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The Location of Purine Phosphoribosyltransferase Activities in *Escherichia coli*

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During the preparation of spheroplasts, adenine phosphoribosyltransferase (EC 2.4.2.7) and hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) were released in parallel with cytidine deaminase (EC 3.5.4.5) and uridine phosphorylase (EC 2.4.2.3), which, on other evidence, are considered to be located intracellularly. The two phosphoribosyltransferases and uridine phosphorylase were not significantly associated with purified membrane fractions as was purine nucleoside phosphorylase (EC 2.4.2.1). The effects of the poorly permeable enzyme-inactivating reagents, 4-diazoniumbenzenesulphonate, 7-diazonium-1,3-naphthalenedisulphonate and 2,4,6-trinitrobenzenesulphonate, on *Escherichia coli* indicate that all the above-mentioned enzymes and also the xanthine–guanine phosphoribosyltransferase [Miller, Ramsey, Krenitsky & Elion (1972) *Biochemistry* **11**, 4723–4731] are located intracellularly.

Appropriate phosphoribosyltransferases and the purine nucleoside phosphorylase facilitate the transport of adenine and hypoxanthine bases into *Escherichia coli*, although they are not essential for a limited transport to occur (Burton, 1977). A group-translocation mechanism involving phosphoribosyltransferases has been advocated (Hochstadt-Ozer & Stadtman, 1971*b*; Hochstadt, 1974), but the evidence for this process is now not considered compelling (Roy-Burman & Visser, 1975; Burton, 1977). Group translocation would require the enzymes to be situated in the plasma membrane, where they could be more readily attacked by inactivating probes or partially released by osmotic shock. Although Hochstadt-Ozer & Stadtman (1971*c*) have described the latter phenomenon for adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase, further study of the location of these enzymes by other methods seemed to be desirable.

Experimental

Bacterial growth

E. coli strain B was grown in minimal medium containing (per litre): 7g of K_2HPO_4, 3g of KH_2PO_4, 0.5g of trisodium citrate, 0.1g of $MgSO_4$, 1g of $(NH_4)_2SO_4$ and 0.2% glucose (Davis & Mingioli, 1950), except that a medium of 80mM-NaCl / 2μM-$FeCl_3$ / 20mM-KCl / 120mM-Tris / 0.14mM-sodium glycerophosphate / 0.2mM-$CaCl_2$ / 0.05% casein hydrolysate / 0.5mM-Na_2SO_4 / 0.2% glucose was used to induce high concentrations of alkaline phosphatase (Garen & Levinthal, 1960). Freshly grown bacteria, harvested in mid-exponential phase, were used in all experiments.

Chemicals and enzymes

Lysozyme, pancreatic deoxyribonuclease I, ribonuclease A and sodium 5-phosphoribosyl 1-pyrophosphate were from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey KT2 7BH, U.K.). Xanthine oxidase, 2,4,6-trinitrobenzenesulphonic acid and 4-aminobenzenesulphonic acid were from BDH (Poole, Dorset, U.K.). 7-Amino-1,3-naphthalenedisulphonic acid (technical grade; Aldrich Chemical Co., Gillingham, Dorset, U.K.) was recrystallized from aq. 80% (v/v) ethanol before use.

Radiochemicals

These were supplied by The Radiochemical Centre (Amersham, Bucks., U.K.), except for [8-^{14}C]-xanthine, which was prepared by deamination of [8-^{14}C]guanine with HNO_2 and purification by paper electrophoresis in 7% (v/v) formic acid.

Measurement of enzyme activity

A number of enzyme activities were measured spectrophotometrically, including alkaline phosphatase (Garen & Levinthal, 1960), uridine phosphorylase (Pardee & Watanabe, 1968) (20μl of a sonicated extract being substituted for toluene and bacterial cells), cytidine deaminase (Cohen & Wolfenden, 1971), ferricyanide reductase (Futai, 1974), succinate dehydrogenase [with 3mM-potassium ferricyanide as electron acceptor (King, 1963)] and purine nucleoside phosphorylase. The latter was assayed by following the increase in A_{286} in a mixture of 1ml of potassium phosphate buffer, pH 8.0 (47.35mM-K_2HPO_4/2.65mM-KH_2PO_4), 10μl of xanthine oxidase suspension (2 enzyme units per ml, where 1 unit

converts 1 μmol of hypoxanthine into urate/min at 25°C in phosphate buffer, pH 8.2), 20 μl of inosine (5 mg/ml) and 50 μl of sonicated extract. The inosine solution was freshly prepared and controls omitting sonicated extract were performed. Cytochrome concentrations were determined spectrophotometrically (Osborn *et al.*, 1972), but without deoxycholate treatment. In all assays, the response was proportional to the volume of extract used.

Uridine phosphorylase activity was also measured with 20 μl of 1 mM-[2-^{14}C]uridine (0.5 Ci/mol) in a mixture with 10 μl of potassium phosphate buffer, pH 6.6 (37.5 mM-K_2HPO_4/62.5 mM-KH_2PO_4), and 10 μl of extract. Samples (10 μl) were taken at intervals and added to tubes containing 10 μl of 4M-formic acid and 10 μg each of uridine and uracil. After 20 min at 0°C, the contents of each tube were spotted on to Whatman no. 1 paper and developed overnight by descending chromatography in butan-1-ol/water (43:7, v/v). The paper was dried and uracil and uridine were detected by u.v. light. The spots were cut out and radioactivity was measured by liquid-scintillation counting in 5 ml of toluene, which contained 0.6% 2,5-diphenyloxazole and 0.005% 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Phosphoribosyltransferase activities were determined at 37°C either (*a*) by the method of Hochstadt-Ozer & Stadtman (1971*a*), except that the concentrations were 0.08 M-Tris (adjusted to pH 7.8 with 1 M-HCl), 0.08 M-$MgCl_2$, 2 mM-sodium 5-phosphoribosyl 1-pyrophosphate and 0.5 mM of either [8-^{14}C]adenine or [^{14}C]-hypoxanthine (1.4 Ci/mol), AMP and IMP being separated by descending paper chromatography in 1 M-ammonium acetate, or (*b*) by the method of Schmidt *et al.* (1976), but with 0.2 M-Tris (adjusted to pH 7.5 with 1 M-HCl), 10 mM-$MgCl_2$, 1 mM-sodium 5-phosphoribosyl 1-pyrophosphate and 0.4 mM-radioactive base (1.4 Ci/mol) in 50 μl. For the xanthine–guanine phosphoribosyltransferase, procedure (*b*) was used, but with 60 μM-[8-^{14}C]xanthine (1.1 Ci/mol). Radioactivity in the chromatogram spots from procedure (*a*) or in filters from procedure (*b*) was measured, after drying, by liquid-scintillation counting as above.

Spheroplast formation and membrane purification

Bacteria were converted into spheroplasts by incubation with lysozyme and EDTA. Cells were first plasmolysed in 0.75 M-sucrose containing lysozyme, then diluted with 2 vol. of an EDTA solution as described by Osborn & Munson (1974). For the experiment described in Fig. 2, spheroplast preparations were aged by pipetting the diluted suspension into 5 vol. of 0.25 M-sucrose/0.06 M-Tris/0.1 M-NaCl/5 mM-$MgCl_2$, which had been adjusted to pH 7.5 with 1 M-acetic acid, and incubating the mixture at 21°C for 15 min. Spheroplasts were collected by centrifugation at 12000*g* for 15 min at 4°C, and the pellet was resuspended in the lysis buffer indicated in the legends to the Figures.

The course of spheroplast formation was followed by phase-contrast microscopy and preparations with less than 80% of the cells in the form of spheroplasts were discarded, except in the experiments described in Fig. 2.

Membrane fractions were purified by lysing spheroplasts with ice-cold water and spinning the membrane fraction across a discontinuous sucrose gradient as described by Osborn & Munson (1974). To prepare membrane vesicles as described by Kaback (1971), spheroplasts were centrifuged at 12000*g* for 15 min at 4°C and the pellet was resuspended in an equal volume of potassium phosphate buffer, pH 7.5 (81 mM-K_2HPO_4/19 mM-KH_2PO_4 adjusted with 1 M-KOH), containing 20% (w/v) sucrose, 20 mM-$MgSO_4$ and 5 mg of each of deoxyribonuclease and ribonuclease/ml. Resuspension was carried out as gently as possible with a wide-bore (no. 10) syringe needle. The resulting suspension was poured into approx. 500 vol. of 50 mM-potassium phosphate buffer, pH 6.6 (18.8 mM-K_2HPO_4/31.2 mM-KH_2PO_4), and further fractionated by the procedure of Kaback (1971).

Inverted membrane vesicles were prepared as described by Futai (1974) from bacteria broken in a French pressure cell. All membrane preparations were microscopically examined for contaminating bacteria and spheroplasts and only preparations that were free from both contaminants were used. Bacteria could be removed by resuspending the sample in the appropriate wash solution and centrifuging at 1500*g* for 30 min at 4°C.

Chemical probes

4-Diazoniumbenzenesulphonate was prepared by the method of Dilley *et al.* (1972) and 7-diazonium-1,3-naphthalenedisulphonate as described by Pardee & Watanabe (1968). Both solutions were diluted with phosphate-buffered saline (11 mM-KH_2PO_4/24 mM-Na_2HPO_4/0.1 mM-NaCl/0.04 mM-$MgCl_2$, pH 6.9) to give 12.5 mM-diazonium compound. The diazoniumnaphthalenedisulphonate was diluted only immediately before use and the pH was finally adjusted to 6.0 with 1 M-KOH.

Bacteria were resuspended in phosphate-buffered saline at 5.5 mg dry wt./ml and 2 vol. of the 12.5 mM-diazonium salt solution was added. The suspension was incubated at either 6 or 28°C and samples were taken at various times, diluted 10-fold with 0.05 M-Tris/acetate, pH 8.0 (0.25 M-sucrose being present for experiments involving spheroplasts), and centrifuged at 1500*g* for 15 min at 4°C. The pellet thus obtained was washed with 50 mM-potassium phosphate buffer, pH 8.0, and resuspended in 9 vol. of the buffer. The

sample was sonicated with an MSE 100 W ultrasonic disintegrator for 3×15s with intermediate cooling for 30s periods in an ice bath and usually clarified by centrifuging at 12000*g* for 30min at 4°C.

For each experiment with a diazonium reagent, two other solutions were prepared. One consisted of the reagents used for the diazotization, but with the appropriate amino compound added after the destruction of the HNO_2. The other was a sample of the diazonium reagent that had been heated to destroy any activity (e.g. towards phenol and 1,3-dihydroxybenzene). When diluted in the same manner as the fresh reagent, neither of these two solutions caused any inactivation of the enzyme activities examined.

Bacteria (2.2mg dry wt./ml) were treated with 2,4,6-trinitrobenzenesulphonate (15mM) dissolved in 120mM-$NaHCO_3$/40mM-NaCl and adjusted to pH 8.6 with NaOH. The reaction mixture was incubated at 6°C and samples were taken at various times, diluted 100-fold with the $NaHCO_3$/NaCl and centrifuged at 1500*g* for 15min at 4°C. The pellet was washed, resuspended and sonicated as above.

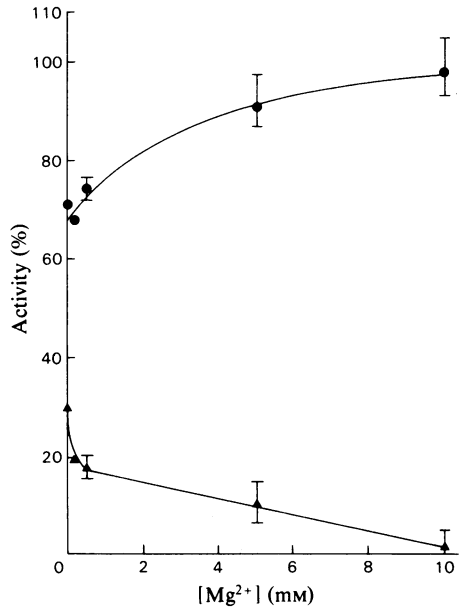

Fig. 1. *Release of cytidine deaminase activity by osmotic shock as a function of the Mg^{2+} concentration in the shock medium*

●, Activity retained in cells after osmotic shock by the procedure of Nossal & Heppel (1966); ▲, activity recovered from shock fluid. Mean values from five experiments are plotted as a percentage of the mean activity recovered from unshocked cells. The bars represent the range of values obtained.

Results

Location of reference enzyme activities

This study is concerned with the localization of the purine phosphoribosyltransferase activities, but a number of other enzymes have been examined, of which the location is fairly certain. The criteria for the assumed positions of these activities are given below.

Cytidine deaminase and uridine phosphorylase have been identified as intracellular by their low release after osmotic shock (Munch-Petersen & Mygind, 1976), by their lack of reaction with chemical probes in intact bacteria (Pardee & Watanabe, 1968; Taketo & Kuno, 1972), by their retention during spheroplast formation (Beacham *et al.*, 1971; Taketo & Kuno, 1972) and by their position relative to a genetically determined permeability barrier (Munch-Petersen & Mygind, 1976). Some uridine phosphorylase and cytidine deaminase activity is released on osmotic shock (Taketo & Kuno, 1972) and, in particular, cytidine deaminase is released in large amounts when the shock medium is water (Munch-Petersen & Mygind, 1976). This phenomenon has been noted for other proteins that are not thought to be located in the periplasm (Jacobson *et al.*, 1976) and would appear to be the result of damage to the inner membrane, resulting in the loss of cytoplasmic constituents. The presence of Mg^{2+} in the shock medium greatly decreases the release (Fig. 1). Osmotic-shock data would appear to be a reliable indicator of periplasmic localization only if 80% or more of the enzyme is released in the presence of Mg^{2+}.

Purine nucleoside phosphorylase is indicated to be intracellular because it is not released to any great extent during osmotic shock and because it is not accessible to external substrates in mutants that are defective in nucleoside transport (Munch-Petersen & Mygind, 1976).

Alkaline phosphatase is generally recognized as being extracellular and it is readily lost during osmotic shock and spheroplast formation (Neu & Heppel, 1965; Jacobson *et al.*, 1976).

Release during spheroplast formation

When spheroplasts are made by digestion with lysozyme in the presence of a metal-ion-chelating reagent, a number of the periplasmic enzymes (Heppel, 1971) are released during the course of the incubation (Neu & Heppel, 1965; Kaback, 1971). This release has been used as a less-severe method than osmotic shock for examining the location of enzyme activities in the bacterial cell (Beacham *et al.*, 1971; Taketo & Kuno, 1972; Jacobson *et al.*, 1976).

Although free from the tendency to cause marked release of intracellular enzymes, periplasmic enzymes may be only partially released by this method (Table 1). Care must be taken to ensure that a good yield of spheroplasts has been achieved, while avoiding undue lysis.

Neither the adenine nor the hypoxanthine phosphoribosyltransferase was released to a considerable extent during the formation of spheroplasts or during prolonged incubation in the presence of lysozyme and EDTA (Table 1, Fig. 2). Adenine phosphoribosyltransferase activities (Table 1) were measured in cell extracts prepared in a French press because sonication gave low activities of this enzyme in samples that contained considerable numbers of whole cells.

Purine nucleoside phosphorylase is not normally released during spheroplast formation, yet selective release can occur when lysis is promoted by aging at room temperature (21°C) for 15 min (Fig. 2).

Table 1. *Recovery of enzyme activities from spheroplast preparation*

Spheroplasts were prepared and harvested as described in the Experimental section. Harvested samples were resuspended in a medium containing 0.01 M-Tris, 5 mM-$MgCl_2$, 10 μg of deoxyribonuclease/ml and 10 μg of ribonuclease/ml adjusted to pH 7.8 with 1 M-HCl, lysed by passage through a French pressure cell at 207 MPa, and then centrifuged at 12000*g* for 15 min at 4°C. Adenine phosphoribosyltransferase was assayed as described by Schmidt *et al.* (1976). Uridine phosphorylase was assayed spectrophotometrically. Results are means of two samples.

Enzyme	Recovery from supernatant of the lysozyme/EDTA digestion (% of recovered activity)
Adenine phosphoribosyltransferase	4
Uridine phosphorylase	6
Cytidine deaminase	5
Alkaline phosphatase	86

Table 2. *Enzyme activities recovered from purified membranes*

Spheroplasts were lysed by the addition of water and the membrane fraction was collected by centrifugation as described by Osborn & Munson (1974). The pellet was resuspended in ice-cold 0.25 M-sucrose/3.3 mM-Tris/1 mM-EDTA, pH 7.8, and re-centrifuged. The membranes were resuspended in 1% Triton X-100/0.05 M-potassium phosphate, pH 8.0, for assay. Phosphoribosyltransferase activities were assayed by the method of Hochstadt-Ozer & Stadtman (1971*a*) and uridine phosphorylase with [2-^{14}C]-uridine. Duplicate assays were performed on each fraction.

Enzyme	Recovery from membrane fraction (% of total recovered activity)
Purine nucleoside phosphorylase	38
Uridine phosphorylase	1.3
Cytidine deaminase	0
Adenine phosphoribosyltransferase	0.3
Hypoxanthine phosphoribosyltransferase	0.2

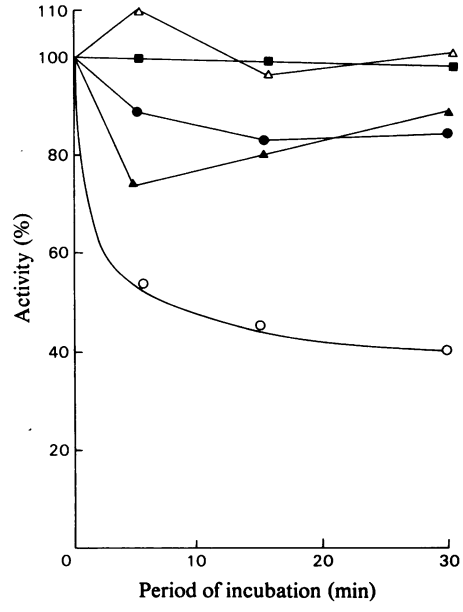

Fig. 2. *Retention of enzyme activities during spheroplast formation and subsequent aging at* 21°C

Bacteria were treated with lysozyme and EDTA in sucrose at 0°C. At intervals, the treated cells were collected by centrifuging either immediately or after dilution and incubation at 21°C for 15 min (for aging, see the text). The pellets were suspended in nine times their volume of 47 mM-K_2HPO_4/3 mM-KH_2PO_4, pH 8.0, and sonicated with an MSE 100 W ultrasonic disintegrator for 3 × 15 s periods with intermediate cooling in ice. ■, Cytidine deaminase, purine nucleoside phosphorylase and hypoxanthine phosphoribosyltransferase in samples not aged at 21°C. Activities in aged samples: ●, cytidine deaminase; ○, purine nucleoside phosphorylase; ▲, hypoxanthine phosphoribosyltransferase; △, uridine phosphorylase. Values are means from duplicate activity measurements plotted against period of incubation with lysozyme and EDTA.

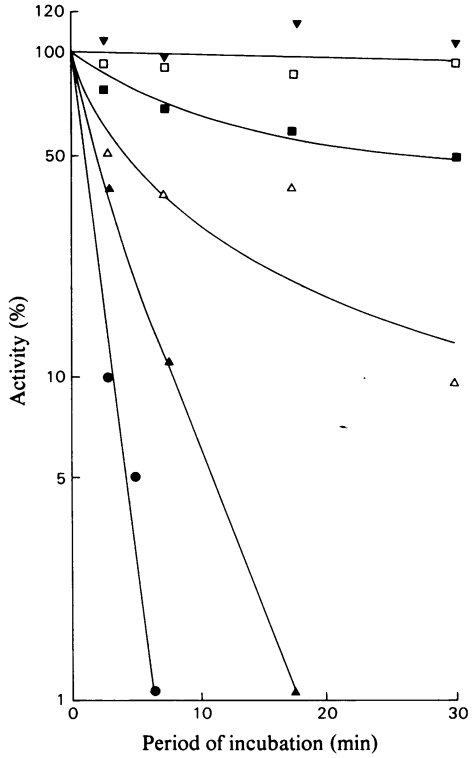

Fig. 3. *Inactivation of enzymes in bacteria by 4-diazoniumbenzenesulphonate*

Activities after treatment with 8mM-diazonium salt: uridine phosphorylase in cells treated at 6 (□) and 28°C (■); purine nucleoside phosphorylase in cells treated at 6 (△) and 28°C (▲); adenine phosphoribosyltransferase in cells treated at 6°C (▼). Activity of hypoxanthine phosphoribosyltransferase (●) in cells treated with 0.08mM-diazonium salt at 6°C. Activities are given as a percentage of those in untreated bacteria.

Retention by membrane fractions

A low release during spheroplast formation could be due to either an intracellular location or a specific attachment to the cytoplasmic membrane. The adenine and hypoxanthine phosphoribosyltransferases did not show any marked affinity for membrane preparations obtained by lysis of spheroplasts (Tables 2 and 3). Purine nucleoside phosphorylase showed greater affinity for the membrane fractions, and especially for the inner-membrane fractions (Table 3). The membrane location of this enzyme may perhaps explain the selective release by aging seen in Fig. 2.

Membrane-vesicle preparations with either the outer surface (Kaback, 1971) or the inner surface (Futai, 1974) of the cytoplasmic membrane predominantly exposed, retain less than 0.4% of the adenine phosphoribosyltransferase activity (Table 4). Particular care was taken to exclude unbroken bacteria or spheroplasts from the preparations. The salt washes used normally remove non-specifically bound activities without damaging the membrane structure (Kaback, 1971; Dewald *et al.*, 1974).

Reaction with chemical probes

4-Diazoniumbenzenesulphonate and 7-diazonium-1,3-naphthalenedisulphonate have been used as non-penetrating probes for enzyme localization in erythrocytes, chloroplasts, mitochondria and bacteria (Pardee & Watanabe, 1968; Berg, 1969; Taketo & Kuno, 1972; Dilley *et al.*, 1972; Tinberg *et al.*, 1974). It has been argued that those enzymes that react rapidly with the reagents are exposed on the outside of the cytoplasmic membrane, whereas others (the non-reactive set) are protected by the membrane structure (Pardee & Watanabe, 1968). 4-Diazoniumbenzenesulphonate slowly inactivates some intracellular activities, presumably because of a degree of

Table 3. *Localization of enzyme activities from purified membrane fractions*

Membranes were prepared by the method of Osborn & Munson (1974) as described in the Experimental section. H is the dense outer membrane, and L_1 and L_2 are the less-dense inner-membrane fractions, identified by their position in the density gradient after centrifugation for 20h. Phosphoribosyltransferases and uridine phosphorylase were measured as for Table 2. Duplicate assays were performed on each fraction. Membrane fractions were resuspended in 1% Triton X-100/0.05mM-potassium phosphate, pH 8.0. Values in parentheses indicate the percentages of the enzyme activity that were subjected to the density-gradient step and subsequently found in the particular fraction.

Membrane fraction	Activity (nmol/min per μg of protein)				Cytochrome (% of total protein in fraction)
	Purine nucleoside phosphorylase	Uridine phosphorylase	Adenine phosphoribosyltransferase	Hypoxanthine phosphoribosyltransferase	
L_1	2.3 (32)	0.004 (6)	0.045 (4)	0.03 (6)	10
L_2	1.8 (35)	—	0.0625 (8)	—	7.9
H	0.12 (3)	0.0007 (3)	0.03 (5)	0.0075 (3)	0.5

Table 4. *Concentrations of adenine phosphoribosyltransferase in membrane preparations*
Method 1 was that of Kaback (1971) in which the vesicle preparation was washed twice with 0.1M-potassium phosphate/10mM-EDTA, pH 6.6. In method 2, the unwashed vesicle preparation was resuspended in the first wash solution (0.25M-sucrose/0.15M-NaCl/5mM-Tris, adjusted to pH 8.0 with 1M-HCl), shaken for 5min at 4°C and then re-centrifuged at 108500*g* for 90min. The pellet was then resuspended in the second wash solution (0.1M-potassium phosphate/10mM-EDTA, pH 6.6), shaken and re-centrifuged. For method 3, everted vesicles were prepared by the method of Futai (1974), but washed as for Method 2. Adenine phosphoribosyltransferase was assayed by the method of Hochstadt-Ozer & Stadtman (1971*a*). The values in parentheses represent the percentages of total activity in the original cell suspension. Each value is the mean from two experiments.

Method of preparation	Enzyme activity in pellet (nmol/min per mg of protein)		
	Unwashed	After wash 1	After wash 2
1	—	—	5.3 (0.4)
2	—	21.3 (0.88)	7.0 (0.3)
3	24.2 (1.1)	9.8 (0.4)	6.3 (0.2)

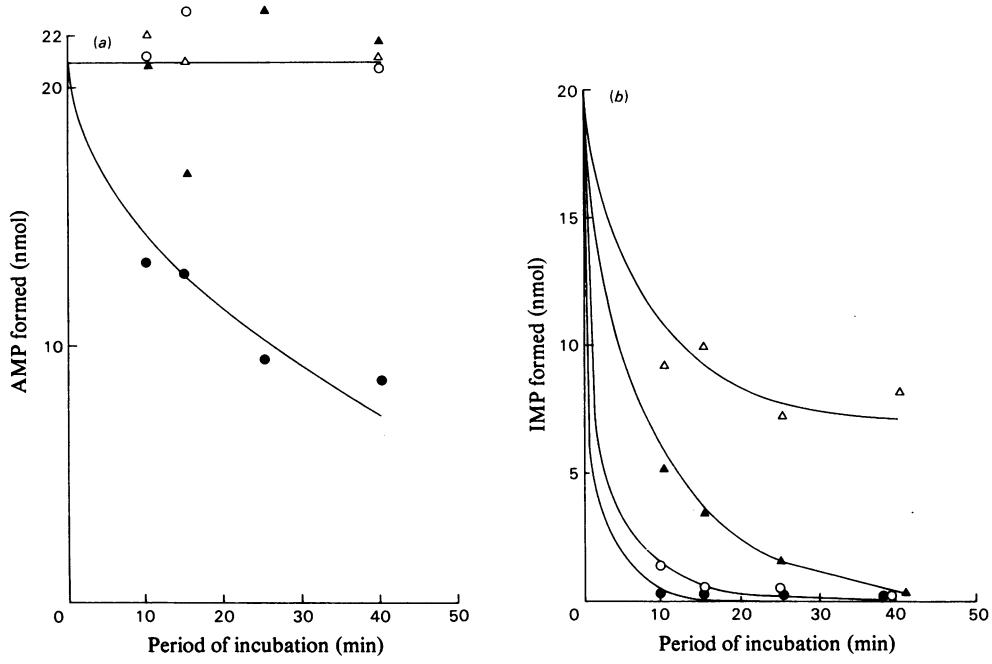

Fig. 4. *Inactivation of purine phosphoribosyltransferases in a bacterial extract by 4-diazoniumbenzenesulphonate*
A sonicated extract, equivalent to 22mg dry wt. of bacteria/ml, was treated with 2 vol. of diazonium salt solution and incubated on ice, for the time shown. A sample was taken and added to the appropriate phosphoribosyltransferase assay mixture [method (*b*) in the text]. The reaction mixture was incubated for 8min before quenching with 5ml of the appropriate wash solution (1mM-ammonium acetate containing 100μM-purine at pH 5.0). Final concentrations of diazonium salt were: ●, 8mM; ○, 0.8mM; ▲, 16μM; △, 0.8μM. (*a*) Adenine phosphoribosyltransferase; (*b*) hypoxanthine phosphoribosyltransferase.

penetration of the membrane, since the larger more highly charged naphthalene derivative causes less inactivation in whole bacteria at 6°C (Pardee & Watanabe, 1968). Nevertheless, neither reagent can be considered to be completely non-penetrating, since all the enzyme activities studied could be destroyed by both reagents in intact cells at 37°C.

Although cytidine deaminase, uridine phosphorylase and adenine phosphoribosyltransferase are completely immune to 4-diazoniumbenzenesul-

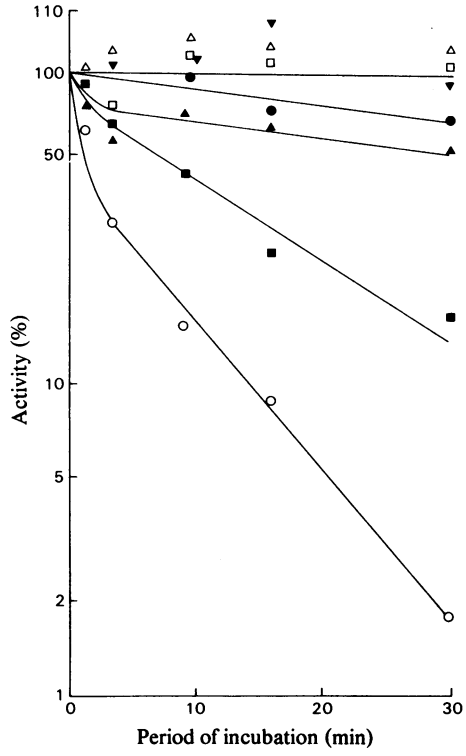

Fig. 5. *Inactivation of enzymes in bacteria by 7-diazonium-1,3-naphthalenedisulphonate*

Activities after treatment with 8mM-diazonium salt: purine nucleoside phosphorylase in cells treated at 6 (△) and 28°C (▲); adenine phosphoribosyltransferase in cells treated at 6°C (▼); xanthine–guanine phosphoribosyltransferase in cells treated at 6 (□) and 28°C (■); hypoxanthine phosphoribosyltransferase in cells treated at 6 (●) and 28°C (○). Results are expressed as percentages of the activity in untreated cells and are plotted on a logarithmic scale.

phonate in intact bacteria at low temperatures, purine nucleoside phosphorylase is slowly destroyed and hypoxanthine phosphoribosyltransferase activity is rapidly abolished (Fig. 3). This would suggest an external localization for the hypoxanthine enzyme were it not for the fact that, in a sonicated extract, this enzyme is inactivated by the reagent at least 500 times more readily than is adenine phosphoribosyltransferase (Fig. 4). The loss of purine nucleoside phosphorylase activity from intact bacteria shows that some reagent must penetrate the membrane even at 6°C and this could therefore attack intracellular hypoxanthine phosphoribosyltransferase.

In whole bacteria, at 6°C, 7-diazonium-1,3-naphthalenedisulphonate hardly reacts at all with the three purine phosphoribosyltransferases or with purine nucleoside phosphorylase. At 28°C there is marked progressive inhibition of hypoxanthine phosphoribosyltransferase activity and, more slowly, of the xanthine–guanine phosphoribosyltransferase (Fig. 5). These results indicate an internal localization for all four enzymes, the inactivation of the 6-hydroxypurine enzymes in whole cells at 28°C being explained by a small penetration of the membrane by the reagent. The xanthine–guanine phosphoribosyltransferase (Miller *et al.*, 1972) would appear to resemble the hypoxanthine enzyme in that it is very susceptible to inactivation by the diazonium reagents.

The protection of enzyme activities, particularly

Table 5. *Exposure of enzyme activities to diazonium compounds in spheroplasts*

Spheroplasts were prepared as described in the Experimental section. Samples were taken when the yield was at least 90% and treated for 10min at 6°C with the diazonium salt in the presence of 0.25M-sucrose. Controls consisted of samples with a solution lacking the diazonium salt. Uridine phosphorylase was assayed spectrophotometrically and phosphoribosyltransferase activities were assayed as described by Hochstadt-Ozer & Stadtman (1971*a*). Adenine phosphoribosyltransferase activities were reproducibly higher in diazonium-treated spheroplast preparations than in untreated samples from the same preparation. Values are the percentage losses of enzyme activity resulting from treatment with the reagent.

	4-Diazoniumbenzenesulphonate		7-Diazonium-1,3-naphthalenedisulphonate	
	Spheroplasts	Whole cells	Spheroplasts	Whole cells
Purine nucleoside phosphorylase	19.5	30	5.6	6.1
Uridine phosphorylase	0	0	4.0	0
Cytidine deaminase	0	0	0	0
Hypoxanthine phosphoribosyltransferase	57.3	60.6	15	5
Adenine phosphoribosyltransferase	−30	0	−25	0

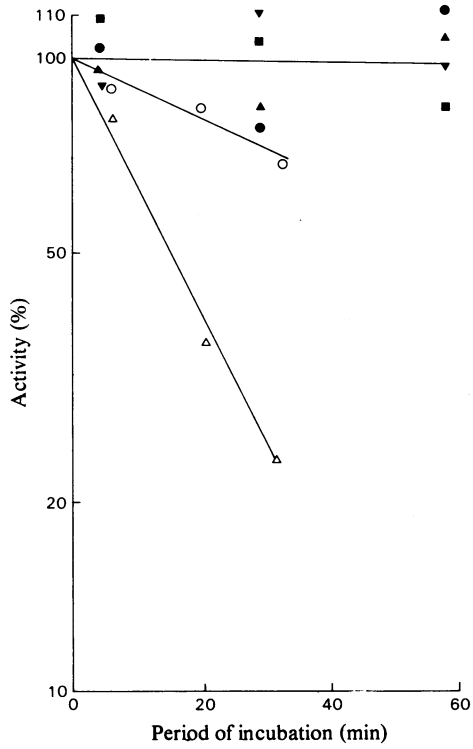

Fig. 6. *Time course of the reaction of* 2,4,6-*trinitrobenzenesulphonate with intact cells and a sonicated extract*
The reaction in the sonicated extract was carried out as described in the legend to Fig. 4. Results are expressed as percentage of the activity in the corresponding untreated sample. Activities in intact cells treated with 15mM-trinitrobenzenesulphonate: ●, hypoxanthine phosphoribosyltransferase; ▼, adenine phosphoribosyltransferase; ■, uridine phosphorylase; ▲, purine nucleoside phosphorylase. Hypoxanthine phosphoribosyltransferase activities in a sonicated extract treated with 1mM- (○) or 10mM- (△) trinitrobenzenesulphonate.

against the larger naphthalene derivative, might be ascribed to the outer lipopolysaccharide layer decreasing the penetration of the reagent (Beacham *et al.*, 1973; Decad & Nikaido, 1976; Gustafsson *et al.*, 1973). However, the relevant enzyme activities are also protected in spheroplasts (Table 5), suggesting that the lipopolysaccharide has no important effect.

2,4,6-Trinitrobenzenesulphonic acid has been used as a label for the primary amino groups of lipids and proteins (Rothman & Kennedy, 1977; Gordesky *et al.*, 1975). At 6°C it does not penetrate the bacterial membrane, although it may do so at higher temperatures (Rothman & Kennedy, 1977). In a sonicated extract 15mM-trinitrobenzenesulphonate removed hypoxanthine phosphoribosyltransferase activity (Fig. 6) and incubation for 1h with 1.5mM-trinitrobenzenesulphonate completely destroyed activity of the hypoxanthine and adenine phosphoribosyltransferases and of the two nucleoside phosphorylases. When whole cells were treated with 15mM-trinitrobenzenesulphonate at 6°C, no inhibition of the enzyme activities was observed (Fig. 6), which is consistent with an internal localization for both adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase.

Discussion

Despite the evidence from release by osmotic shock, the examination of release during spheroplast formation and the action of chemical probes shows that the purine phosphoribosyltransferases are located internally. Osmotic shock causes partial, and occasionally major, release of other enzymes, which are shown to be internally located by more compelling evidence (Taketo & Kuno, 1972; Jacobson *et al.*, 1976). Our studies give no substantial evidence for any membrane association of the three purine phosphoribosyltransferase activities, but purine nucleoside phosphorylase showed a marked association with the cytoplasmic membrane fractions. Purine nucleoside phosphorylase has been reported to lie behind a permeability barrier that is genetically determined (Munch-Peterson & Mygind, 1976). These facts, coupled with its behaviour during spheroplast formation and aging and its low susceptibility to diazonium reagents in whole cells, support the suggestion of Nygaard (1977) that purine nucleoside phosphorylase may normally be organized on the internal surface of the cytoplasmic membrane.

Some residual membrane-bound phosphoribosyltransferase activity does occur, but this is easily removed by salt or EDTA washes that do not remove the activity of ferricyanide reductase or succinate dehydrogenase. Thus the purine phosphoribosyltransferases appear to be located in the bacterial cytoplasm. The findings do not support the group-translocation proposals of Hochstadt-Ozer & Stadtman (1971*b*) and Hochstadt (1974) for purine transport in *E. coli*. An alternative possibility, namely that the phosphoribosyltransferases are physically associated with the appropriate transport systems and so control purine uptake, is also not supported.

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