***Salmonella typhimurium* HfrA, a Mutant in which Adenosine Triphosphate Can Drive Amino Acid Transport but not Energy-Dependent Nicotinamide Nucleotide Transhydrogenation**

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In contrast with wild-type *Salmonella typhimurium* LT2, strain HfrA did not have ATP-driven energy-dependent transhydrogenase activity, although ATP-dependent quenching of atebrin fluorescence was normal. Respiration-dependent and energy-independent transhydrogenase, and Ca^{2+} -activated ATPase (adenosine triphosphatase) activities were similar in both strains. Purified ATPases from the two strains had similar specific activities, similar subunit polypeptides, and were equally effective in restoring energy-dependent transhydrogenase activities to membrane particles of strain LT2 from which the ATPase had been stripped. The purified ATPases from both strains could restore respiration-dependent but not ATP-dependent transhydrogenation to stripped particles of strain HfrA. Both strains grew aerobically equally well on salts media containing glucose, malate, succinate, citrate, acetate, pyruvate, fumarate, lactate or aspartate as substrates. Growth on glucose under anaerobic conditions was similar. Strains LT2 and HfrA were equally effective in the accumulation under both aerobic and anaerobic conditions of the amino acids proline, phenylalanine, histidine, lysine, isoleucine and aspartic acid. Inhibition of amino acid accumulation by KCN and dicyclohexylcarbodi-imide occurred to the same extent in both strains. The complete inhibition by dicyclohexylcarbodi-imide of amino acid uptake under anaerobic conditions suggested that ATP could drive amino acid uptake in both strains. The ability of strain HfrA to carry out ATP-dependent transport or quenching of atebrin fluorescence but not ATP-dependent transhydrogenation is different from the wild-type strain and from any previously described energy-coupling mutant. It is difficult to reconcile the properties of this mutant with the chemiosmotic hypothesis.

During the past few years mutants of *Escherichia coli* and *Salmonella typhimurium* defective in oxidative phosphorylation have been isolated (Cox & Gibson, 1974). These mutants have become of increasing importance in determining the function of the components of the various energy-linked systems found in the membranes of these organisms. At present most of the mutants may be grouped into one or other of two main classes. By the nomenclature of Cox & Gibson (1974) these are the *unc A*⁻ and *unc B*⁻ types. Mutants of the *unc A*⁻ type are *unc A*⁻ (Butlin *et al.*, 1971), A103c (Schairer & Haddock, 1972), DL54 (Simoni & Shallenberger, 1972), N₁₄₄ (Kanner & Gutnick, 1972), NR70 (Rosen, 1973) and *unc*-405 (Cox *et al.*, 1974). They are characterized by the presence of an inactive ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-activated ATPase‡ (*unc A*⁻) (Cox *et al.*, 1974) or by the loss of this enzyme (DL54, NR70, *unc*-405) (Futai, 1974;

Rosen, 1973; Cox *et al.*, 1974). By use of these mutants it has been shown that the ATPase is involved in oxidative phosphorylation (Butlin *et al.*, 1971; Gutnick *et al.*, 1972), ATP-dependent transhydrogenation of NADP^+ from NADH (Kanner & Gutnick, 1972; Cox *et al.*, 1971; Schairer & Gruber, 1973; Bragg *et al.*, 1973), transport of β -galactosides and certain amino acids under anaerobic conditions, and ATP-dependent membrane energization as indicated by studies with fluorescent dyes (Schairer & Haddock, 1972; Or *et al.*, 1973; Van Thienen & Postma, 1973; Nieuwenhuis *et al.*, 1973).

Mutants of the *unc B*⁻ class are *unc B*⁻ (Butlin *et al.*, 1973), B_{v5} (Gutnick *et al.*, 1972) and *unc* 253 (Schairer & Gruber, 1973). In these mutants the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-activated ATPase is at normal activity, but despite this these cells cannot carry out the processes outlined above for *unc A*⁻ cells. Thus the ATPase must in some way be not linked to these energy-requiring processes in the membranes of *unc B*⁻ cells.

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‡ Abbreviation: ATPase, adenosine triphosphatase.

Like *unc A*⁻ cells, *unc B*⁻ mutants can by substrate oxidation through the respiratory chain energize the fluorescence of certain dyes, drive transhydrogenation of NADP⁺ by NADH, and actively transport amino acids and β -galactosides. Evidence obtained by use of *unc A*⁻ and *unc B*⁻ mutants clearly supports the hypothesis of Klein & Boyer (1972) that the *E. coli* membrane can be energized either by the respiratory chain or by ATP.

There is a third group of mutants which may or may not be related to one another. The *etc* mutants of *E. coli* and *S. typhimurium* (Hong & Kaback, 1972), and *E. coli* mutants *K*₁₁ and *A*₁₄₄ (Van Thienen & Postma, 1973; Nieuwenhuis *et al.*, 1973) differ from *unc A*⁻ and *unc B*⁻ mutants. They have about half the ATPase activity found in the parent strain and have less ability to carry out respiration-dependent amino acid transport and transhydrogenation (Van Thienen & Postma, 1973; Nieuwenhuis *et al.*, 1973; Hong & Kaback, 1972; Bragg *et al.*, 1973).

In the present paper we describe the properties of a fourth type of mutant, *S. typhimurium* HfrA, in which loss of one ATP-dependent energy-linked function (transhydrogenation) has occurred without loss of other ATP-dependent energy-linked functions (amino acid transport, growth on intermediates of the tricarboxylic acid cycle and quenching of atebriin fluorescence).

Materials and Methods

Organisms

S. typhimurium strains LT2 and HfrA (*his* D23⁻, *gal* 50⁻) were obtained from the collection of K. E. Sanderson (University of Calgary). Strain HfrA was originally derived by Zinder (1960) by crossing a strain of *S. typhimurium* LT2 with the *E. coli* HfrA strain Y-92 (*his*⁻, *met*⁻, *T*_{1,5}^R, *azi*^R). Definition of genetic symbols is that of Taylor & Trotter (1972).

The strains were kept as freeze-dried cultures and maintained as a routine on nutrient agar slants.

Determination of growth yields

Cells were grown in the minimal medium of Davis & Mingioli (1950) and were adapted by serial transfer in this medium with the appropriate carbon source. Growth curves at various carbon source concentrations were followed turbidimetrically with a Klett colorimeter equipped with a no. 42 filter. The readings were converted into dry weight by the use of a calibration curve. Anaerobic-growth curves were followed as above with the use of 13 mm screw-cap tubes equipped with silicone septa. After inoculation the culture was gassed with N₂ for 10 min before incubation. These cultures were agitated with a magnetic stirrer during incubation.

Amino acid accumulation

Cells were grown on minimal media with 15 mM-glucose to late exponential phase, harvested, washed twice, resuspended to 0.355 mg dry weight/ml in the same medium, and kept at 0°C before the transport experiment. Transport was followed for 10 min at 37°C after the addition of 10 μ M-amino acid containing ¹⁴C-labelled amino acid at 0.05 μ Ci/ml. Samples were removed at intervals and filtered through a Sartorius membrane filter (0.45 μ m). Cells were washed with 5 ml of minimal medium and assayed for radioactivity in a dioxan-base scintillation fluid (PCS; Amersham/Searle Corp., Don Mills, Ont., Canada) with a Nuclear-Chicago Mark II liquid-scintillation spectrometer. Maximum accumulation was achieved by this time and was calculated as a concentration ratio of internal to external amino acid. To avoid incorporation of amino acid into cellular protein, as a routine the cells were incubated with chloramphenicol (100 μ g/ml) for 15 min before the initiation of transport. Chromatography demonstrated that none of these amino acids was significantly metabolized over this time-interval, except for aspartate, approx. 80% of which was converted into glutamate by 10 min.

Transport experiments under anaerobic conditions were performed as above except that sealed serum capped vessels were used, and both the radioactive isotope and reaction mixture were flushed with N₂ for 10 min before the addition of labelled amino acid with a hypodermic syringe.

Preparation of membrane particles, coupling factor and purified ATPase

Cells were grown with vigorous aeration on a minimal salts medium containing 22 mM-glucose and 12 μ M-ferric citrate (Bragg *et al.*, 1972). The cells were harvested in the late exponential phase and converted into washed respiratory particles as previously described (Bragg & Hou, 1972). The washed particles were suspended in, and dialysed overnight at 4°C against, 1 mM-Tris-HCl buffer, pH 7.5, containing 0.5 mM-EDTA, 0.1 mM-dithiothreitol and 10% glycerol. The dialysed particle suspension was centrifuged (120 000g; 2 h) to yield a pellet of 'stripped' particles. The supernatant fluid was re-centrifuged to remove residual particulate material and then concentrated to about one-tenth of its volume by ultrafiltration through a PM 10 membrane (Amicon Corp., Lexington, Mass., U.S.A.) to yield the 'coupling factor' (protein, 8–11 mg/ml) (Bragg & Hou, 1973).

ATPase was purified to homogeneity from the coupling factor by chromatography on DEAE-cellulose followed by sucrose-gradient centrifugation. The procedure followed the method used previously (Bragg & Hou, 1972) except that chromatography

on Sepharose 6B was omitted since it did not improve the purity of the enzyme.

Reconstitution of transhydrogenase activity

The stripped particles were suspended in 50mM-Tris-H₂SO₄ buffer, pH 7.8, containing 10mM-MgCl₂, and 1% bovine serum albumin, at a concentration of 10–20mg of protein/ml. The particles (50μl) were preincubated for 5min at 37°C with various amounts of dicyclohexylcarbodi-imide, coupling factor, or purified ATPase in a cuvette with 1 ml of Tris-H₂SO₄ buffer, pH 7.8, containing 10mM-MgCl₂, 0.1% bovine serum albumin, 0.1mM-dithiothreitol and 0.16M-sucrose. After preincubation, the transhydrogenase assay was carried out directly on the contents of the cuvette. The final volume of the complete assay mixture was 1.2ml.

Enzyme assays

Protein, Ca²⁺-activated ATPase, respiration and ATP-driven energy-dependent transhydrogenase activities were measured exactly as described by Bragg *et al.* (1972) except that the final concentration of sucrose in the transhydrogenase assays was 0.133M. ATPase activity was determined in the presence of 5mM-CaCl₂. Energy-independent transhydrogenation of 3-acetylpyridine-NAD⁺ by NADPH was assayed as described by Singh & Bragg (1974). Energy-linked reduction of NAD⁺ by succinate, DL-α-glycerophosphate and D-lactate was measured by using membrane particles prepared from cells grown on the medium described by Poole & Haddock (1974) with 0.5% glycerol as the carbon source. The method used to prepare the membrane particles and for the assay also followed their procedure. All enzyme activities were measured at 37°C.

Energy-dependent quenching of atebrin fluorescence

The method was based on that of Haddock & Downie (1974). Atebrin fluorescence was measured at 22°C with a Turner model 420 spectrofluorimeter connected to a Varian strip chart recorder. The

reaction mixture in a final volume of 2.0ml contained 300mM-KCl, 5mM-MgCl₂, 10mM-Hepes-[2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid]-KOH buffer, pH 7.5, 7.5–10μM-atebrin and membrane particles; 0.05ml of NADH, 0.05ml of ATP and 0.025ml of dicyclohexylcarbodi-imide were added to give final concentrations of 1mM, 1mM and 0.116mM respectively. Atebrin fluorescence was excited by light at 430nm and emission was measured at 505nm.

Gel electrophoresis

Analytical disc gel electrophoresis was performed either on 5% (w/v) polyacrylamide gels prepared and run in 0.05M-Tris-glycine buffer, pH 8.7, or on 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate. For the latter system the protein sample was depolymerized at 37°C for 1h, and then at 100°C for 3min, in 4M-urea-sodium dodecyl sulphate–1% 2-mercaptoethanol, and run in system 1 as described previously (Bragg *et al.*, 1973). Protein bands were stained with Coomassie Blue.

Materials

All radioactive isotopes were obtained from Amersham/Searle Corp. and were checked for purity by chromatography and radioautography before use. Dicyclohexylcarbodi-imide and amino acids were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Results

Energy-dependent transhydrogenase and ATPase activities of membrane particles

Washed membrane particles of *S. typhimurium* LT2 and HfrA contained similar energy-independent and respiration-dependent transhydrogenase activities. However, in contrast with strain LT2, ATP-dependent transhydrogenase activity was absent from strain HfrA (Table 1). The Ca²⁺-activated ATPase, which has been shown to be involved in the ATP-

Table 1. *Specific activities of Ca²⁺-activated ATPase and transhydrogenase in washed and stripped membrane particles of S. typhimurium LT2 and HfrA*

Particle	Enzymic activity	Specific activity(nmol/min per mg of protein)	
		Strain LT2	Strain HfrA
Washed	ATPase	348	426
	Energy-independent transhydrogenase	249	177
	Respiration-dependent transhydrogenase	11.5	11.9
	ATP-dependent transhydrogenase	16.2	0
Stripped	ATPase	34	13
	Energy-independent transhydrogenase	222	296
	Respiration-dependent transhydrogenase	0	8.6
	ATP-dependent transhydrogenase	0	0

dependent transhydrogenase (Cox *et al.*, 1971), was present in both strains (Table 1). Thus the absence of ATP-dependent transhydrogenase activity from strain HfrA cannot be due to lack of the ATPase.

Coupling-factor activity of ATPases

Previous work with *E. coli* had shown that the

Ca²⁺-activated ATPase could restore both respiration and ATP-driven energy-dependent transhydrogenase activities to membrane particles which had been stripped of the ATPase by washing with a buffer of low ionic strength (Bragg & Hou, 1972). Membrane particles of both strains submitted to the washing procedure lost most of their ATPase activity.

Fig. 1. Reconstitution of energy-dependent transhydrogenase activities in stripped membrane particles of *S. typhimurium* LT2 (a–c) and *S. typhimurium* HfrA (d–f) by dicyclohexylcarbodi-imide (a, d), or coupling factor from *S. typhimurium* LT2 (b, e) or HfrA (c, f)

○, Respiration-dependent transhydrogenase activity; ●, ATP-dependent transhydrogenase activity. The horizontal broken and solid lines indicate respectively the activities of the respiration- and ATP-dependent transhydrogenases in washed particles. Specific activity of the transhydrogenase is expressed as nmol/min per mg of particle protein. The amount of dicyclohexylcarbodi-imide and of coupling factor added to the system is expressed as μmol and as units ($\mu\text{mol}/\text{min}$) of ATPase activity respectively. Particle protein: strain LT2, 0.91 mg; strain HfrA, 0.70 mg. Specific activity of ATPase of coupling factor: strain LT2, 4.3 nmol/min per mg of protein; strain HfrA, 5.8 nmol/min per mg of protein.

There was little change in energy-independent transhydrogenase activity of strain LT2. The increase in the specific activity of this enzyme in stripped HfrA particles may have been due to the removal of cytoplasmic proteins included in the washed particles (Table 1). Stripped particles of strain LT2 were unable to carry out energy-dependent transhydrogenation, whereas those of strain HfrA still retained some respiration-dependent transhydrogenase activity (Table 1). The partial retention of the latter activity by particles of strain HfrA is not considered to be a significant difference between the two strains. Thus in Fig. 2 LT2 particles have retained some of this activity. The percentage of this activity which was lost was variable with particles of both strains but was generally 40%, and appeared to be related to the completeness with which the ATPase had been stripped from the particles (P. D. Bragg, unpublished work). The washings obtained during the stripping procedure were concentrated to give 'coupling factor' which contained the ATPase activity released from the particles of both strains. Addition of the coupling factor to the stripped particles resulted in restoration of the energy-dependent transhydrogenase activities (Fig. 1). The coupling factor from strain LT2 when added to stripped particles of strain LT2, restored both respiration- and ATP-driven transhydrogenation to values found in washed particles (Fig. 1b). Only the former activity was restored with stripped particles of strain HfrA (Fig. 1e). Similar results were obtained with the coupling factor of strain HfrA (Figs. 1c and 1f). Also, as previously observed with *E. coli* (Bragg & Hou, 1973), dicyclohexylcarbodi-imide when added to stripped particles of both strains could restore respiration-driven transhydrogenase activity (Figs. 1a and 1b).

Properties of purified ATPases

The experiments above suggested that the defect in the ATP-dependent transhydrogenase of strain HfrA was associated with the membrane of this strain, since the coupling factor from this strain when added to stripped particles of strain LT2 apparently had normal reconstitutive properties. The ATPases of strains LT2 and HfrA were purified to establish that the activity of the coupling factor was due to the ATPase present in it, and to show that the enzymes were identical. The purification procedure was that which had been found to be effective with *E. coli* (Bragg & Hou, 1972). The purified enzymes both gave a single protein-staining band of similar mobility on gel electrophoresis in Tris-glycine buffer, pH 8.7. On gel electrophoresis in the presence of sodium dodecyl sulphate both enzymes yielded five subunits with molecular weights of 56800, 51800, 30900, 21500 and 13200 (Bragg & Hou, 1975). The specific activities of the purified ATPases of strains LT2 and HfrA were 30.2 ± 1.5 (three preparations) and

32.2 ± 2.0 (three preparations) nmol/min per mg of protein respectively.

As shown in Fig. 2 the purified ATPases of strains LT2 and HfrA could react with stripped membrane particles of strain LT2 to reconstitute both respiration- and ATP-driven energy-dependent transhydrogenase activities. Similar amounts of each enzyme ($6.0 \mu\text{g}$ of ATPase protein/mg of LT2 particle protein) were required for half-maximal stimulation of the transhydrogenase activities.

The data presented above suggest that the ATPases of strains LT2 and HfrA are identical, and that the defect in the ATP-driven transhydrogenase of strain HfrA cannot be due to modification of the ATPase. The properties described so far suggest that strain HfrA might be an *unc B*⁻ mutant. The *unc B*⁻ mutant of *E. coli* had normal respiration-driven transhydrogenase activity and lacked ATP-driven transhydrogenase activity. A crude coupling-factor preparation has been obtained from membrane particles which would reconstitute the later activity in normal stripped membrane particles of *E. coli* (Cox *et al.*, 1973). The experiments in the succeeding sections show that strain HfrA is not a mutant of the *unc B*⁻ type.

Growth yields on different substrates

The *unc A*⁻ and *unc B*⁻ mutants of *E. coli* are characterized by an inability to grow on acetate,

Fig. 2. Reconstitution of energy-dependent transhydrogenase activities in stripped particles of *S. typhimurium* LT2 by purified ATPase of *S. typhimurium* HfrA (a) or LT2 (b).

○, Respiration-dependent transhydrogenase activity; ●, ATP-dependent transhydrogenase activity. Specific activity of transhydrogenase is expressed as nmol/min per mg of particle protein. The amount of ATPase added is expressed as units ($\mu\text{mol/min}$) of activity. Specific activity of ATPase (units/mg of protein): strain HfrA, 29.6; strain LT2, 30.9. Particle protein, 0.96 mg.

Table 2. Amino acid transport in *S. typhimurium* LT2 and HfrA

The final concentrations of KCN and dicyclohexylcarbodi-imide were 3mM and 1mM respectively. The amino acid accumulation was measured as the ratio of internal to external concentration at maximum accumulation.

Strain	Conditions	Amino acid accumulation					
		Proline	Phenylalanine	Histidine	Lysine	Isoleucine	Aspartic acid
LT2	Aerobic	533	86	106	206	47	604
	Anaerobic	224	79	267	149	80	244
	Aerobic+KCN	86	53	40	117	35	50
	Aerobic+dicyclohexylcarbodi-imide	438	58	138	84	110	134
	Anaerobic+dicyclohexylcarbodi-imide	2	7	2	8	5	7
HfrA	Aerobic	401	82	116	185	43	346
	Anaerobic	160	49	264	203	48	183
	Aerobic+KCN	144	41	27	102	33	97
	Aerobic+dicyclohexylcarbodi-imide	478	63	69	182	107	213
	Anaerobic+dicyclohexylcarbodi-imide	2	4	2	32	4	17

Fig. 3. Effect of dicyclohexylcarbodi-imide (a) and KCN (b) on proline accumulation in *S. typhimurium* LT2 and HfrA

The cells were incubated with the inhibitor for 15 min before initiation of transport. Proline accumulation is expressed as the ratio of internal to external concentration when the rate of proline taken up was at its maximum. Accumulation under aerobic and anaerobic conditions is shown by open and closed points respectively. ○, ●, Strain LT2; △, ▲, strain HfrA.

malate or succinate (Butlin *et al.*, 1971, 1973), although growth on glucose or glycerol can occur since ATP can be formed by glycolysis on the latter substrates. *S. typhimurium* HfrA and wild-type strain LT2 showed similar growth rates and yields on media containing succinate, malate, acetate,

lactate, pyruvate, citrate, fumarate or aspartate as carbon sources. Further, strains HfrA and LT2 showed similar growth yields when grown anaerobically on glucose. This separates strain HfrA from *uncA*⁻ mutants, which will not grow under these conditions (Butlin *et al.*, 1973).

Fig. 4. Atebrin fluorescence (traces 1, 2) and energy-dependent transhydrogenase (traces 3, 4) in membrane particles of strain LT2 (traces 1, 3) and strain HfrA (traces 2, 4)

The experiments were carried out as described in the Materials and Methods section. In traces 1 and 2 changes in fluorescence are expressed in arbitrary units. In traces 3 and 4 reduction of NADP⁺ is measured as ΔE_{340} . Additions made at the arrows in traces 1 and 2: A, atebrin (10 μ M and 7.5 μ M in traces 1 and 2 respectively); NADH (1 mM); ATP (1 mM); DCCD (dicyclohexylcarbodi-imide) (0.116 mM). Additions made at the arrows in traces 3 and 4: NADP⁺ (0.68 mM); ATP (0.5 mM). The same preparations of membrane particles of strains LT2 and HfrA were used both for fluorescence and transhydrogenase experiments. Concentration of membrane particles (mg of protein): trace 1, 1.4; trace 2, 2.0; trace 3, 0.7; trace 4, 1.0.

The data in this section distinguish *S. typhimurium* HfrA from *unc B*⁻ and *unc A*⁻ mutants of *E. coli*.

Amino acid transport

Endogenous substrates could be used aerobically and anaerobically by intact cells of strains LT2 and HfrA to drive the accumulation of proline, phenylalanine, histidine, lysine, isoleucine and aspartic acid (Table 2). Under all conditions strains LT2 and HfrA gave similar results. Aerobic transport was effectively inhibited by 3 mM-KCN, whereas, except for lysine and aspartic acid, accumulation of the amino acids was not markedly inhibited by 1 mM-

dicyclohexylcarbodi-imide. In contrast with these results, there was marked inhibition by dicyclohexylcarbodi-imide of transport under anaerobic conditions. The effect of these inhibitors on proline accumulation was analysed more fully (Fig. 3). Transport of proline under aerobic conditions was completely abolished in both strains by 1 mM-KCN but was unaffected by 10 mM-dicyclohexylcarbodi-imide. Anaerobically accumulation of proline was completely prevented in both strains by 1 mM-dicyclohexylcarbodi-imide.

Dicyclohexylcarbodi-imide is an inhibitor of the Ca²⁺-activated ATPase (Evans, 1970) and KCN

inhibits at the level of the cytochrome oxidases in *S. typhimurium* (Singh & Bragg, 1974). Therefore these results are consistent with the hypothesis (Klein & Boyer, 1972; Parnes & Boos, 1973) that transport under aerobic conditions was energized primarily by substrate oxidation through the respiratory chain, whereas ATP was the prime energy donor under anaerobic conditions. Of significance is the fact that strains LT2 and HfrA showed identical behaviour, indicating that amino acid transport can be energized in these strains by both routes. This result is in sharp contrast with the behaviour of *E. coli* *unc A*⁻ and *unc B*⁻ mutants, where anaerobic conditions or the presence of KCN completely abolishes the transport of proline or β -galactosides although still permitting significant accumulation in the parent strains (Schairer & Haddock, 1972; Schairer & Gruber, 1973; Or *et al.*, 1973).

Quenching of atebrin fluorescence

The inability of ATP to drive transhydrogenation in membrane particles of strain HfrA, which in whole cells showed normal ATP-dependent transport of amino acids, could be due to damage of the particles brought about by disruption of the cells. Transport studies using membrane vesicles instead of whole cells to study the effect of cell disruption are not feasible, since a vesicle system which can use ATP as an energy source has not been obtained (Kaback & Hong, 1973). However, two other systems able to use ATP as an energy source have been described in membrane particles of *E. coli*, namely the energy-dependent quenching of atebrin fluorescence and the ATP-dependent reduction of NAD⁺ by succinate, DL- α -glycerophosphate and D-lactate (Haddock & Downie, 1974; Poole & Haddock, 1974). Our attempts to demonstrate the presence of the latter system in membrane particles of strains LT2 and HfrA were not successful, although the cells were grown, and the membrane particles prepared and assayed by the methods described by Poole & Haddock (1974). Energy-dependent quenching of atebrin fluorescence by the use of membrane particles of both strains is shown in Fig. 4 (traces 1 and 2). Oxidation of NADH resulted in the quenching of fluorescence, which then returned to near the starting value when the oxygen in the cuvette had been consumed. Then addition of ATP produced fluorescence quenching which could be reversed by dicyclohexylcarbodi-imide, an inhibitor of the (Mg²⁺+Ca²⁺)-activated ATPase (Evans, 1970). The behaviour of particles from strains LT2 and HfrA was similar, ATP being an effective energy donor for this reaction in both cases. This result contrasts with the ineffectiveness of ATP in driving the ATP-dependent transhydrogenase reaction in the same preparation of HfrA particles (Fig. 4, trace 4). The same preparation of particles from strain LT2 as

used in the fluorescence experiments showed ATP-dependent transhydrogenation of NADP⁺ by NADH (Fig. 4, trace 3).

Discussion

S. typhimurium HfrA is defective in one of the energy-dependent processes of the cell membrane, since it cannot carry out the ATP-dependent transhydrogenation of NADP⁺ by NADH. This reaction is also defective in *unc A*⁻ and *unc B*⁻ mutants of *E. coli*, but strain HfrA can be distinguished from this type of mutant, since it can grow on intermediates of the tricarboxylic acid cycle (Butlin *et al.*, 1971, 1973). Further, although strain HfrA resembles an *unc B*⁻ mutant in having a reconstitutively active ATPase it differs from this type in being able to carry out ATP-dependent ('anaerobic') transport of amino acids (Schairer & Gruber, 1973; Or *et al.*, 1973).

The defect in strain HfrA is associated with the membrane and not with the (Mg²⁺+Ca²⁺)-activated ATPase, since this enzyme appears to be like that of the wild-type strain, LT2. The ATPase appears to fulfil two roles, a structural and a catalytic role, in *S. typhimurium* as it does in *E. coli* (Rosen, 1973; Bragg & Hou, 1973) and in mitochondria (Fessenden-Raden, 1969). The structural function can be mimicked by dicyclohexylcarbodi-imide, whereas the catalytic role requires the active enzyme. Of interest is the fact that although the ATPase can interact with the membrane of strain HfrA to fulfil the structural role, as shown by the restoration of respiration-dependent transhydrogenation, and the catalytic role in the anaerobic transport of amino acids and in growth on tricarboxylic acid cycle acids, it cannot use ATP to drive transhydrogenation. This behaviour is different from the results of Schairer & Haddock (1972), Or *et al.* (1973), Prezioso *et al.* (1973) and Schairer & Gruber (1973), with *unc A*⁻ and *unc B*⁻ mutants. It seems unlikely that the inability of ATP to drive the transhydrogenase reaction in strain HfrA was due to disruption of this system during the preparation of the membrane particles, since the ATP-dependent quenching of atebrin fluorescence was unimpaired in this strain. Although it is not yet clear if quenching of the fluorescence of acridine dyes such as atebrin measures changes in a transmembrane pH gradient (Casadio *et al.*, 1974), or alterations in the interaction of the dye with the membrane (Fiolet *et al.*, 1974), it is believed to monitor energization of the membrane. Thus energization of the membrane by ATP as measured by fluorescence quenching or by active transport appears to involve a different pathway or mechanism from that for ATP-dependent transhydrogenation in spite of the fact that the (Ca²⁺+Mg²⁺)-activated ATPase is involved in all of these processes (Schairer & Haddock, 1972; Nieuwenhuis *et al.*, 1973; Cox *et al.*, 1971). This

result is difficult to reconcile with the chemiosmotic hypothesis of Mitchell (Mitchell, 1966; Harold, 1972) which postulates that ATP generates by one reaction pathway an energized state (protonmotive force) which can be used for all of the above processes.

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