Biochem. J. (1973) **134**, 1015–1021
Printed in Great Britain

Reconstitution of Oxidative Phosphorylation and the Adenosine Triphosphate-Dependent Transhydrogenase Activity by a Combination of Membrane Fractions from *uncA*$^-$ and *uncB*$^-$ Mutant Strains of *Escherichia coli* K12

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(*Received* 3 *April* 1973)

1. Membrane preparations from both *uncA*$^-$ and *uncB*$^-$ mutant strains of *Escherichia coli* K12, in which electron transport is uncoupled from phosphorylation, were fractionated by washing with a low-ionic-strength buffer. The fractionation gave a '5mM-Tris wash' and a 'membrane residue' from each strain. This technique, applied to membranes from normal cells, separates the Mg^{2+},Ca^{2+}-stimulated adenosine triphosphatase activity from the membrane-bound electron-transport chain and the non-energy-linked transhydrogenase activity. 2. Reconstitution of both oxidative phosphorylation and the ATP-dependent transhydrogenase activity was obtained by a combination of the 'membrane residue' from strain AN249 (*uncA*$^-$) with the '5mM-Tris wash' from strain AN283 (*uncB*$^-$). 3. Valinomycin plus NH_4^+ inhibited oxidative phosphorylation both in membranes from a normal strain of *E. coli* and in the reconstituted membrane system derived from the mutant strains. 4. The electron-transport-dependent transhydrogenase activity was located in the membrane residue and was de-repressed in both the mutant strains. 5. The spatial and functional relationships between the proteins specified by the *uncA* and *uncB* genes and the transhydrogenase protein are discussed.

Mutant strains of *Escherichia coli* K12 lacking the membrane-bound Mg,Ca-ATPase* have been isolated in a number of laboratories (Butlin *et al.*, 1971; Kanner & Gutnick, 1972*a*; Schairer & Haddock, 1972; Simoni & Shallenberger, 1972). The loss of Mg,Ca-ATPase activity has, in those strains in which it has been tested, been accompanied by the loss of oxidative phosphorylation and the ATP-dependent transhydrogenase activity (Butlin *et al.*, 1971; Cox *et al.*, 1971; Gutnick *et al.*, 1972; Kanner & Gutnick, 1972*b*). Those mutations affecting Mg,Ca-ATPase activity that have been mapped occur near minute 74 on the *E. coli* genome (Butlin *et al.*, 1971; Kanner & Gutnick, 1972*a*; Schairer & Haddock, 1972), and the gene in which one of these mutations occurs has been designated the *uncA* gene (Butlin *et al.*, 1971).

The ATP-dependent transhydrogenase activity has been successfully reconstituted in membranes from the *uncA*$^-$ mutant by using partially purified Mg,Ca-ATPase from a normal strain (Cox *et al.*, 1973). However, membranes from the *uncA*$^-$ mutant strain had to be first treated by the method used to solubilize the Mg,Ca-ATPase from the normal strain before reconstitution could be achieved. Experiments on the reconstitution of the ATP-dependent transhydrogenase activity (Bragg & Hou, 1973) by using the mutants isolated by Kanner & Gutnick (1972*a*) and Simoni & Shallenberger (1972) indicate that these mutants differ from the *uncA*$^-$ mutant (see the Discussion section). Reconstitution of oxidative phosphorylation in membranes of any of the mutants lacking Mg,Ca-ATPase activity has not yet been reported.

A second class of mutants of *E. coli* K12 has been isolated (Gutnick *et al.*, 1972; Butlin *et al.*, 1973), in which phosphorylation is also uncoupled from electron transport, but in which the Mg,Ca-ATPase activity is normal. The mutations in these strains also map near minute 74 on the *E. coli* genome (Butlin *et al.*, 1973; see Gutnick *et al.*, 1972) and the gene in which one of these mutations occurs has been designated the *uncB* gene (Butlin *et al.*, 1973). No reconstitution studies have been reported involving this second class of uncoupled mutants.

In the present work, experiments on the reconstitution of oxidative phosphorylation and the ATP-dependent transhydrogenase activity have been carried out on membrane fractions from strains carrying mutations in the *uncA* and *uncB* genes. The electron-transport-dependent transhydrogenase activity was also examined in the membrane fractions from the mutant strains.

* Abbreviation: Mg,Ca-ATPase, Mg^{2+},Ca^{2+}-stimulated adenosine triphosphatase activity.

Experimental

Materials

Chemicals. Chemicals generally were of the highest purity available commercially and were not further purified. Lactate dehydrogenase and hexokinase were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. and glutathione reductase and alcohol dehydrogenase were from Calbiochem (Australia) Pty. Ltd., Carlingford, N.S.W., Australia.

Organisms. All the bacterial strains used were derived from *E. coli* K12 and are described in Table 1.

Methods

Media and growth of organisms. The medium used was that described by Monod *et al.* (1951) as medium 56. Growth supplements were added as sterile solutions to the sterilized mineral salts base. The appropriate L-amino acids were added to give a final concentration of 0.2mM. Thiamin was added to give a final concentration of 0.2μM and 2,3-dihydroxybenzoate was added to give a final concentration of 40μM. Glucose was added to give a final concentration of 30mM.

Cells were grown in 7-litre New Brunswick fermenters as described by Cox *et al.* (1970).

Preparation and fractionation of cell membranes. The preparation and fractionation of membranes was carried out as described previously (Cox *et al.*, 1973). Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator and the 'membranes' were separated by ultracentrifugation and resuspended in a 0.1M-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (Tes) buffer system (pH7.0) containing magnesium acetate, sucrose and ethanedioxybis(ethylamine)tetra-acetate (EGTA). The membranes were then washed with 50mM-Tris–HCl buffer (pH7.4) and subsequently subjected to two low-ionic-strength washes with 5mM-Tris–HCl buffer (pH7.4) containing 0.25mM–EDTA and 0.5mM-dithiothreitol. After centrifugation at 160000*g* for 2h the supernatant was decanted and then concentrated by using a Diaflo XM-50 ultrafilter to give the '5mM-Tris wash'. The pellet was resuspended in the 5mM-Tris buffer system to give the 'membrane residue'.

Protein concentrations were determined by using Folin's phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard.

Transduction technique. The technique used for transduction experiments, in which the generalized transducing bacteriophage P1KC was used, is described by Pittard (1965).

Measurement of the non-energy-linked transhydrogenase activity. Reduction of NAD^+ by NADPH was assayed by coupling the reaction to the NADH-dependent reduction of pyruvate by lactate dehydrogenase and measuring the decrease in E_{340}. Details of the technique used are described by Cox *et al.* (1971).

Measurement of the ATP-dependent transhydrogenase activity. The reduction of $NADP^+$ by NADH was assayed by coupling the reaction to the NADPH-dependent glutathione reductase and measuring the decrease in E_{340} (Ernster & Lee, 1967). Details of the technique used are described by Cox *et al.* (1971).

Measurement of the electron-transport-dependent transhydrogenase activity. The technique used was based on that described by Fisher & Sanadi (1971). The reaction mixture in a cell of 1cm light-path contained, in a final volume of 2.7ml, the following components (final concentrations): membrane fraction (0.2–0.4mg of protein), Tris–HCl buffer (70mM, pH7.5), $MgCl_2$ (13mM), ethanol (30mM), NAD^+ (0.07mM), alcohol dehydrogenase (25μg) and $NADP^+$ (0.17mM).

Alcohol dehydrogenase was added to a mixture of buffer, $MgCl_2$, ethanol and NAD^+ at 30°C and the E_{340} measured. After equilibrium was reached (about 2min) the membrane fraction was added and a new equilibrium was established (about 2min). The transhydrogenase reaction was then initiated by the addition of $NADP^+$ and the rate of increase in E_{340} used to determine the electron-transport-dependent transhydrogenase activity.

Oxidative phosphorylation. The oxygen uptake by

Table 1. *Strains of Escherichia coli K12 used*

Genes coding for enzymes in various biosynthetic pathways are denoted as follows: *ilv*, isoleucine–valine; *arg*, arginine; *ent*, enterochelin. The *uncA* and *uncB* genes code for proteins required in the coupling of phosphorylation to electron transport.

Strain	Relevant genetic loci	Other information
AN248	*ilvC*$^-$, *argH*$^-$, *entA*$^-$	Butlin *et al.* (1973)
AN120	*argE*$^-$, *uncA*401	Butlin *et al.* (1971)
AN249	*uncA*401, *argH*$^-$, *entA*$^-$	Isolated after transduction with strain AN120 as donor and strain AN248 as recipient
AN283	*uncB*401, *argH*$^-$, *entA*$^-$	Butlin *et al.* (1973)
AN259	*argH*$^-$, *entA*$^-$	Butlin *et al.* (1973)

the membrane preparations was measured by using an oxygen electrode as described previously (Cox *et al.*, 1970). The reaction mixture contained, in a final volume of 2.5ml, 0.1M-Tes buffer (pH7.0), 0.25M-sucrose, 0.02M-magnesium acetate, 0.25mM-EGTA, 0.15mM-ADP, 0.75mM-AMP, 3mM-glucose, 50 EC units of hexokinase and $^{32}P_i$ to give a specific radioactivity of approx. 100c.p.m./nmol of phosphate. Membranes (about 0.8mg of protein) were preincubated with the above mixture at 30°C for 2min and the reaction was initiated by the addition of D-lactate at a final concentration of 0.5mM.

The reaction was stopped by transferring 2ml of the reaction mixture into 1ml of ice-cold 20% (v/v) trichloroacetic acid. Denatured protein was removed by centrifugation and P_i was extracted after addition of molybdate by the method of Avron (1960). A sample of aqueous residue (2.5ml) was transferred to a scintillation vial and the volume made up to 10ml with water. The Čerenkov radiation was measured in a Packard liquid-scintillation counter. Incubation mixtures without added D-lactate were included as controls and vials containing known concentrations of $^{32}P_i$ were used as standards.

For the measurement of oxidative phosphorylation in the reconstituted membrane system, the membrane residue was resuspended in 0.1M-Tes buffer (pH7.0) rather than the Tris buffer system used to resuspend the membrane residue for the reconstitution of the ATP-dependent transhydrogenase reaction. The '5mM-Tris wash' was diluted tenfold in 0.1M-Tes buffer (pH7.0) plus 0.5mM-dithiothreitol and then concentrated, by filtration through a Diaflo XM-50 ultrafilter, to the original volume.

Results

Oxidative phosphorylation by membranes from normal and mutant strains

Measurements of oxygen uptake and the esterification of P_i by membranes from the normal strain AN259, with D-lactate as the oxidizable substrate, gave a P/O ratio of 0.12. Membranes from strains AN249 (*uncA*$^-$) and AN283 (*uncB*$^-$) showed little or no ability to esterify P_i when tested under the same conditions (Table 2). The inability of other strains carrying the *uncA*401 and *uncB*401 alleles to carry out oxidative phosphorylation has been described previously (Butlin *et al.*, 1971, 1973). The phosphorylation coupled to electron transport in the membranes from the normal strain AN259 was inhibited by valinomycin plus NH_4^+, but not by either alone (Table 2).

Reconstitution of oxidative phosphorylation by using membrane fractions from mutant strains

Membranes from the mutant strains AN249 (*uncA*$^-$) and AN283 (*uncB*$^-$) were fractionated by washing with low-ionic-strength buffer to give a '5mM-Tris-wash' fraction and a 'membrane-residue' fraction from each strain. The '5mM-Tris-wash' fraction from a normal strain contains the Mg,Ca-ATPase activity and the 'membrane-residue' fraction contains the oxidase activities (Cox *et al.*, 1973). Various combinations of the membrane fractions were then used in an attempt to reconstitute oxidative phosphorylation.

The results (Table 3) show that the only effective combination of membrane fractions is the 'membrane residue' from strain AN249 (*uncA*$^-$) and the '5mM-Tris wash' from strain AN283 (*uncB*$^-$). With this reconstituted system the P/O ratio obtained was about half the value obtained with normal membranes. The phosphorylation obtained with the reconstituted system was sensitive to valinomycin plus NH_4^+ (Table 3). The addition of increasing amounts of '5mM-Tris wash' from strain AN283 (*uncB*$^-$) to a constant amount of 'membrane residue' from strain AN249 (*uncA*$^-$) showed (Fig. 1) that the 'membrane residue' could be saturated by the '5mM-Tris wash' with respect to the P/O ratio obtained.

Table 2. *Oxidative phosphorylation by membranes from normal and mutant strains of Escherichia coli*

Details are given in the Experimental section.

Strain	Additions to basal system and final concn.	Oxygen uptake (ng-atoms of O/min per mg of protein)	Glucose 6-phosphate formed (nmol/min per mg of protein)	P/O ratio
AN259	—	360	43	0.12
	Valinomycin (1μM)	352	38	0.11
	Valinomycin (1μM)+ ammonium acetate (40mM)	370	4	0.01
	Ammonium acetate (40mM)	346	36	0.11
AN249 (*uncA*$^-$)	—	355	<0.1	<0.001
AN283 (*uncB*$^-$)	—	305	1.5	0.005

Table 3. *Reconstitution of oxidative phosphorylation with membrane fractions from mutant strains of Escherichia coli*

Details are given in the Experimental section. The '5mM-Tris-wash' preparations added contained 1–2mg of protein.

Membrane fraction		Additions to assay system and final concn.	Oxygen uptake (ng-atoms of O/min per mg of residue protein)	P_i esterified (nmol/min per mg of residue protein)	P/O ratio
Residue	'5mM-Tris wash'				
AN283 (*uncB*$^-$)	AN283 (*uncB*$^-$)	None	565	<0.1	<0.001
AN283 (*uncB*$^-$)	AN249 (*uncA*$^-$)	None	575	<0.1	<0.001
AN249 (*uncA*$^-$)	AN249 (*uncA*$^-$)	None	870	<0.1	<0.001
AN249 (*uncA*$^-$)	AN283 (*uncB*$^-$)	None	775	39	0.05
AN249 (*uncA*$^-$)	AN283 (*uncB*$^-$)	Valinomycin (1μM)	790	32	0.04
AN249 (*uncA*$^-$)	AN283 (*uncB*$^-$)	Valinomycin (1μM) +ammonium acetate (40mM)	855	<0.1	<0.001
AN249 (*uncA*$^-$)	AN283 (*uncB*$^-$)	Ammonium acetate (40mM)	725	30	0.04

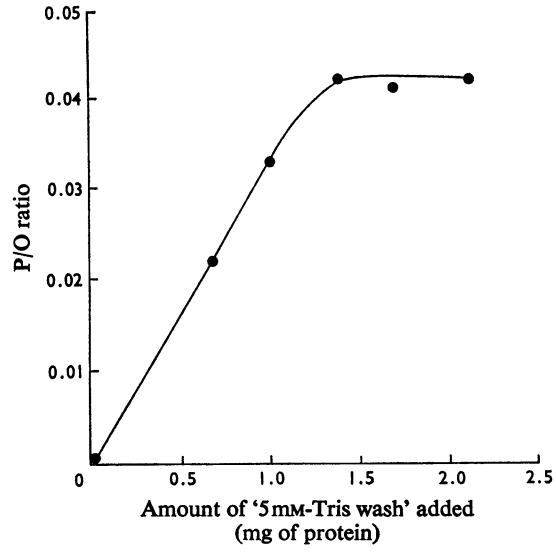


Fig. 1. *Reconstitution of oxidative phosphorylation by the addition of increasing amounts of '5mM-Tris wash' from strain AN283 (uncB$^-$) to a constant amount (0.7mg of protein) of 'membrane residue' from strain AN249 (uncA$^-$)*

Details are given in the Experimental section.

Reconstitution of the ATP-dependent transhydrogenase activity by using fractionated membranes

The reconstitution of the ATP-dependent transhydrogenase activity in the 'membrane residue' from an *uncA*$^-$ strain with a partially purified Mg,Ca-ATPase preparation from a normal strain of *E. coli* has been shown previously (Cox *et al.*, 1973). In the present experiments the 'membrane residue' from strain AN249 (*uncA*$^-$) was reactivated by the '5mM-Tris wash' from strain AN283 (*uncB*$^-$) (Table 4). These results are consistent with the results given above for the reconstitution of oxidative phosphorylation.

Electron-transport-dependent transhydrogenase activity in membranes and membrane fractions

Examination of membrane fractions from strains AN259, AN249 (*uncA*$^-$) and AN283 (*uncB*$^-$) showed (Table 5) that the electron-transport-dependent transhydrogenase activity is greater in the mutants than in the normal strain. This activity is located in the 'membrane-residue' fraction (Table 5) along with the non-energy-linked transhydrogenase activity (Cox *et al.*, 1973).

Table 4. *ATP-dependent transhydrogenase activities of membranes and various combinations of membrane fractions from mutant strains of Escherichia coli*

Details are given in the Experimental section.

Membrane preparation (mg of protein)						ATP-dependent transhydrogenase activity (nmol of NADPH formed/min)
Strain AN249 (*uncA*⁻)			Strain AN283 (*uncB*⁻)			
Membrane	'Membrane residue'	'5mM-Tris wash'	Membrane	'Membrane residue'	'5mM-Tris wash'	
1.18	—	—	—	—	—	9
—	0.7	—	—	—	—	11
—	—	1.08	—	—	—	0
—	—	—	1.14	—	—	11
—	—	—	—	0.9	—	9
—	—	—	—	—	0.65	0
—	0.7	1.08	—	—	—	11
—	—	—	—	0.9	0.65	2
—	—	1.08	—	0.9	—	4
—	0.7	—	—	—	0.65	132

Table 5. *Electron-transport-dependent transhydrogenase activities of membranes and membrane fractions from normal and mutant strains of Escherichia coli*

Details are given in the Experimental section.

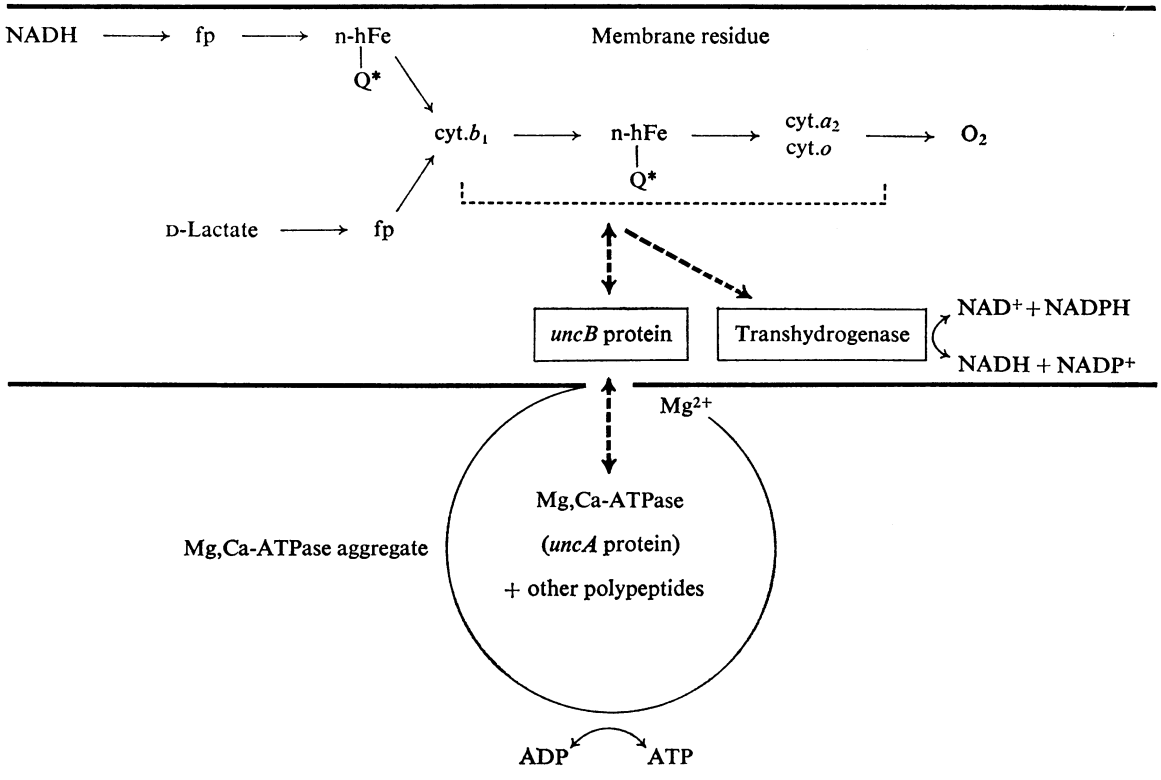
Strain	Membrane fraction	Electron-transport-dependent transhydrogenase (nmol of NADPH formed/min per mg of protein)
AN259	Membrane	93
AN259	'Membrane residue'	86
AN259	'5mM-Tris wash'	1
AN249 (*uncA*⁻)	Membrane	116
AN249 (*uncA*⁻)	'Membrane residue'	187
AN249 (*uncA*⁻)	'5mM-Tris wash'	18
AN283 (*uncB*⁻)	Membrane	120
AN283 (*uncB*⁻)	'Membrane residue'	221
AN283 (*uncB*⁻)	'5mM-Tris wash'	11

Discussion

The above results provide information about the localization, within the membrane, of the effect of a mutation in the *uncB* gene, and of the metabolic effects of such a mutation. Thus membranes from the *uncB*⁻ strain have lost both the ability to couple phosphorylation to electron transport and to carry out the ATP-dependent transhydrogenase reaction. However, the electron-transport-dependent transhydrogenase and the Mg,Ca-ATPase remain active in the *uncB*⁻ mutant. The Mg,Ca-ATPase activity, when separated from the membrane of the *uncB*⁻ strain by a low-ionic-strength wash, appears to be normal, since it will reconstitute oxidative phosphorylation and the ATP-dependent transhydrogenase in the 'membrane residue' from the *uncA*⁻ strain. This also indicates that the 'membrane residue' from the *uncA*⁻ strain is normal and implies that the effect of a mutation in the *uncB* gene is localized in this fraction.

The results of the above experiments, taken in conjunction with those of previous experiments with *E. coli* K12 (Cox *et al.*, 1970; Butlin *et al.*, 1971; Cox *et al.*, 1971; Butlin *et al.*, 1973), are summarized in Scheme 1. This scheme serves as a working hypothesis for the distribution and role of the proteins affected by mutations in the *uncA* and *uncB* genes.

The Mg,Ca-ATPase is bound to the membrane residue by Mg^{2+} and appears to be an aggregate of polypeptides separable by gel electrophoresis (Bragg & Hou, 1972; G. B. Cox, unpublished work). Further work is still required to show which polypeptide in the Mg,Ca-ATPase aggregate is affected by a mutation in the *uncA* gene. The NADH oxidase system, the



Scheme 1. *Relationships between some components involved in energy-linked reactions in the Escherichia coli membrane*

D-lactate oxidase system and the protein responsible for transhydrogenase activity are found in the membrane residue (Cox *et al.*, 1973). The observation that a mutation in the *uncB* gene impairs ATP-dependent transhydrogenase activity (Butlin *et al.*, 1973), while not affecting the electron-transport-dependent transhydrogenase activity, indicates that there is probably some common factor involved in energy transfer lying between the electron-transport chain and the *uncB* protein. This common factor is presumably not ubiquinone, since the ATP-dependent transhydrogenase activity is normal in ubiquinone-deficient mutants (Cox *et al.*, 1971).

Experiments on the reconstitution of the energy-dependent transhydrogenase activity in normal strains and the Mg,Ca-ATPase-deficient mutants of *E. coli* isolated by Kanner & Gutnick (1972*a*) and by Simoni & Shallenberger (1972) have given results (Bragg & Hou, 1973) which indicate that these mutants are different from those described in the present paper. Bragg & Hou (1973) found that the membranes from the mutant isolated by Simoni & Shallenberger (1972) (strain DL-54) could be reactivated to some extent by a preparation of coupling factor without any prior treatment of the membranes with low-ionic-strength buffer (cf. Cox *et al.*, 1973). The mutant strain (N144) isolated by Kanner & Gutnick (1972*a*) could not be reactivated with the preparation of coupling factor even after treatment with low-ionic-strength buffer.

The P/O ratios obtained with the reconstituted system approached those found by using intact membranes and, in addition, the oxidative phosphorylation in both cases was sensitive to valinomycin plus NH_4^+. The amount of protein in the '5mM-Tris wash' required to saturate the 'membrane residue' for reconstitution of oxidative phosphorylation in the present experiments was about three times that required to reconstitute fully the ATP-dependent transhydrogenase activity in previous experiments (Cox *et al.*, 1973). This difference can be accounted for by the fact that the '5mM-Tris wash' used previously

was obtained from a strain forming about three times the activity of Mg,Ca-ATPase found in the '5mM-Tris wash' from strain AN283 (*uncB*$^-$).

We thank Mrs. J. McDonald and Miss B. Pfeiffer for skilled technical assistance.

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