The Activity and Specificity of the Proline Permease in Wild-type and Analogue-resistant Strains of *Escherichia coli*

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SUMMARY

The main characteristics of the previously described proline-specific transport mechanism (permease) of *Escherichia coli* were confirmed in strain C4. The same permease was responsible for entry of a number of proline analogues, including 3,4-dehydroproline, 4-methyleneproline, *cis*- and *trans*-4-chloroprolines, thiazolidine-4-carboxylic acid (thioproline) and the lower homologue, azetidine-2-carboxylic acid. These analogues also entered the cells by an exchange reaction between extracellular analogue and previously accumulated intracellular proline. Growth of the parent (C4) strain was inhibited by 3,4-dehydroproline and azetidine-2-carboxylic acid, both of which were incorporated into cellular protein. Several classes of mutants, selected for resistance to either dehydroproline or azetidine, failed to incorporate one or both analogues into protein. Some of these mutants owed their resistance to failure to produce a functional proline permease. At least one strain, resistant to azetidine but not to dehydroproline, possessed an altered permease with little affinity for azetidine-2-carboxylic acid but still capable of transporting proline and 3,4-dehydroproline; the permease of this strain could no longer promote exchange between intracellular proline and extracellular proline or proline analogues.

INTRODUCTION

Exposure of bacterial cells to a wide variety of structural analogues of 'protein' amino acids results in inhibition of growth, frequently accompanied by incorporation of the analogue into the proteins of the organism (Cohen & Munier, 1959; Munier & Cohen, 1959; Richmond, 1962; Fowden, Lewis & Tristram, 1967). Recently, two analogues of proline have been investigated, namely, azetidine-2-carboxylic acid (a 'non-protein' amino acid occurring naturally in certain higher plants) and 3,4-dehydroproline. These compounds are strongly growth-inhibitory to *Escherichia coli* and some higher plants, inhibition being specifically annulled by proline. Both analogues are incorporated into the proteins of these organisms, stoichiometrically replacing proline residues (Smith, Ravel, Skinner & Shive, 1962; Fowden & Richmond, 1963; Fowden, Neale & Tristram, 1963; Peterson & Fowden, 1965). Incorporation of dehydroproline into the alkaline phosphatase of *E. coli* has been shown to result in a marked increase in thermolability of the enzyme (Neale & Tristram, 1964). Thiazolidine-4-carboxylic acid (thioproline) also inhibits growth and is incorporated into the proteins of *E. coli* (Beerstecher, 1950; Unger & DeMoss, 1966). The biological activi-

ties of other proline analogues have recently been reviewed (Mauger & Witkop, 1966; Fowden *et al.* 1967).

Numerous examples of the development of resistance to growth-inhibitory amino acid analogues have been described (Moyed, 1964; Richmond, 1965; Fowden *et al.* 1967). The mechanisms whereby resistance is acquired vary; one type of resistance involves mutation leading to failure of the analogue to penetrate into the cell, thereby excluding the analogue from the site (or sites) at which toxicity is exerted. Several investigators have described the isolation of analogue-resistant bacterial strains which failed to accumulate either the analogue or the corresponding natural amino acid (Schwartz, Maas & Simon, 1959; Ferroluzzi-Ames, 1964; Shifrin, Ames & Ferroluzzi-Ames, 1966; Lester, 1966). These observations not only provide an explanation of the mechanism of resistance, but also demonstrate that the analogues enter the cell by the specific transport mechanisms normally responsible for entry of the corresponding natural amino acids. The latter conclusion was also reached in a study of the uptake of phenylalanine and its analogue, *p*-fluorophenylalanine by *Escherichia coli* (Kempner & Cowie, 1960).

A specific energy-dependent transport mechanism (permease) permitting the accumulation of proline by *Escherichia coli* has been described (Britten & McClure, 1962; Britten, 1965; Kessel & Lubin, 1962). Strains deficient in proline permease activity have been obtained (Lubin, Kessel, Budreau & Gross, 1960; Kessel & Lubin, 1962). Evidence will be presented that 3,4-dehydroproline, azetidine-2-carboxylic acid and some other proline analogues are accumulated in the cell by the proline permease and that, compared with the parent strain, some of the mutants resistant to the two named analogues have an impaired ability to concentrate proline. A preliminary account of some of these experiments has already been presented (Neale & Tristram, 1965).

METHODS

Chemicals. L-Proline, *N*-acetyl-DL-proline and glycyl-L-proline were obtained from the Sigma Chemical Company; D-proline, L- and D-azetidine-2-carboxylic acid from Calbiochem; L-thiazolidine-4-carboxylic acid, DL-pipecolic acid, 4,5-dehydro-DL-pipecolic acid, 4-methylene-DL-proline, *cis*- and *trans*-4-chloro-L-proline were the gift of Professor L. Fowden, F.R.S.; 3,4-dehydro-DL-proline was a gift from Dr B. Witkop and Professor A. V. Robertson. Labelled amino acids were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. All amino acids and analogues were checked for purity by chromatography. Where possible, all other chemicals were 'Analar' grade, obtained from British Drug Houses Ltd.

Media. The basal glucose mineral salts medium (designated 232G) described by Tristram (1960) was used throughout the investigation. For selection of analogue-resistant mutants, the medium was supplemented with 25 μg. 3,4-dehydro-DL-proline/ ml. (232G/DHP), or 25 μg. L-azetidine-2-carboxylic acid/ml. (232G/Az). Solid media were prepared by addition of 2 % (w/v) Bacto agar. The effect of analogues on growth was measured in shaken cultures at 37° as described by Neale & Tristram (1963).

Organisms. Escherichia coli C4 (a strain derived from K-10), isolated and described by Echols, Garen, Garen & Torriani (1961), was used as the source of analogue-resistant strains described below. Strains resistant to 3,4-dehydroproline were isolated by plating between 10^8 and 10^9 bacteria of strain C4 (without exposure to mutagens) on

232 G/DHP agar. Similar attempts to isolate azetidine-resistant strains by plating on 232 G/Az resulted, after a lag, in a sparse 'lawn' of growth which covered the plates. Accordingly, strains resistant to this analogue were obtained by repeated passage of *E. coli* C4 through 232 G/Az liquid medium, followed by plating on 232 G/Az agar to obtain individual colonies. Resistant colonies were picked from 232 G/DHP and 232 G/Az plates, purified by streaking on the same media and maintained on slopes of 232 G/DHP or 232 G/Az agar in which the glucose concentration was reduced to 0·1 % (w/v) to prevent development of highly acid conditions. Mutants were allocated numbers prefixed by 'DHP' or 'AZ', denoting strains selected for resistance to 3,4-dehydroproline or azetidine-2-carboxylic acid respectively.

Excretion of proline by analogue-resistant mutants was detected on double-layer plates consisting of a lower layer of 232 G agar covered by a thin layer of the same medium in which was suspended about 10^{10} organisms of *Escherichia coli* strain H 208 (a proline-dependent derivative of K 12, kindly provided by Dr K. Fisher). Mutants were streaked on such plates and proline excretion detected by cross-feeding of the auxotrophic strain suspended in the agar.

Accumulation of amino acids; measurement of exchange between intra- and extra-cellular amino acids. The procedure for measurement of the uptake of amino acids was essentially that described by Britten & McClure (1962). Unless otherwise stated, uptake was followed in continuously aerated suspensions containing 125 μg. dry wt. organism/ml. and maintained at 10°. In those experiments involving uptake in the presence of chloramphenicol, exponential cultures growing in 232 G at 37° were cooled to 10° and, when necessary, diluted with 232 G medium, followed by addition of chloramphenicol (in 232 G medium) to give a final concentration of 150 μg./ml. The suspensions were distributed in suitable amounts in Pyrex tubes, aerated with a stream of water-saturated air and used within 15 min. of addition of chloramphenicol. For experiments not involving addition of chloramphenicol, cultures growing exponentially at 37° were transferred to 10° and aeration continued for 2 hr before dilution and distribution as already described. Additions of amino acids and/or analogues were made in volumes not exceeding 1 % (v/v) of the total volume of suspensions. Total intracellular radioactivity was measured by filtration of 1 ml. samples through Millipore filters (type HA; 23 mm. diameter) held in stainless steel holders (Tracerlab) and covered, immediately prior to receiving the sample, with 2 ml. ice-cold 232 G medium. After filtration, which occupied about 5 sec., filters were transferred to aluminium or stainless steel planchets, held in position by a slip-on retaining ring (Tracerlab), dried over calcium chloride and counted by use of a thin end-window G-M tube (Isotope Developments Ltd., England). In all instances at least 1000 counts over background were obtained and, where necessary, counts were converted into μmoles amino acid by calculation from specific radioactivities and the known efficiency of the counting equipment. For kinetic studies, the amount of radioactivity taken up by suspensions could be determined at 20 sec. intervals and the initial rates of uptake were calculated by the method of Algranati (1963).

In earlier experiments involving exchange between accumulated radioactive proline and extracellular unlabelled proline or analogue, the bacteria were 'loaded' by exposure to $1·22 \times 10^{-7}$ M- or 2×10^{-6} M-L-[U-^{14}C]proline for 20 min. or 40 min., respectively, at 10° in aerated 232 G medium containing chloramphenicol (150 μg./ml.). Later exchange experiments involved exposing bacteria, suspended in 232 G

medium containing chloramphenicol, to 1.22×10^{-7} M-L-[U-^{14}C]proline for 10 min. at 25°. The suspensions were then cooled to 0° with continuous aeration for 15 min., followed by centrifugation at 0° (10,000 g; 10 min.) or rapid filtration through HA Millipore membrane filters (47 mm. diameter) and resuspension of the cells in fresh 232G medium, previously cooled to 0° containing chloramphenicol (150 μg./ml.). Aeration at 0° was continued for at least a further 30 min. before exchange commenced. In both procedures the exchange reaction was initiated by rapid addition of a suitable sample of preloaded bacteria to Pyrex tubes already containing a solution of the compound to be tested for exchange activity. Samples were filtered and intracellular radioactivity measured as described above.

Incorporation of amino acids and analogues into protein; extraction of free amino acid pool. Incorporation of analogues into protein was determined after acid hydrolysis of the 'principal protein fraction' (Roberts *et al.* 1955) of dehydroproline-containing protein or, for azetidine-containing protein, by alkaline hydrolysis, as described previously (Neale & Tristram, 1963). Proline in hydrolysates was assayed after electrophoresis on Whatman 3MM paper (6000 V.; 30 mA.; formic + acetic acid buffer, pH 2, containing 61·2 ml. formic acid and 97·2 ml. acetic acid per litre of solution), followed by spraying with isatin and comparison of spot intensities with those of standard proline solutions, using a 'Chromoscan' (Joyce-Loebl and Co. Ltd., Newcastle-on-Tyne, England) fitted with filter 595 (A. Hussain, personal communication). 3,4-Dehydroproline was assayed on the same electrophoretogram by over-spraying with Ehrlich's reagent (Fowden, Neale & Tristram, 1963) and scanning as described above. Azetidine-2-carboxylic acid in hydrolysates was determined after two-dimensional chromatography, as described by Fowden & Richmond (1963).

Before extraction of the free amino acid 'pool', bacterial suspensions were centrifuged (20,000 g; 15 min.) at the temperature at which amino acid accumulation occurred (to avoid changes in 'pool' size due to temperature shock). The pellet of bacteria was resuspended in 70 % (v/v) ethanol in water to extract the amino acid 'pool' and, after overnight storage at 4°, cell debris was removed by centrifugation and the constituents of the ethanol-soluble fraction separated and detected by conventional chromatography or by electrophoresis as already described. Radioautography was carried out on 'Kodirex' X-ray film.

Incorporation of radioactivity into trichloroacetic acid-insoluble material was determined as described by Neale & Tristram (1963).

<center>RESULTS</center>

<center>*Growth in the presence of analogues*</center>

In common with many amino acid analogues (see reviews by Richmond, 1962; Fowden *et al.* 1967), addition of 3,4-dehydro-DL-proline (25 μg./ml.) or L-azetidine-2-carboxylic acid (20 μg./ml.) to cultures of *Escherichia coli* strain C4 growing exponentially in 232G medium resulted in 'linear' growth, i.e. whereas the extinction of control cultures increased exponentially, in the presence of the analogue increase in extinction was arithmetic. In cultures containing azetidine, onset of linear growth was virtually immediate. However, in cultures receiving dehydroproline there was little effect on growth for the first 40 min. of exposure, after which linear increase in optical density was established (Fig. 1 *a, b;* see also Fowden *et al.* 1963).

Addition of analogues to exponentially growing cultures of suitable bacterial concentration was advocated by Cohen & Munier (1959) and allows a sufficient increase in dry weight of organism to facilitate isolation and study of incorporation of analogues into cell protein. When either 3,4-dehydro-DL-proline (25 μg./ml.) or L-azetidine-2-carboxylic acid (20 μg./ml.) were added to 232G medium at the same time as inoculation with a small inoculum (about 5 × 10⁵ bacteria/ml.), then detectable growth was completely inhibited for at least 12 hr. Addition of much higher (400 μg./ml.) concentrations of L-thiazolidine-4-carboxylic acid yielded a curve similar to that

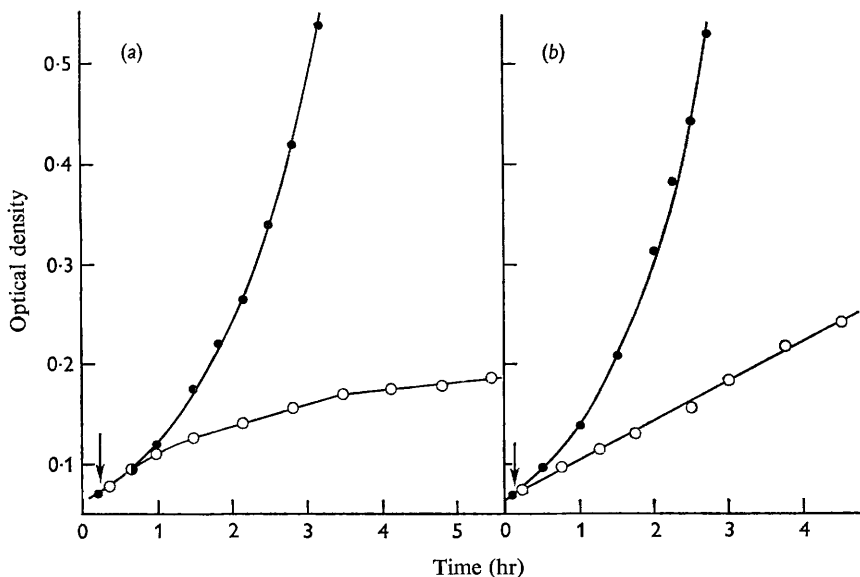Fig. 1. The effect of addition of (*a*) L-azetidine-2-carboxylic acid (20 μg./ml.) and (*b*) 3,4-dehydro-DL-proline (25 μg./ml.) to exponential cultures of *Escherichia coli* C4 growing in 232G medium at 37°. Analogues were added at points indicated by arrows. ●—●, Control (no analogue); ○—○, plus analogue.

obtained on addition of 3,4-dehydroproline, thus confirming the observations of Unger & DeMoss (1966). *Cis-* or *trans-*4-chloro-L-prolines did not inhibit growth of *Escherichia coli* C4 at concentrations up to 200 μg. analogue/ml., nor was growth inhibited by DL-pipecolic acid or 4,5-dehydro-DL-pipecolic acid, in agreement with earlier observations (Fowden & Richmond, 1963).

In studying growth of mutants resistant to 3,4-dehydroproline or azetidine, 232G, 232G/DHP and 232G/Az media were inoculated with bacteria growing exponentially in 232G medium to give an initial bacterial concentration equiv. to about 10 μg. dry wt bacteria/ml. Dehydroproline-resistant strains grew in the presence of 3,4-dehydroproline or azetidine at rates comparable with that displayed by the parent (C4) strain in the absence of analogue. All dehydroproline-resistant strains tested displayed cross-resistance to both analogues. However, although fewer resistant strains were isolated when using azetidine as selective agent, all those tested (fourteen in all) were completely resistant to azetidine (judged by their capacity to grow in 232G/Az medium at rates comparable with that of the parent strain in 232G medium), but were still

sensitive to growth inhibition by dehydroproline when inoculated into 232G/DHP medium.

Incorporation of analogues into proteins of parent and mutant strains

The 'principal protein fraction' (Roberts *et al.* 1955) isolated from strains grown in 232G, 232G/DHP and 232G/Az was examined for the presence of incorporated analogues (see Methods). As previously reported (Fowden & Richmond, 1963; Fowden *et al.* 1963), both 3,4-dehydroproline and azetidine were incorporated into proteins of wild-type *Escherichia coli*, with a corresponding decrease in the proline content (Table 1). Although not all the analogue-resistant strains isolated were tested,

Table 1. *Incorporation of 3,4-dehydroproline and azetidine-2-carboxylic acid into the* '*principal protein fraction*' *of Escherichia coli c4 and analogue-resistant derivatives*

Organisms were grown at 37° in 232G, 232G/DHP and 232G/Az media. The 'principal protein fraction' was isolated, hydrolysed and the content of proline and, where appropriate, 3,4-dehydroproline and azetidine-2-carboxylic acid determined (see Methods). Cultures grown in the presence of analogues were harvested after a 2·5- to 3-fold increase in extinction. Figures reported are not corrected for the proline content of analogue-free protein present in cells before addition of analogues (see Fowden & Richmond, 1963; Fowden *et al.* 1963). The proline content of all strains harvested during exponential growth in 232G medium was about 24 μg. proline/mg. 'principal protein fraction'.

| | Growth in 232G/DHP | | Grown in 232G/Az | |
| | 3,4-Dehydro-proline (μg./mg. protein) | Proline (μg./mg. protein) | Azetidine-2-carboxylic acid (μg./mg. protein) | Proline (μg/mg. protein) |
Strain				
c4	13·1	10·8	5·4	18·1
DHP 15	0	22·8	0	23·2
DHP 27	0	23·9	0	24·1
DHP 29	0	24·4	0	23·8
AZ 3	12·9	11·0	0	22·7
AZ 642	13·3	10·6	0	24·2
AZ 643	14·6	9·4	0	24·0

the results presented in Table 1 show that dehydroproline-resistant strains (also cross-resistant to azetidine) did not incorporate either dehydroproline or azetidine into protein, whereas strains which had been selected for resistance to azetidine did not incorporate this analogue but still incorporated dehydroproline into cell proteins. Moreover, the degree of incorporation of dehydroproline by these azetidine-resistant strains was comparable with that observed in strain c4 (Table 1).

Excretion of proline by analogue-resistant mutants

Excretion of proline was detected by ability to cross-feed proline auxotrophs (see Methods). Whereas many strains selected for resistance to dehydroproline excreted proline, most azetidine-resistant strains studied were unable to cross-feed proline-dependent organisms. The significance of proline excretion will be discussed below (see Discussion).

Proline uptake by the parent strain

Addition of 10^{-6} M-L-[U-^{14}C]proline to a suspension of *Escherichia coli* C4 maintained at $10°$ in the absence of chloramphenicol resulted in rapid accumulation of radioactivity in the cells (Fig. 2). Incorporation into TCA-insoluble material was linear for about 40 min., the rate of incorporation subsequently decreasing with approaching exhaustion of added proline. The proline 'pool' (obtained as the difference between
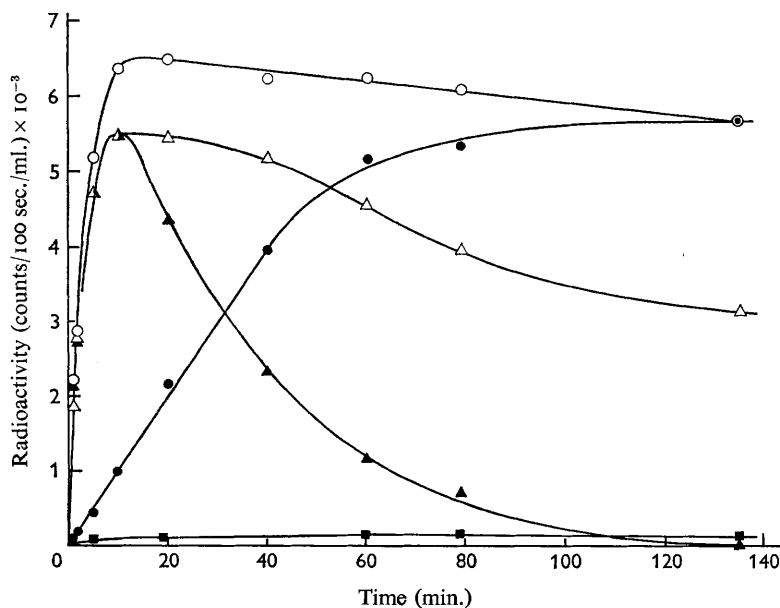

Fig. 2. Total uptake and incorporation of radioactivity into trichloroacetic acid-insoluble material by *Escherichia coli* C4 in the presence of 10^{-6} M-L-[U-^{14}C]proline (31·25 μc./μmole). Bacteria (equiv. 125 μg. dry wt/ml.) were suspended in 232G medium, with or without addition of chloramphenicol (150 μg./ml.) and aerated at $10°$. In the absence of chloramphenicol: O—O, total uptake; ●—●, incorporation into the trichloroacetic acid-insoluble fraction; ▲—▲, calculated proline 'pool' (total uptake minus trichloroacetic acid-insoluble fraction). In the presence of chloramphenicol: △—△, total uptake (= 'pool'); ■—■, incorporation into the trichloroacetic acid-insoluble fraction.

total uptake and TCA-insoluble incorporated material) rose to a maximum after 10 min. and then decreased as the limited amount of proline supplied passed into protein. In the presence of chloramphenicol, incorporation into protein was suppressed, and the total uptake may be taken as representing incorporation into the 'pool'. Although under these conditions some radioactivity was lost from the bacteria, the initial rate of accumulation in the 'pool' was identical in the presence or absence of chloramphenicol (Fig. 2). This overall picture of proline uptake in strain C4 is similar to that described for *E. coli* B by Britten & McClure (1962).

The addition of relatively high concentrations of labelled proline to *E. coli* strain C4 at $10°$ in the presence of chloramphenicol resulted in rapid uptake of radioactivity which reached a maximum and subsequently decreased due to leakage of accumulated proline. This effect was less pronounced at lower initial proline concentrations. For

example, in the presence of 2×10^{-6} M-L-proline about 50 % of the accumulated proline was lost in 150 min. In the same time-period leakage accounted for 38 %, 32 %, 25 % and 17 % of the maximum 'pool' size after addition of 10^{-6} M, $2 \cdot 6 \times 10^{-7}$ M, 10^7 M and $0 \cdot 33 \times 10^{-7}$ M-L-[U-^{14}C]proline, respectively. This 'overshoot' phenomenon has been noticed by W. Maas (personal communication) during investigation of the arginine permease of *E. coli*, and has been studied and discussed in detail with respect to sulphate transport in *Salmonella typhimurium* (Dreyfuss & Pardee, 1966).

Following uptake of L-[U-^{14}C]proline in the presence of chloramphenicol, extraction of the 'pool' followed by chromatography and radioautography (see Methods) revealed that virtually all the accumulated radioactivity could be accounted for as proline. Similarly, after uptake of the labelled amino acid in the absence of chloramphenicol followed by isolation, and hydrolysis and electrophoresis of the 'principal protein fraction', only the proline spot was labelled.

Inhibition of proline accumulation by analogues

Since radioactively labelled analogues were not available, their uptake could not be measured directly. A measure of the affinity of the proline permease for proline analogues was obtained by investigating inhibition of L-[U-^{14}C]proline uptake by the

Table 2. *Inhibition of uptake of L-[U-^{14}C]proline by proline analogues in Escherichia coli strain C4*

Bacterial suspensions (equiv. 125 μg. dry wt bacteria/ml.) in 232G medium containing chloramphenicol (150 μg./ml.) at 10° were simultaneously exposed to $1 \cdot 22 \times 10^{-7}$ M-L-[U-^{14}C]proline and $1 \cdot 1 \times 10^{-4}$ M-analogue (or unlabelled proline), as indicated below. Analogue concentrations were calculated on the basis of the content of L-isomer. Results are expressed as percentage inhibition of the initial rate of uptake in the presence of $1 \cdot 22 \times 10^{-7}$ M-L-[U-^{14}C]proline only.

Addition	Inhibition (%)
L-Proline (unlabelled)	95·7
3 4-Dehydro-DL-proline	94·8
L-Thiazolidine-4-carboxylic acid	86·7
L-Azetidine-2-carboxylic acid	77·5
4-Methylene-DL-proline	75·9
cis-4-Chloro-L-proline	76·1
trans-4-Chloro-L-proline	56·9
Glycyl-L-proline	13·9
D-Proline	0
D-Azetidine-2-carboxylic acid	0
N-Acetyl-DL-proline	0
DL-Pipecolic acid	0
4,5-Dehydro-DL-pipecolic acid	0

analogues. Table 2 (line 1) reveals that the presence of excess unlabelled proline inhibited accumulation of radioactive proline. Many analogues, including 3,4-dehydro-DL-proline, L-thiazolidine-4-carboxylic acid and a number of 4-substituted prolines had a similar effect. The lower homologue of proline, L-azetidine-2-carboxylic acid also inhibited the uptake of labelled proline, though to a lesser degree; on the other hand, the higher homologue of proline, DL-pipecolic acid, together with 4,5-dehydro-DL-pipecolic acid and N-acetyl-DL-proline were without effect (Table 2).

When studied as a function of initial proline concentration, the initial rates of uptake in the presence of chloramphenicol obeyed Michaelis–Menten kinetics (Fig. 3), with

a K_m of 6.4×10^{-7} M. Inhibition of proline uptake into the amino acid pool by 3,4-dehydroproline and azetidine was studied in detail. Inhibition by both analogues was competitive (Fig. 3). Calculation of K_i (see Dixon & Webb, 1964) yielded a value of 2.6×10^{-6} M and 2.4×10^{-5} M for 3,4-dehydroproline and azetidine, respectively. It should be noted that, since only racemic (DL) 3,4-dehydroproline was available, the calculation of K_i for this analogue was based on the assumption that only the L-isomer was active in inhibiting uptake of proline. Thus, in the parent strain *Escherichia coli* C4, the affinity of the proline transport system for 3,4-dehydroproline was comparable with that for proline itself, but affinity of the system for azetidine was considerably lower (see also Table 2).
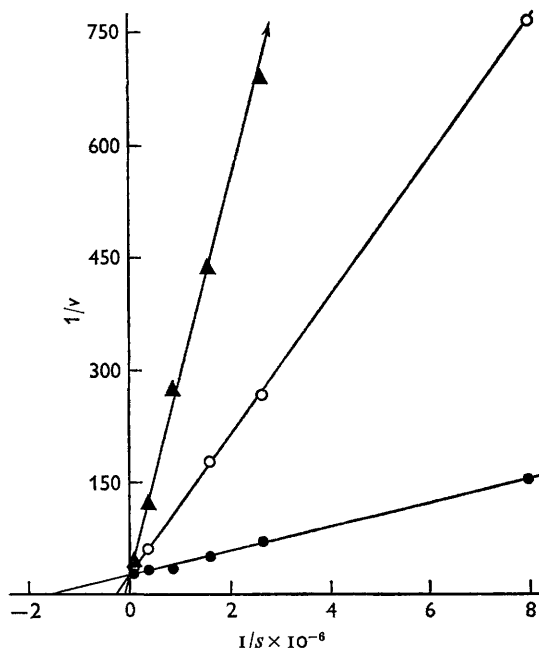

Fig. 3. Lineweaver-Burk plot of the initial rate of uptake of radioactivity as a function of L-[U-^{14}C]proline concentration in the presence and absence of 5×10^{-5} M-3,4-dehydro-DL-proline or 10^{-4} M-L-azetidine-2-carboxylic acid. *Escherichia coli* C4, suspended in 232G medium containing chloramphenicol (150 μg./ml.), were aerated at 10°. Various concentrations of substrate were obtained by addition of suitable amounts of carrier L-proline to L-[U-^{14}C]proline (130 μc./μmole). ●—●, L-proline only; ○—○, L-proline+L-azetidine-2-carboxylic acid; ▲—▲, L-proline+3,4-dehydro-DL-proline.

Accumulation of proline by analogue-resistant mutants

A wide range of strains selected for resistance to either 3,4-dehydroproline or azetidine were tested for their ability to accumulate labelled proline. Some resistant strains appeared to take up labelled proline at rates comparable to that displayed by the parent strain *Escherichia coli* C4. However, in many other mutants the capacity to accumulate proline was impaired. A representative group of results are shown in Fig. 4. The activity of the impaired permease varied from virtually complete absence to values with which the initial rate of proline uptake was about 25 % of that measured in the parent strain C4. In many of the strains selected on 3,4-dehydroproline, the

kinetics of proline uptake were abnormal (see strain DHP 21, Fig. 4) in that radio-activity entered the bacteria at a high rate, but was quickly lost. These strains also excreted proline in considerable amounts. The significance of this behaviour will be considered in a later publication.
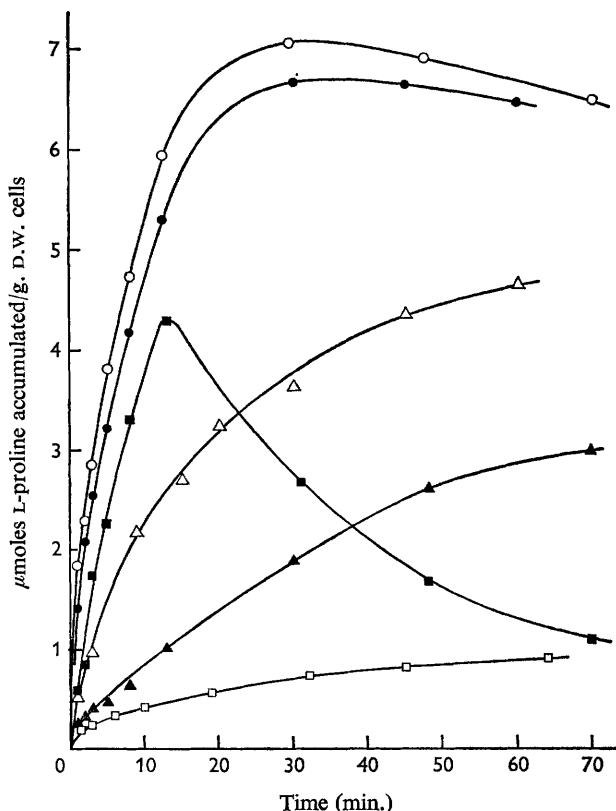

Fig. 4. Uptake of radioactivity by *Escherichia coli* wild-type c4 and analogue-resistant strains in the presence of 2×10^{-6} M-L-[U-^{14}C]proline ($31 \cdot 25$ $\mu c/\mu$mole). Bacteria (equiv. 125 μg. dry wt/ml.) were suspended in 232G medium containing chloramphenicol (150 μg./ml.). Azetidine-resistant strains: ▲—▲, AZ3; △—△, AZ642; ●—●, AZ643. 3,4-Dehydroproline-resistant strains: ■—■, DHP21; □—□, DHP27. ○—○, strain c4.

The presence of 10^{-3} M-2,4 dinitrophenol almost completely suppressed the uptake of 2×10^{-6} M-L-[U-^{14}C]proline by the parent strain and by those mutants having some-what impaired permease activity. However, the inhibitor was without significant effect on the very low intracellular levels of those strains which were virtually devoid of permease activity (see also Britten & McClure, 1962; Kessel & Lubin, 1962).

The uptake of $1 \cdot 65 \times 10^{-6}$ M-L-[U-^{14}C]arginine ($12 \cdot 5$ mμc/ml. suspension) by the parent and all mutant strains tested was identical, suggesting that the impaired per-mease activity of the mutants was specific for the proline permease.

The mutants selected for azetidine-resistance were of particular interest since the majority of these strains were not cross-resistant to 3,4-dehydroproline, and the latter compound was still incorporated into protein (see Table 1). The affinity of the per-

mease of one of these strains (AZ642) for proline ($K_m = 9{\cdot}0 \times 10^{-7}$ M) and 3,4-dehydro-proline ($K_i = 3{\cdot}6 \times 10^{-6}$ M) differed only slightly from that found for the parent strain. However, in strain AZ 642, uptake of proline was much less sensitive to inhibition by L-azetidine-2-carboxylic acid compared with the parent (C4) strain (Fig. 5). The K_i for azetidine determined for AZ 642 was $3{\cdot}0 \times 10^{-4}$ M, an approximately tenfold increase over the corresponding value for strain C4. As in the parent strain, both analogues competitively inhibited uptake of proline in strain AZ 642.
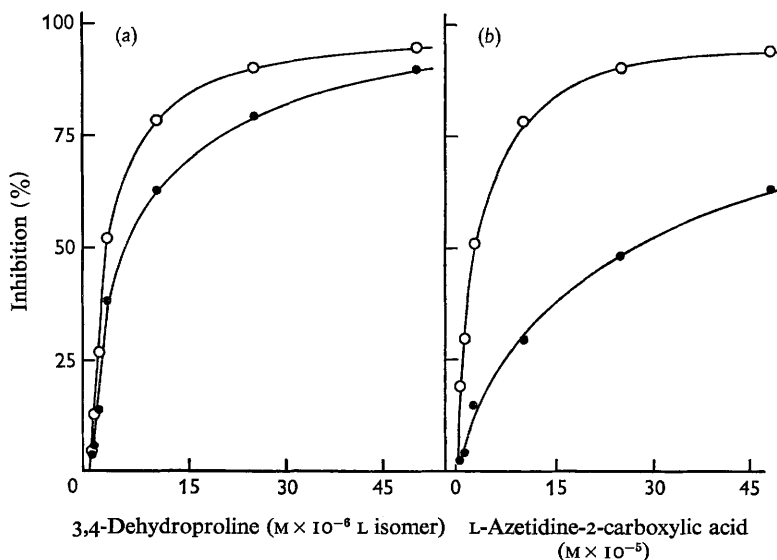

3,4-Dehydroproline (M × 10⁻⁶ L isomer)   L-Azetidine-2-carboxylic acid
(M × 10⁻⁵)

Fig. 5. Inhibition of uptake of labelled proline by varying concentrations of (*a*) 3,4-dehydroproline and (*b*) azetidine-2-carboxylic acid by *Escherichia coli* C4 and the azetidine-resistant strain AZ642. Bacteria (equiv. 125 μg. dry wt/ml.) were aerated at 10° in 232G medium containing chloramphenicol (150 μg./ml.), to which was added $1{\cdot}22 \times 10^{-7}$ M-L-[U-¹⁴C]proline (130 μc/μmole) and, where necessary, either 3,4-dehydro-DL-proline or L-azetidine-2-carboxylic acid at concentrations indicated. Uptake of radioactivity was measured at 20 sec. intervals over the first 80 sec. and results are expressed as % inhibition of the initial rate of proline uptake in the absence of inhibitor. ○—○, Strain C4; ●—●, azetidine-resistant strain AZ642.

Exchange between intracellular proline and extracellular proline or analogues

Several investigators have shown that a variety of metabolites, once accumulated in the intracellular 'pool', can exchange with extracellular metabolite (Cohen & Kepes, 1962; Kessel & Lubin, 1962; Britten & McClure, 1962; Britten, 1965). Experiments with *Escherichia coli* strain C4 confirmed the results obtained with *E. coli* B by Britten & McClure (1962). Proline accumulation did not occur at 0°, though proline previously accumulated at higher temperatures could still exchange with extracellular proline at 0°. At 0° proline pools of moderate size were stable for at least 3 hr. When a stable pool at 0° was established by exposure to $1{\cdot}22 \times 10^{-7}$ M-L-[U-¹⁴C]proline (see Methods), the rate of exchange between intracellular and unlabelled extracellular proline was independent of the initial external proline concentration in the range 10^{-7} M to $7{\cdot}5 \times 10^{-4}$ M. At 10° the rate of exchange was not markedly dependent on external proline concentration, but the final value of intracellular radioactivity

observed was concentration-dependent, due to the establishment of a new equilibrium between internal and external proline. Further, when the logarithm of the residual intracellular radioactivity was expressed as a function of time, the exchange process was revealed as an initial rapid phase followed by a period during which exchange was slower (see Britten & McClure, 1962; Kessel & Lubin, 1962). These workers interpreted this as indicating the presence of at least two pool components, one capable of rapid exchange with extracellular proline, the other a slowly exchanging component.

Several proline analogues also exchanged with intracellular radioactive proline. A direct correlation was demonstrated between ability of an analogue to inhibit proline uptake in the presence of chloramphenicol (see Table 2) and ability to effect exchange. When tested at 5×10^{-5} M- (based on the concentration of L-isomer), 3,4-dehydro-DL-proline, L-azetidine-2-carboxylic acid, L-thiazolidine-4-carboxylic acid and 4-methylene-DL-proline exchanged readily at 10° with the pool established by prior exposure
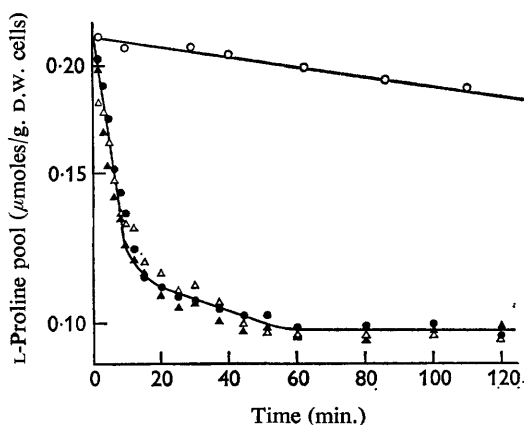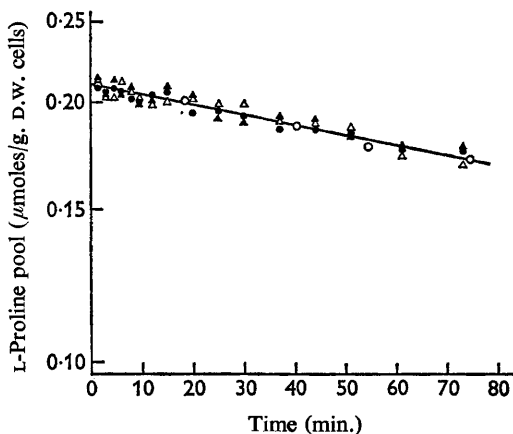

Fig. 6. Exchange between labelled L-proline in the amino acid pool of *Escherichia coli* c4 with extracellular 3,4-dehydro-DL-proline, L-azetidine-2-carboxylic acid or (unlabelled) L-proline at 0°. The amino acid pool of bacteria maintained at 25° in 232G medium containing chloramphenicol (150 μg./ml.) was 'loaded' by exposure to 1.22×10^{-7} M-L-[U-^{14}C] proline (130 μc/μmole) for 10 min., followed by cooling the bacterial suspension to 0° and resuspension in fresh 232G medium containing 150 μg. chloramphenicol/ml. Exchange was initiated by adding samples of this bacterial suspension to tubes containing either L-proline, 3,4-dehydro-DL-proline or L-azetidine-2-carboxylic acid solutions to yield final concentrations of 5×10^{-5} M (based on the content of L-isomer). O—O, Control; △—△, L-proline; ▲—▲, 3,4-dehydro-DL-proline; ●—●, L-azetidine-2-carboxylic acid.

Fig. 7. Exchange between labelled L-proline in the amino acid pool of azetidine-resistant *Escherichia coli* strain AZ642 with extracellular 3,4-dehydro-DL-proline, L-azetidine-2-carboxylic acid and unlabelled L-proline at 0°. Details and symbols as described for Fig. 6, except that, owing to the diminished permease activity of *E. coli* strain AZ642, time of 'loading' of the pool at 25° was extended to 30 min.

to 1.22×10^{-7} M-L-[U-^{14}C]proline. Under the same conditions glycyl-L-proline supported very little exchange, while DL-pipecolic acid, 4,5-dehydro-DL-pipecolic acid, D-proline and D-azetidine-2-carboxylic acid were unable to exchange with intracellular L-proline.

The exchange reaction between accumulated labelled proline and extracellular 3,4-

dehydroproline or azetidine-2-carboxylic acid was studied in detail at 0° and 10°. At 0° not only the rates of exchange but also the final steady state pool values after exchange between a labelled pool (established by exposure to 1.22×10^{-7} M-L-[U-^{14}C] proline) and external L-proline, 3,4-dehydro-DL-proline or L-azetidine-2-carboxylic acid (5×10^{-5} M, based on the content of L-isomer) were virtually identical (Fig. 6). At 10° both analogues exchanged with intracellular labelled proline, but in the presence of L-azetidine-2-carboxylic acid loss of radioactivity from the pool ceased after the first (rapid) phase of exchange. Other experiments, in which the steady-state pool values were measured following simultaneous addition of 1.22×10^{-7} M-L-[U-^{14}C] proline and L-azetidine-2-carboxylic acid at concentrations ranging from 5×10^{-5} M to 2.5×10^{-3} M, established that this behaviour was due to the preferential re-accumulation of labelled proline molecules which had left the pool by exchange with azetidine. The final pool values found in these experiments were dependent on the
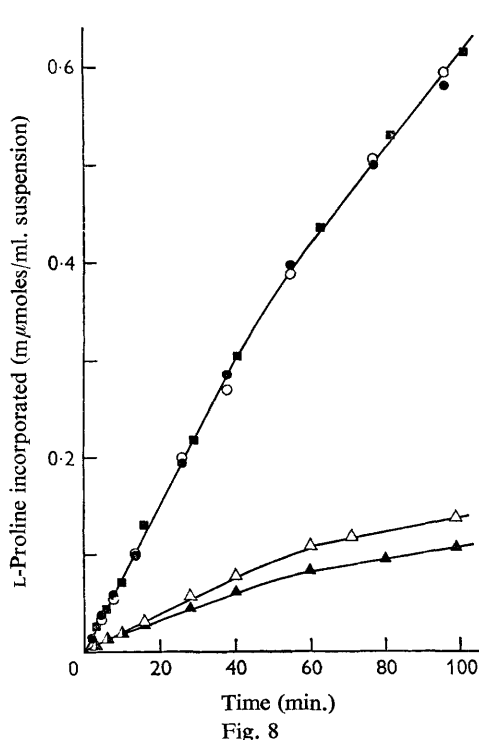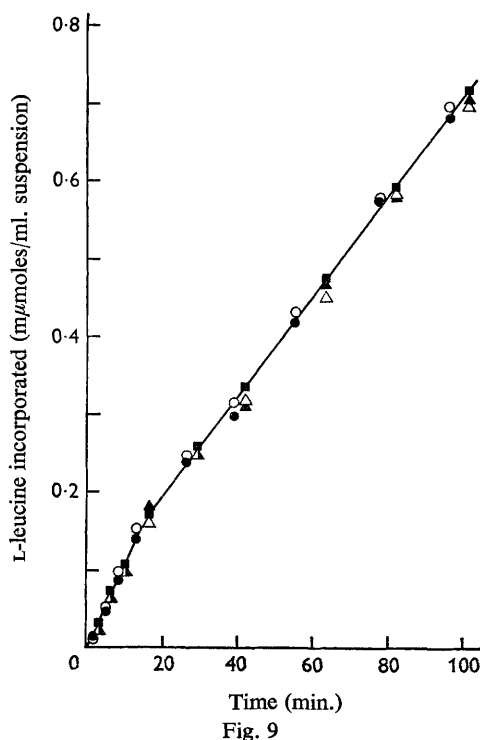

Fig. 8   Fig. 9

Fig. 8. Incorporation of labelled L-proline into the trichloroacetic acid-insoluble fraction of *Escherichia coli* wild-type C4 and analogue-resistant strains. Cultures growing exponentially at 37° in 232G medium were cooled and maintained at 10° for 2 hr. L-[U-^{14}C]Proline (31·25 μc/μmole) and L-leucine were added to give final concentrations of 2×10^{-6} M. Samples were withdrawn at intervals for determination of total uptake of labelled proline and radioactivity incorporated into trichloroacetic acid-insoluble material (see Methods). Azetidine-resistant strains: ●—●, AZ3; O—O, AZ642. 3,4-Dehydroproline-resistant strains: ▲—▲, DHP27; △—△, DHP29. ■—■, strain C4.

Fig. 9. Incorporation of labelled L-leucine into the trichloroacetic acid-insoluble fraction of *Escherichia coli* wild-type C4 and analogue-resistant strains. Details and symbols as described for Fig. 8, except that L-[U-^{14}C]leucine (50 μc/μmole) and L-proline replaced L-leucine and L-[U-^{14}C]proline.

azetidine-2-carboxylic acid concentration and were identical with those observed when the same amounts of azetidine were added to comparable bacterial suspensions in which a pool had previously been established by exposure to 1.22×10^{-7} M-L-[U-^{14}C] proline.

The exchange reaction could not be conveniently studied in many analogue-resistant mutants possessing very low permease activities. An azetidine-resistant strain (AZ 642) displaying a diminished permease activity with much reduced affinity for azetidine (see Fig. 5) completely lacked the ability to promote exchange between intracellular radioactive proline and external (unlabelled) proline, 3,4-dehydroproline or azetidine (Fig. 7).

Incorporation of proline and leucine into TCA-insoluble material of analogue-resistant strains

Compared with the parent strain *Escherichia coli* C4, the capacity to incorporate exogenous L-[U-^{14}C]proline into TCA-insoluble material was markedly decreased in mutants selected for 3,4-dehydroproline resistance (Fig. 8). This diminution in proline incorporation was not due to a decrease in the rate of overall protein synthesis in the mutants, since the rate of incorporation of L-[U-^{14}C]leucine into protein was identical in both the parent strain and in all the mutants studied (Fig. 9). This suggests that those mutants with a decreased capacity for the uptake of exogenous proline continue to synthesize (unlabelled) proline and preferentially use this endogenous source for synthesis of protein. Although the rate of uptake of proline from the medium was decreased in most of the azetidine-resistant mutants tested, the residual permease activity was still capable of ensuring a sufficient supply of proline for protein synthesis since the rate of incorporation of exogenous labelled proline into protein was identical in the parent strain and in at least two azetidine-resistant mutants (Fig. 8).

DISCUSSION

Earlier experiments on the proline permease of *Escherichia coli* strains B and W (Britten & McClure, 1962; Kessel & Lubin, 1962) have been confirmed with *E. coli* strain C4, a derivative of *E. coli* K-10. The observations were extended to include a study of the specificity of the permease towards proline analogues. Inhibition of proline accumulation by 3,4-dehydroproline and azetidine-2-carboxylic acid, the two analogues studied in detail, was competitive, suggesting that both analogues enter the cell by the mechanism responsible for transport of proline. That 3,4-dehydro-proline and azetidine actually enter the cell is indicated by two types of observation: (i) both analogues are incorporated into the proteins of *E. coli* (Fowden & Richmond, 1963; Fowden *et al.* 1963); (ii) both analogues exchanged with previously accumulated proline (Fig. 6).

Other analogues capable of inhibiting proline uptake (Table 2) also compete with proline for sites leading to accumulation within the cell. The proline permease of the parent culture displayed affinity for a number of 4-substituted proline derivatives. The analogues *cis*- and *trans*-4-chloroprolines are of particular interest since the former inhibited the uptake of proline to about the same extent as azetidine, but the latter possessed far less inhibitory action (Table 2). This is in contrast to the observations of Gottlieb, Fujita, Udenfriend & Witkop (1965), who showed that incor-

poration of *cis*-4-fluoroproline into TCA-insoluble material exceeded that of the *trans* isomer in a cell-free system from *Escherichia coli*. Whereas azetidine, the lower homologue of proline, markedly inhibited uptake of proline, the higher homologue, pipecolic acid, was devoid of inhibitory action, an effect presumably attributable to the larger ring structure of the latter compound.

A large number of strains were obtained from *Escherichia coli* C4 following selection for resistance to the growth-inhibitory analogues 3,4-dehydroproline or azetidine-2-carboxylic acid. In general, strains selected for resistance to 3,4-dehydroproline were also resistant to azetidine and failed to incorporate either analogue into protein. Some of these strains displayed impaired ability to take up exogenous proline; others excreted proline, but possessed normal permease activity. In many of the proline-excreting mutants the accumulation of proline showed anomolous behaviour in that labelled proline passed into the cells, but uptake was followed by rapid loss of radio-activity (see, for example, strain DHP 21, Fig. 4). Mutants of this type will be discussed fully in a later publication.

Several workers have shown changes in permeability in mutants selected for resistance to amino acid analogues. Failure of a toxic analogue to enter the cell serves to exclude it from the site (or sites) at which toxicity is exerted and accounts fully for resistance to the analogue. It is commonly found that failure to transport an analogue is accompanied by failure of the corresponding natural amino acid to enter the cell (Schwartz *et al.* 1959; Ferroluzzi-Ames, 1964; Lester, 1966; Shifrin *et al.* 1966; see also reviews by Moyed, 1964; Fowden *et al.* 1967). In the present investigation the impairment of the proline permease in dehydroproline-resistant strains was substantiated by decreased incorporation of exogenous proline into protein (Fig. 8). Presumably 3,4-dehydroproline and azetidine-2-carboxylic acid were excluded from protein by the same mechanism.

Strains selected for resistance to azetidine did not show cross-resistance to 3,4-dehydroproline. In some strains the proline permease was normal; proline excretion, if it occurred at all, was weak. The mechanism of resistance in these strains is under investigation. The majority of the azetidine-resistant strains isolated possessed an impaired capacity for uptake of proline (Fig. 4), though the residual permease activity was sufficient to meet the needs of protein synthesis, at least at 10° (Fig. 8). The behaviour of these mutants is of interest since growth was inhibited by 3,4-dehydro-proline yet they still incorporated this analogue into protein. It was shown that the affinity of the proline permease of one of these strains (AZ 642) for proline and 3,4-dehydroproline was comparable with that of the parent strain, but affinity for azetidine was considerably reduced. Thus, strain AZ 642 displayed a highly selective effect towards proline analogues, owing its resistance to azetidine to the capacity to exclude this compound from the cell. Other isolates behaved similarly, but it should be noted that the method of selection of azetidine-resistant strains (see Methods) makes it possible that most of these cultures were derived from a single clone and hence genetically identical.

Although in the parent organism affinity of the proline permease for 3,4-dehydro-proline was comparable with that for proline itself, its affinity for azetidine was somewhat lower. This difference, however, was not apparent in the exchange of intra-cellular labelled proline with extracellular (unlabelled) proline or proline analogues. The rates of exchange of proline, 3,4-dehydroproline and azetidine were virtually

identical (Fig. 6). The azetidine-resistant strain AZ 642 failed to promote the exchange reaction between intracellular proline and extracellular proline, 3,4-dehydroproline or azetidine (Fig. 7). Kessel & Lubin (1962) described an *Escherichia coli* mutant lacking proline permease activity which also lacked the capacity to exchange internal and external proline at 0°. They concluded that uptake and exchange are closely related phenomena. The isolation of AZ 642, a strain unable to promote exchange yet possessing a permease which, though impaired, was still capable of proline uptake, suggests that the two processes may not be closely interrelated. Other experiments (Britten & McClure, 1962; Britten, 1965) lead to the same conclusion. For example, uptake could not occur at 0° or in the absence of an energy source, though exchange was rapid under these conditions.

Excretion of proline implies failure of the metabolic control of proline biosynthesis. Proline-excreting dehydroproline-resistant derivatives of *Escherichia coli* K-12 and azetidine-resistant strains of *Salmonella typhimurium* SW 1061 which excrete proline have also been obtained, but not yet studied in detail (Tristram, unpublished observations). In *E. coli* proline is formed from glutamate, via glutamic γ-semialdehyde and its spontaneous cyclization product, Δ^1-pyrroline-5-carboxylic acid. The overall conversion of glutamate to Δ^1-pyrroline-5-carboxylic acid is inhibited by proline, 3,4-dehydroproline and, to a lesser extent, by azetidine (Strecker, 1957; Baich & Pierson, 1965; Tristram & Thurston, 1966). Formation of Δ^1-pyrroline-5-carboxylic acid by a proline-excreting 3,4-dehydroproline-resistant mutant had lost its sensitivity to proline (Baich & Pierson, 1965). At least the earlier enzymes of proline biosynthesis are subject to enzyme repression by the final product of the pathway (Tristram & Thurston, 1966). Preliminary experiments have shown that some of the analogue-resistant strains which excrete proline are no longer subject to repression; in others the formation of Δ^1-pyrroline-5-carboxylic acid is insensitive to end-product inhibition (Neale & Tristram, unpublished observations). The genetic study of dehydroproline- and azetidine-resistant strains has commenced and should lead to recognition of proline regulatory genes and also the gene (or genes) determining the proline permease.

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