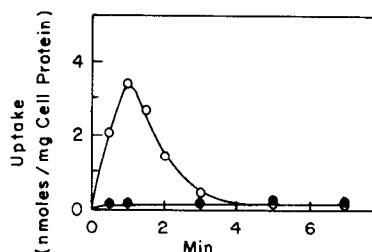


FIG. 7. Effect of cyanide upon transport in MAL300 at 42°. Glucose-grown MAL300 was prepared and assayed for transport as described in the legend to Fig. 4. Cyanide-treated cells were prepared by adding KCN to 3 mM and incubating at 25° for 5 min. The cells were stored on ice until assayed. Symbols: nontreated cells + 0.4 mM glucose (○—○); cyanide-treated cells + 0.4 mM glucose (●—●).

*tial*—Of prime importance in the process of membrane energization is the establishment of a transmembrane potential, interior negative, via proton translocation (9, 24–26). We examined the ability of MAL300 to generate and maintain a membrane potential under nonpermissive conditions. As an indicator of the potential we used triphenylmethylphosphonium ion (TPMP<sup>+</sup>), a lipid-soluble cation. The use of this ion as an indicator of the membrane potential has been well established (9). At the nonpermissive temperature, in the absence of glucose, only 5 to 10% of the wild type level of TPMP<sup>+</sup> was accumulated by the cells (Fig. 8A). In the presence of low concentrations of glucose, spikes of TPMP<sup>+</sup> uptake were observed, indicating a transient establishment of a membrane potential. For all glucose concentrations examined, the kinetics of TPMP<sup>+</sup> accumulation (Fig. 8B) paralleled that of amino acid uptake. This result indicates that the inability to transport is most probably due to a loss of the energized membrane state, as measured by a loss of membrane potential.<sup>4</sup>

It was possible to estimate the efficiency in generating and maintaining a membrane potential in the mutant relative to the wild type. The wild type strain utilized, MAL310 (JSH210 with an *uncA* mutation added) was chosen because it could not energize the membrane via the ATPase pathway. In this manner both the mutant (MAL300) and wild type (with respect to *ecf*) cannot generate a membrane potential via the hydrolysis of ATP. Furthermore, we starved MAL310 with dinitrophenol (11) to deplete endogenous energy sources such that in the absence of glucose both the mutant and wild type do not transport appreciably at 43°. Fig. 9 shows the uptake of proline and TPMP<sup>+</sup> at 25° and 42° by the mutant and wild type (MAL310) upon addition of glucose. It is clear that at both temperatures glucose, at a concentration as low as 0.4 mM, can stimulate both membrane potential generation and active transport in starved MAL310, as well as allow the cells to maintain the potential for at least 12 min. In contrast, the addition of glucose to nonstarved MAL300, at a concentration as high as 1.2 mM, resulted in only transient accumulation of

both proline and TPMP<sup>+</sup>. Thus, MAL300 cannot adequately couple energy to the maintenance of a membrane potential even when the energy available is 3 times greater than that available for wild type, which can maintain its potential.

*Reduced Efficiency in Energy Coupling*—The efficiency of energy coupling in starved MAL310 and MAL312 (MAL300 with an *uncA* mutation present) was also measured. The efficiency is defined as the initial rate of transport (stimulated by glucose) divided by the rate of oxygen consumption (also stimulated by glucose). We used starved cells in order to reduce the rate of uptake due to endogenous energy sources. The data, expressed as the ratio of efficiencies, 38.5°:25.5°, is summarized in Table IV. Clearly, the ratios are very low for MAL312 as compared to MAL310 reflecting the fact that under nonpermissive conditions transport is only slightly stimulated although oxygen is continuously consumed (these experiments were done after a 5-min preincubation with glucose in order to bolster energy levels within the cell<sup>5</sup>). These results indicate that the efficiency with which respiratory energy is coupled to active transport in the mutant is greatly diminished. It therefore appears that the defect in the mutant is due to both a reduction in the efficiency with which energy is coupled to generation of a membrane potential and an inability to maintain a membrane potential under conditions in which energy supplies are low.

*Defective Oxidative Phosphorylation*—The ability of the mutant to perform oxidative phosphorylation, which is dependent upon a membrane potential, was also investigated. We used intact cells that had been depleted of ATP by starvation in the presence of dinitrophenol and then assayed for the amount of ATP synthesized after the addition of the artificial electron donor, ascorbate-phenazine methosulfate. The results are shown in Fig. 10. Preheating wild type cells at 43° did not reduce the net amount of ATP formed as compared to nonheated cells. Preheating the mutant cells, however, reduced the net amount of ATP formed, as compared to nonheated cells, by 100%. Both CCCP and KCN completely inhibit ATP synthesis in both JSH210 and MAL300 (data not shown), indicating that the ATP was being formed through oxidative phosphorylation.

To show that the electron transfer chain was functioning in the mutant, oxygen consumption was followed before and after the addition of ascorbate-phenazine methosulfate. In the absence of ascorbate-phenazine methosulfate no detectable oxygen consumption was observed in either strain (data not shown). As seen in Table V, on addition of ascorbate-phenazine methosulfate oxygen consumption is initiated. With wild type (JSH210) preheating cells at 43° results in an increase of 42% in the rate of oxygen consumption. Similar results were obtained with the mutant. However, the initial nonheated rate in the mutant is 42% higher than that of the wild type. Thus, electron transfer is normal in starved mutant cells, under both permissive and nonpermissive conditions.

*Membrane Permeability to Protons*—In order to determine whether the markedly reduced efficiency in energy coupling in MAL300 at 42° was due to an increased membrane permeability to protons, this parameter was measured. An increased proton permeability would explain the loss of the membrane potential. Membrane proton permeability was examined in both Tris/EDTA-treated and non-Tris/EDTA-treated cells. The Tris/EDTA-treated cells were prepared in exactly the same manner as for TPMP<sup>+</sup> uptake measurements. The re-

<sup>4</sup> Padan *et al.* (25) have shown that *Escherichia coli* invariably maintains an internal pH of 7.5; Ramos *et al.* (26) have also demonstrated this for *E. coli* membrane vesicles. As all of the experiments reported in this paper were performed at pH 7.1 to 7.2, the contribution of  $\Delta\text{pH}$  to the total proton motive force is rather small. Ramos and Kaback (manuscripts submitted) have shown that at pH 7.5 all transport systems (in vesicles) are operative and driven solely by a membrane potential, even those substrates which, at pH 5.5, are solely driven by  $\Delta\text{pH}$ . We shall, therefore, consider that under our conditions the  $\Delta\Psi$  is the major part of the proton motive force. We have also been able to demonstrate (unpublished results) that at pH 6.4 a  $\Delta\text{pH}$  of the correct orientation exists in the mutant under nonpermissive conditions; this eliminates the possibility of MAL300 establishing an inverted  $\Delta\text{pH}$ .

<sup>5</sup> A 5-min preincubation with glucose is necessary in order to obtain a maximal stimulation of transport.



FIG. 8. Uptake of [ $^3\text{H}$ ]TPMP $^+$  by intact cells. Glucose-grown cells were prepared and assayed as described under "Materials and Methods." A, uptake of TPMP $^+$  at 42° in the absence of an external energy source. The concentration of TPMP $^+$  was 0.4 mM (28.5 mCi/mmol). Symbols: MAL300 (○—○); JSH210 (●—●); MAL301 (a Class II revertant) (△—△). B, uptake of TPMP $^+$  in MAL300 at 42° in the presence of various amounts of glucose. Symbols: 0.2 mM glucose, (○—○); 0.4 mM glucose (●—●).

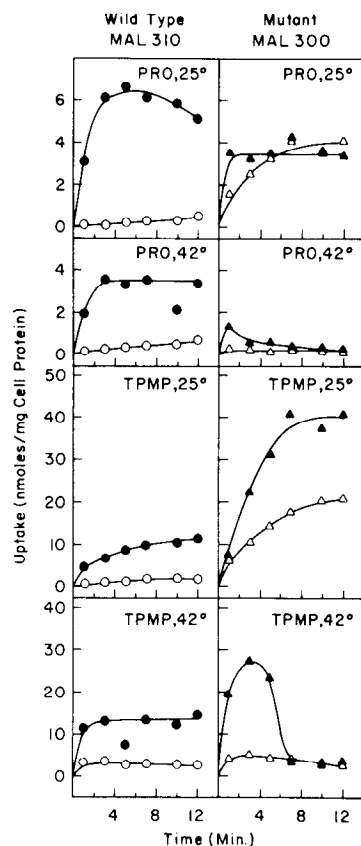
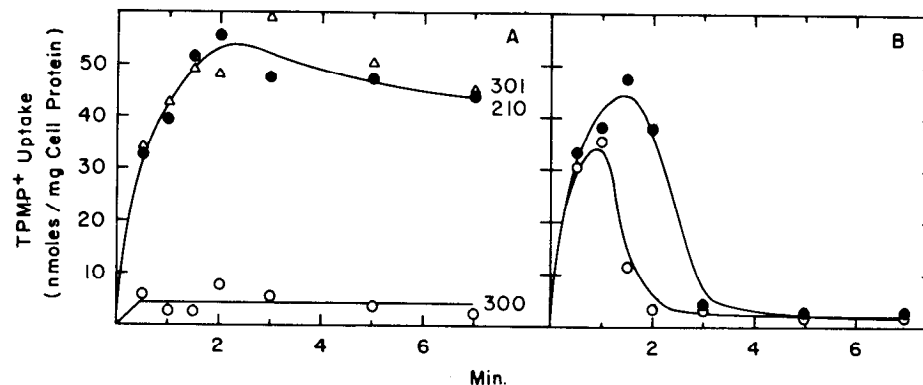


FIG. 9. Effects of exogenous glucose on starved MAL310 and non-starved MAL300. Glucose-grown MAL310 (*ecf<sup>+</sup> uncA*) was starved (Method I), Tris/EDTA-treated, and prepared for transport as described under "Materials and Methods." MAL300 was prepared similarly except not subjected to starvation. Cells were preincubated for 5 min at either 25° or 42° at which time either 0.4 mM glucose (MAL310) or 1.2 mM glucose (MAL300) was added. For MAL300 the [ $^{14}\text{C}$ ]proline or [ $^3\text{H}$ ]TPMP (0.4 mM, 80 mCi/mmol) was added immediately after glucose addition; for starved MAL310 a 5-min incubation preceded the addition of radioactive substrate. Symbols: MAL310, endogenous (○—○); MAL310 + 0.4 mM glucose (●—●); MAL300, endogenous (△—△); MAL300 + 1.2 mM glucose (▲—▲).

sults are summarized in Table VI. As shown, there was approximately a 20% increase in membrane proton permeability observed with wild type cells (JSH210) after heat or Tris/EDTA treatment. A larger increase (40%) in the proton permeability was observed with MAL300 cells following either of the treatments. It is noteworthy that once the membrane proton permeability in MAL300 had been altered by Tris/EDTA treat-

TABLE IV  
Efficiency of energy utilization

Glucose-grown cells were processed and starved as described under "Materials and Methods." Experiments at 38° were done with cells which had been preheated for 10 min at 42° and cooled in ice. For uptake experiments the cells were preincubated for 5 min and then another 5 min with the energy source before the initial rate of uptake (determined at 20 s) was measured. Oxygen consumption was monitored as described under "Materials and Methods." In all cases oxygen consumption continued for at least 8 min after glucose addition.

	Initial rate of amino acid up-take		Rate of oxygen consumption	Efficiency ( $\times 10^{-2}$ )	
	Pro	Ser		Pro	Ser
	nmol/min/mg cell protein		ng atoms/min/mg cell protein	nmol/ng atom $\text{O}_2$	
25°					
MAL310					
+1.2 mM Glu	9.36	8.13	89.3	10.5	9.1
+20 mM Glu	9.22	8.97	85.0	10.8	10.5
MAL312					
+1.2 mM Glu	3.63	3.12	79.8	4.6	3.9
+20 mM Glu	2.72	2.35	78.2	3.5	3.0
38°					
MAL310					
+1.2 mM Glu	4.64	4.09	31.6	14.6 (1.40) <sup>a</sup>	12.9 (1.42)
+20 mM Glu	2.75	2.64	26.3	10.5 (0.97)	10.0 (0.95)
MAL312					
+1.2 mM Glu	0.12	0.28	53.3	0.2 (0.05)	0.5 (0.14)
+20 mM Glu	0.08	0.18	30.0	0.3 (0.08)	0.6 (0.20)

<sup>a</sup> All numbers in parentheses are the efficiency ratios, 38°/25°.

ment subsequent heat treatment did not affect this parameter further. In all cases the addition of the uncoupler CCCP (12.5  $\mu\text{M}$ ) to the cells resulted in an extremely rapid equilibration of protons across the membrane.

The total loss of transport ability in the heated but non-Tris/EDTA-treated MAL300 cells cannot be due to the observed increase in membrane proton permeability, since the proton permeability of these cells is identical with that observed with the nonheated Tris/EDTA-treated MAL300 cells, and transport activity is observed in the latter case. In addition, the Tris/EDTA-treated MAL300 cells are capable of generating a membrane potential whereas the heated Tris/EDTA-treated cells are not (Fig. 7B), although both have the same membrane permeability to protons.

*Inability of N,N'-Dicyclohexylcarbodiimide to Restore Transport in MAL300*—*N,N'*-Dicyclohexylcarbodiimide, normally an inhibitor of the (Ca,Mg)-ATPase, has been shown to be able to restore transport ability to two *uncA* strains of *E. coli* that are defective in transport. The evidence presented (8, 27) suggested that *N,N'*-dicyclohexylcarbodiimide acts by sealing a "proton hole" which was present in the mutants. In



these mutants, the increased membrane permeability to protons was apparently short-circuiting the membrane potential, resulting in a lowering of transport capability. The possible effect of *N,N'*-dicyclohexylcarbodiimide on transport was ex-

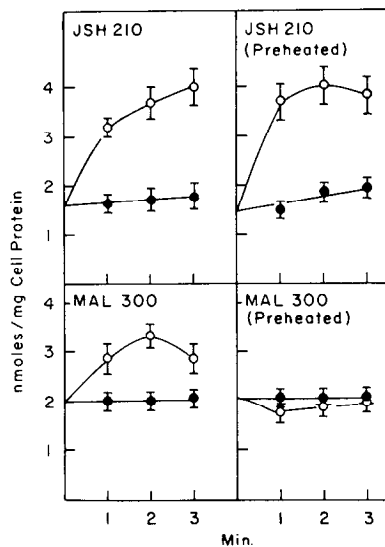


FIG. 10. ATP synthesis by starved cells. Starved cells (JSH210, MAL300) were prepared as described under "Materials and Methods." Ascorbate-phenazine methosulfate (20 mM, 0.1 mM) was added at  $t = 0$  and the ATP synthesized measured as previously described (5). All assays were carried out at 26°. Symbols: with ascorbate-phenazine methosulfate (○—○); without ascorbate-phenazine methosulfate (●—●).

TABLE V

Ascorbate-phenazine methosulfate stimulated oxygen consumption in starved cells

Cells were starved and oxygen consumption determined as described under "Materials and Methods." All assays were done at 26°. Heated cells were prepared by placing the cells at 42° for 5 min, then storing in ice until use. The background rates of the nonenzymatic oxidation of reduced phenazine methosulfate have been subtracted from the stimulated rate, and the difference is reported below.

	Oxygen uptake	
	JSH210 (wild type)	MAL300 (mutant)
	ng atoms $O_2$ /min/mg cell protein	
Nonheated	88	123
Heated	125	195

TABLE VI

Membrane proton permeability

Cells were harvested and Tris/EDTA treated as described in the text. Proton permeability was assayed and the data analyzed as described under "Materials and Methods." Heated cells were prepared by placing cells in a 42° water bath for 7 min, then cooling on ice.

Treatment	$t_{1/2}$ (s) of strain	
	JSH210 (wild type)	MAL300 (mutant)
None	131	70
+CCCP	7	7.5
Heated	106	36
+CCCP	7	6
Tris/EDTA	105	41
+CCCP	5	6
Tris/EDTA, heated	90	37
+CCCP	6	7

amined in MAL300 following the procedure used by Rosen (8). The results in Fig. 11 show that *N,N'*-dicyclohexylcarbodiimide is unable to restore transport ability (proline) to MAL300 at 43°, nor is there any stimulation observed at 25°. In addition, treatment of MAL300 membrane vesicles with *N,N'*-dicyclohexylcarbodiimide (40  $\mu$ M, 10 min, 25°) did not restore transport ability (serine) at either temperature (data not shown).

These results indicate that the transport defects caused by the *ecf<sup>ts</sup>* mutation differ from the defect observed in the *uncA* strains DL54 (27) and NR70 (8).

**Sensitivity of Transport Ability to High pH**—When MAL300 is treated with buffers at pH 8.0 or higher, the transport ability at the permissive temperature (25°) is found to be greatly reduced. The data obtained utilizing Tris/HCl are shown in Fig. 12, A and B. Similar results were obtained with glycylglycine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. In all cases,  $\alpha$ -methylglucoside transport is unaffected (Fig. 12D) and transport at 43° remains defective. The exposure to high pH ( $\geq 8.0$ ) consists of washing cells twice with the buffer prior to a 7-min incubation at 24°. The cells are then washed free of the high pH buffer and placed in  $N^+C^-$  (pH 7.0). Treating cells with Tris at low pH was found to have a general lowering (20 to 40%) of transport ability relative to the control ( $N^+C^-$  treated) for both wild type (JSH210) and mutant (MAL300) cells.

These results indicate that a brief exposure to mild alkaline conditions specifically and irreversibly reduces the ability of MAL300 to transport substrates which require a functional *ecf* gene product. This suggests that the temperature-induced alterations in *ecf* function can also be brought about at low temperature by exposure to base.

**Transport in Isolated Membrane Vesicles**—Vesicles were prepared from glucose-grown cells according to Kaback (28) and transport was assayed using 20 mM D-lactate as the energy source. As shown in Fig. 13, both the initial rate of uptake as well as the steady state level of accumulation for both proline and serine in vesicles prepared from MAL300 are markedly impaired at both 25° and 43°.

In light of the finding that Tris/HCl at pH 8.0 affects energy coupling in MAL300 (Fig. 12) and the fact that the preparation of vesicles by the lysozyme/EDTA method makes use of Tris/

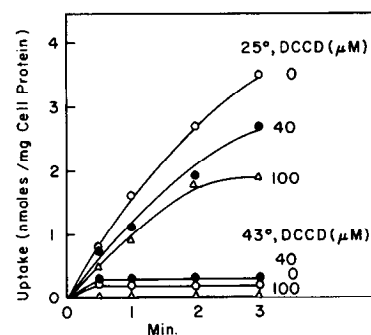


FIG. 11. Effect of *N,N'*-dicyclohexylcarbodiimide (DCCD) upon proline transport in MAL300. Cells were grown in minimal glucose medium and treated with Tris and EDTA as described by Rosen (8). Cells were then diluted into carbon-free salts medium to a cell density of  $2.1 \times 10^9$  cells/ml. *N,N'*-Dicyclohexylcarbodiimide was added as a solution in absolute ethanol and incubated with the cells for 5 min at 25°. Control cells received ethanol alone (0.875%). Symbols: control cells (○—○); 40  $\mu$ M *N,N'*-dicyclohexylcarbodiimide-treated cells (●—●); 100  $\mu$ M *N,N'*-dicyclohexylcarbodiimide-treated cells (△—△).

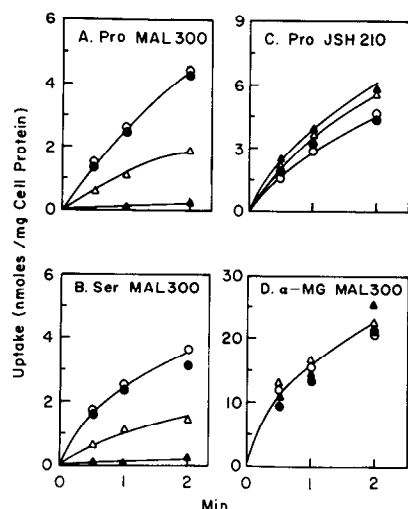


FIG. 12. Effect of Tris-HCl on MAL300. Cells were grown in minimal glucose medium at 25° and harvested in mid-log phase. The culture was divided into 4 parts and washed twice with 0.1 M Tris/HCl, at pH 7.2, 7.7, 8.1, or 8.5. The cells were then resuspended in the appropriate Tris buffer to a cell density of  $1 \times 10^9$  cells/ml. The cells were incubated for 7 min at 24°, spun down, washed once, and resuspended in  $N^+C^-$  to a cell density of  $2.1 \times 10^9$  cells/ml. Transport was assayed as previously described (3). Symbols: cells treated with 0.1 M Tris, pH 7.2 (○—○); pH 7.7 (●—●); pH 8.1 (△—△); pH 8.5 (▲—▲),  $\alpha$ -MG,  $\alpha$ -methylglucoside.

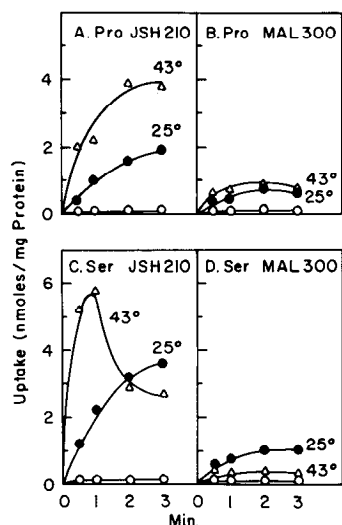


FIG. 13. Uptake of proline and serine by membrane vesicles. Vesicles were prepared utilizing the Tris/EDTA lysozyme procedure of spheroplast formation and assayed as described by Kaback (26). Symbols: No D-lactate added (at either 25° or 42°) (○—○); +20 mM D-lactate, 25° (●—●); +20 mM D-lactate, 42° (△—△).

HCl at pH 8.0, we also prepared vesicles by the penassay broth/penicillin method (28) to avoid exposure to high pH. The transport results with these vesicles, however, are similar to those prepared by the lysozyme/EDTA method (data not shown). Regardless of the method used in the vesicle preparation, the mutant vesicles take up oxygen (in the presence of 20 mM D-lactate) at a similar rate as the vesicles from the wild type. These results indicate that the defect observed in MAL300 vesicles is not due to a defect in respiration, but rather to the energy-coupling process itself.

*Normal AdoMet Synthetase in Mutant*—The gene for

TABLE VII

## Activity of AdoMet synthetase

The assays were carried out on sonicated cell-free extracts as described by Holloway *et al.* (10) except that the assays were allowed to proceed for 45 min before the reactions were terminated.

Assay temperature	JSH1	MAL300
	nmol AdoMet produced/mg protein/45 min	
25°	35	34
43°	123	108

AdoMet synthetase (*metK*) maps very close to the *metC* gene. Cox *et al.* (29) have shown that mutants defective in AdoMet synthetase have reduced transport capabilities relative to wild type. AdoMet also appears to be necessary for chemotaxis (30–32) and conceivably might also play a role in transport. In order to determine whether the transport defect exhibited by our mutant is caused by a temperature-sensitive AdoMet synthetase, assays were carried out on sonicated cell-free extracts. The results shown in Table VII show that this enzyme is normal at 42°. Similar results were obtained when toluenized cells were used (data not shown). Thus, the transport defect of our mutant cannot be caused by a defective AdoMet synthetase.

## DISCUSSION

In the absence of an exogenous energy source, the *metC<sup>\*</sup> ecf<sup>ts</sup>* mutant is completely incapable of transporting amino acids and certain sugars at 43°. Under these conditions a transmembrane potential, as measured by TPMP<sup>+</sup> uptake, is almost nonexistent. Addition of glucose to these cells allows generation of a membrane potential, as well as a stimulation of amino acid transport. For all glucose concentrations examined the extent of stimulation of amino acid transport always paralleled the extent of TPMP<sup>+</sup> accumulated. This is indicative of the importance of a membrane potential to active transport and suggests that the defect in MAL300 lies in the generation and maintenance of the membrane potential. Through comparison with starved wild type cells (which contained an *uncA* mutation in order that energization via the ATPase pathway could not occur) we demonstrated that the ability to maintain a membrane potential, in the presence of exogenous energy, is defective in the mutant (Fig. 9). In addition, the mutant was also shown to be inefficient in the coupling of energy to the generation of a membrane potential (Table IV). This was shown to be true for both the ATPase and respiratory routes of membrane energization.

The mechanism whereby the *metC<sup>\*</sup> ecf<sup>ts</sup>* gene products are responsible for the loss of a membrane potential in MAL300 has not yet been elucidated, nor have these proteins been isolated and characterized. However, it is clear that these proteins play an important role in energy transduction. The transport defect associated with the *metC<sup>\*</sup> ecf<sup>ts</sup>* mutations cannot be attributed to inactive carrier molecules (Table II) or an increased membrane permeability to protons, as a "proton hole" of sufficient magnitude to inactivate transport was not found in the mutant (Table VI). We have previously shown, however, that potassium ion and various glycolytic intermediates are excreted by the mutant at the nonpermissive temperature (5). The possibility exists that at the nonpermissive temperature ion fluxes may cause the observed dissipation of the membrane potential in the mutant.

Considering the effect of glucose on transport, it is possible that glucose metabolism, rather than providing energy, may

simply be providing a needed metabolite which disappears in the absence of glucose. This metabolite may be necessary for transport or may temporarily repair a gross membrane alteration which occurs at 42°. These possibilities are highly improbable. First, ascorbate-phenazine methosulfate, an artificial electron donor which cannot supply carbon atoms for metabolic use, is able to stimulate transport. Second, it has been well established that no glycolytic metabolite of glucose origin is required for active transport in the membrane vesicle system of Kaback (34). Alternatively, glucose metabolism may produce an inhibitor of active transport in the *ecf* mutant. If this is the case, then the inhibitor being produced must disappear rather quickly upon the addition of glucose, as double glucose effects (Fig. 6) have been demonstrated. In addition, the supplying of 0.4 mM glucose to MAL300 cells, which had already been stimulated for transport (at 42°) by the addition of ascorbate-phenazine methosulfate, did not inhibit uptake,<sup>6</sup> implying that an inhibitor of active transport is not formed. If the order of addition is reversed, that is, ascorbate-phenazine methosulfate is added to glucose-stimulated cells, the same results are obtained. Therefore, it is unlikely that glucose metabolites are involved in the transport defects observed in MAL300.

In an earlier report we concluded that membrane vesicles prepared from an *ecf<sup>ts</sup> metC\** mutant (JSH4) were normal in transport activity (3). This paper shows, however, that this conclusion was in error. This was due to an unusually low activity obtained for the wild type (JSH1) to which the activity of the mutant was compared. The data presented in this paper show clearly that membrane vesicles prepared from MAL300 are defective in transport at both 25° and 43° and suggests that the *ecf<sup>ts</sup>* gene product resides in the membrane. The finding that exposure to high pH specifically inactivates the *ecf<sup>ts</sup>* function at the permissive temperature also suggests that the *ecf<sup>ts</sup>* protein resides in the membrane. It must be mentioned, however, that the exact mechanism of the inactivation is not yet known and may reflect some other membrane-related phenomena, such as a base-catalyzed alteration of the membrane rendering the membrane permeable to protons (33), or some other nonmembrane-related effect of which we have, at present, no knowledge.

Our genetic study suggests that two mutations are probably involved in the mutant. This conclusion rests solely on the frequency (89%) with which the phenotype of MAL300 is transduced into JSH210 (*metC*). It is possible that this frequency is erroneous, and is only a minimal figure, despite repeated transductions with freshly prepared phage lysates, all of which gave a co-transduction frequency to 89%. If the *ecf<sup>ts</sup>* mutation is much more closely linked to *metC* the two mutation hypothesis may not be warranted, and one mutation residing in the *metC* gene may be sufficient to explain the data. In view of this possibility our conclusion should be considered tentative until mutational alterations can be demonstrated in isolated protein molecules.

Despite the uncertainty about the number of mutations involved, the phenotypic suppression observed in *metC* (Class II) revertants indicates an interesting involvement of cystathionase in energy coupling. The fact that *metC* is the only auxotrophic revertant found among all revertants, together with the finding that the introduction of *metB* or *metE* mutations into *ecf<sup>ts</sup> metC\** strains does not restore either the ability

to transport or to grow at 42°<sup>7</sup> demonstrate that the cystathionase involvement in energy coupling is indeed specific.

The map position of the *ecf* mutation makes it distinct from all known *unc* mutations, and the normal ATPase activity seen in membrane particles prepared from the mutant make it unlikely that the ECF protein is a part of the (Ca,Mg)-ATPase. Our data, however, do not rule out the possibility that the *metC\* ecf<sup>ts</sup>* gene products are involved in the synthetic aspect of the ATPase. In fact, the finding that both oxidative phosphorylation and the ATPase route of membrane energization are defective in the mutant suggests a likely physical interaction between the ATPase and the *ecf<sup>ts</sup> metC\** gene products *in vivo*.

Our mutant is similar to the one recently reported by Plate (35) with respect to a defect in active transport, the presence of (Ca,Mg)-ATPase activity, and the lack of a membrane proton "hole." This mutant was isolated based on resistance to colicin K. Although not isolated on the same basis our mutant exhibits many of the cellular alterations associated with colicin K action (5), and is also resistant to colicin K.<sup>8</sup> It remains to be determined, however, whether Plate's mutant is another *ecf* mutant.

**Acknowledgment**—We wish to thank Dr. H. R. Kaback for his gift of [<sup>3</sup>H]TPMP-Br<sup>-</sup>.

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<sup>6</sup> M. Lieberman and J.-S. Hong, unpublished results.

<sup>7</sup> M. Lieberman, unpublished results.

<sup>8</sup> J.-S. Hong and D. L. Haggerty, manuscript submitted.

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**Characterization of Escherichia coli mutant incapable of maintaining a transmembrane potential. MetC ecfts mutations.**

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*J. Biol. Chem.* 1977, 252:4056-4067.

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## Additions and Corrections

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Vol. 252 (1977) 4056-4067

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*Michael A. Lieberman, Marcia Simon, and Jen-Shiang Hong*

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The line should read

revertants must have an effect on cystathionase activity. This

Vol. 252 (1977) 4648-4654

**Properties and subunit structure of aspartokinase II from *Bacillus subtilis* VB217.**

*Don Moir and Henry Paulus*

Page 4649, Left hand column, paragraph headed *Bacterial Strain and Growth Conditions*

The twelfth line of this paragraph should say L-phenylalanine, 49.5 mg; and L-tryptophan, 61.2 mg instead of L-phenylalanine, 495 mg; and L-tryptophan, 612 mg. The line should read:

cose, 5 g; L-phenylalanine, 49.5 mg; and L-tryptophan, 61.2 mg. Cells

Vol. 252 (1977) 5622-5629

**GDP-mannose-polyprenyl phosphate mannosyltransferases of the retina.**

*Edward L. Kean*

Page 5624, Left hand column, last two lines of the first paragraph

Due to a printer's error, two other lines were duplicated instead of the correct lines. The paragraph should read:

The effect of variation in the concentration of dolichyl phosphate on the rate of formation of Lipid-I is shown in Fig. 4A. The double reciprocal plot (Fig. 4B) was drawn after the data were analyzed by computer using the programs of Cleland (34). From these studies, an apparent  $K_m$  for dolichyl phosphate was calculated to be 7.3  $\mu$ M. Similar kinetics were observed for Lipid-II and for the residue (data not shown).

Page 5625, Left column under "Lipid II," Line 6

99% ethanol should be chloroform:methanol:water (10:10:3). The line should read:

Ammonium formate in chloroform:methanol:water (10:10:3) at a salt concentration of

Vol. 252 (1977) 6878-6884

**The RNA-dependent DNA polymerase of avian sarcoma virus B77. Binding of viral and nonviral ribonucleic acids to the  $\alpha$ ,  $\beta_1$ , and  $\beta_2$  forms of the enzyme.**

*Amnon Hizi, Jonathan P. Leis, and Wolfgang K. Joklik*

Page 6879, Left hand column, "Experimental Procedures," last two sentences of the second paragraph.

The specific activities were expressed per mg instead of per  $\mu$ g. The correct sentence should read:

DNA at 37° in 30 min. The specific activities of the B77 enzyme preparations were 1600 to 3000 units/ $\mu$ g of protein for the  $\alpha\beta$  and  $\beta_2$  enzyme forms, and 400 to 600 units/ $\mu$ g for the  $\alpha$  form. The specific activity of the AMV polymerase form  $\alpha\beta$  was 32,000 units/ $\mu$ g.

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