# Uptake of Amino Acids by *Salmonella typhimurium*

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The transport systems of *Salmonella typhimurium* for histidine and for the aromatic amino acids have been studied. Each of these amino acids is transported by a highly specific permease. All four amino acids and a wide variety of their structural analogs are also transported by a general permease, the aromatic permease. A mutant for the aromatic permease was isolated and its properties are described. A method for assaying the activity of the permease in growing cells has been devised and Michaelis constants for the substrates were measured using this method. All the affinity constants are quite low (about $10^{-7}\ M$). A new parameter is introduced: the *limit concentration*, which represents the minimal external concentration of an amino acid at which the organism utilizes exclusively the external supply, and completely stops the biosynthetic production of that amino acid. The limit concentration is a measure of the efficiency of a permease *in vivo*. The phenylalanine and the histidine limit concentrations, in minimal medium, are $1.9 \times 10^{-7}\ M$ and $1.5 \times 10^{-7}\ M$, respectively.

## INTRODUCTION

The mechanism of uptake of the amino acids histidine, tryptophan, tyrosine, and phenylalanine was studied in *Salmonella typhimurium*. A *specific* permease (transport system) exists for each of these amino acids. In addition, there is a *general* permease, able to transport all four amino acids as well as a wide variety of structural analogs. This scheme is supported by inhibition studies with amino acid analogs and by the properties of a mutant missing the aromatic permease.

A method for measuring the affinity constant of an amino acid permease in growing cells is introduced and accurate $Km$ values have been measured for the first time. The affinity constants are extremely low, e.g., the $Km$ for histidine is $10^{-7}\ M$. The method can be applied to any amino acid whose rate of uptake into the pool is too fast to be measured under the usual assay conditions (Britten and McClure, 1962).

Recent and very complete reviews of the state of the field have been presented by Holden (1962) and Kepes and Cohen (1962).

## MATERIALS AND METHODS

*Salmonella typhimurium*, wild type (strain LT-2) and histidine-requiring mutants, were obtained from Dr. B. N. Ames. A phenylalanine-requiring mutant was obtained from Dr. M. Demerec. All unlabeled amino acids were purchased from California Corporation for Biochemical Research (Los Angeles, California). L-Histidine-2-C¹⁴ (22 mc. per millimole) was purchased from Volk Radiochemical Corp. (Chicago, Illinois); DL-tryptophan-3-C¹⁴ (10 mc. per millimole), uniformly labeled L-tyrosine (200 mc. per millimole), uniformly labeled L-phenylalanine (200 mc. per millimole) and DL-*p*-fluorophenylalanine-3-C¹⁴ (5 mc. per millimole) were purchased from New England Nuclear Corp. (Boston, Massachusetts). Azaserine and diazo-5-oxo-L-norleucine (DON) were obtained from Dr. E. P. Anderson. L-Carnosine, L-3-methyl histidine, and L-1-methyl histidine were purchased from California Corp. for Biochemical Research (Los Angeles, California). D-Histidine was purchased from Mann Research Lab. (New York, New York). D,L-Thiazole-alanine, D,L-1,2,4,triazole-3-alanine, urocanic acid, imidazolepropionic acid, D,L,-α-methyl-histidine, 3-pyrazolealanine, L-histidinol, and L-histidine hydroxamate were obtained from Dr. B. N. Ames. D,L-β-3-Furylalanine was obtained from Dr. D. E. Lewis (Lewis and Dunn, 1962).

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Overnight cultures were grown in medium E (Vogel and Bonner, 1956), with glucose (0.4%) as carbon source, in a New Brunswick rotary shaker. For experiments with starved cells the overnight cultures were first diluted and regrown in medium E-C, with glucose (0.4%) as carbon source. Medium E-C was essentially the same as medium E, with the omission of citrate (which can be used as a carbon source by *S. typhimurium*) and of $MgSO_4$; HCl was used instead to adjust the pH to 7.0; $MgSO_4 \cdot 7H_2O$ was added to the medium just before use at a final concentration of 0.2 mg. per milliliter. Both media, E and E-C, were supplemented with 1 ml. of trace element mixture per 1000 ml. of medium. The trace element mixture contained per milliliter: $CoSO_4$, 2.6 μg.; $H_3BO_3$, 5.7 μg.; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 15 μg.; $FeCl_3$, 483 μg.; $MnCl_2 \cdot 4H_2O$, 278 μg.; $CuCl_2$, 269 μg.; and $ZnCl_2$, 2.08 mg. When histidine-requiring mutants were used, histidine ($10^{-4}$ M) was added to the growth medium.

## ASSAY FOR UPTAKE WITH STARVED CELLS

An overnight culture was diluted one hundred-fold in medium E-C and incubated with shaking. When the culture had doubled three times it was diluted again in medium E-C to a final bacterial density of $10^8$ cells per milliliter and incubated further. All cultures grew exponentially with a doubling time of about 48 minutes. The cells were harvested by centrifugation at a density of $4 \times 10^8$ cells per milliliter. The density of the cultures was assayed on a Beckman DU spectrophotometer at 650 mμ. A bacterial density of $1.3 \times 10^8$ cells per milliliter corresponds to 75 γ dry weight of cells per milliliter or an optical density (O.D.) of 0.100 on the spectrophotometer used (each instrument gives a different value). Cells rejuvenated in this way had undergone at least 4 divisions and were in early exponential growth. The cells were starved by suspension in medium E-C, without glucose, and incubation, without aeration, at 25°C. for the indicated times. Aliquots were added to tubes containing the desired amount of radioactive amino acid and the indicated additions, and incubated at 25°C. Samples of 1.0 ml. were filtered on a Millipore filter (Millipore Filter Corp., New Bedford, Massachusetts) (pore size: 0.45 μ, 25 mm. diameter) and the filter washed three times with about 3 ml. of cold E-C medium. There was no significant loss of accumulated radioactivity with this washing procedure. The filter was then glued to a planchet with rubber cement and counted in a thin window flow-gas counter (Nuclear-Chicago Corp., Des Plaines, Illinois). Rates are expressed in μmoles per gram dry weight per minute.

## CHROMATOGRAPHY OF ACCUMULATED PRODUCT

Ascending paper chromatography on Whatman No. 1 paper was carried out overnight (approximately 12 hours) in each of two different systems: (1) n-propanol-1 N HCl(3:1, vol./vol.); and (2) n-propanol-1 N NH₃(3:1, vol./vol.). The paper was then dried, cut in strips, and counted in a Vanguard strip counter (Vanguard Instrument Co., New York, New York). A standard of histidine-$C^{14}$, with carrier $C^{12}$-histidine added, was run on each paper. The $R_f$ for histidine was 0.22 in system (1) and 0.48 in system (2).

## ASSAY FOR INCORPORATION INTO PROTEIN

Radioactive amino acid was added to an exponentially growing culture at a density of approximately $4 \times 10^8$ cells per milliliter. One-tenth ml. samples were withdrawn and either filtered directly (total uptake) or added to 1.0 ml. of cold 5% trichloroacetic acid (TCA). Half of each TCA sample was heated at 90°C. for 10 minutes. The TCA samples were then filtered on Millipore filters and washed with about 10 ml. of cold 5% TCA. The filters were glued to planchets and counted. Counts stable to treatment with hot TCA represent incorporation into protein; counts labile to hot TCA (i.e., cold-TCA minus hot-TCA values) presumably represent amino acid bound to s-RNA. The pool is obtained by subtracting the counts precipitable by cold TCA from the total uptake (e.g., see Fig. 3).

When the permease was rendered limiting, e.g., when the concentration of amino acid was very small, the assay was performed as follows: cells growing exponentially were diluted to a calculated density of approximately $6 \times 10^6$ cells per milliliter and incubated for 1 hour to allow the culture to approximately double in density and get out of the lag in growth resulting from the dilution to such a low density. The radioactive amino acid was then added and a number of 1.0-ml. aliquots were transferred to 0.1 ml. of 50% TCA during the first 2 minutes and treated as above. The change in substrate concentration during the assay was not more than 10% at the lowest concentration used. A portion of the culture was incubated further, frequent samples were taken for O.D. readings, and the growth curve was plotted. The O.D. at the time of addition of the amino acid was obtained by extrapolation of the growth curve. Rates are expressed in μmoles per gram dry weight per minute.

## MEASUREMENT OF THE BIOSYNTHETIC POOL OF HISTIDINE

Exponentially growing cells were harvested by centrifugation, in the cold, when the culture had reached a density of about $2 \times 10^8$ cells per milliliter. The pellet from 1 liter of culture was extracted with 5 ml. of 5% TCA at 0° for 45 minutes. The extract was centrifuged, the clear supernatant was extracted with 5 ml. of ether three times, to eliminate the TCA, and finally neutralized with NaOH to pH 7.0. Various aliquots of the neutralized supernatant were added to a culture actively incorporating labeled histidine present in various concentrations. The amount of cold histidine in the extract was calculated from the decrease in the rate of incorporation of labeled histidine after the addition of the TCA extract. Controls were made by adding, at the same time as the extract, aliquots of cold histidine of known concentration. This checked for the presence of compounds in the extract which could inhibit the histidine incorporation. The pool of histidine, assayed in this way, was determined to be 0.06 μmole per gram dry weight. This value could be erroneous if the pool undergoes modifications during the harvesting of the cells. That it does not undergo very big changes is indicated by the following observation: The histidine pool was measured by a more indirect way, by extrapolating back the protein incorporation rates to zero time (Britten and McClure, 1962); at histidine concentrations saturating the permease, the extent of the lag in the incorporation indicated that external histidine was going through a pool of cold histidine which contained about 0.06–0.2 μmole. The value, 0.06 μmole, corresponds to an internal concentration of histidine of $1.5 \times 10^{-5} M$, assuming that 1 g. dry weight of cells corresponds to 4 ml. internal volume (Robert et al., 1955).

### RESULTS

Four types of experiments have been performed:

(a) The accumulation process was studied in cells under starvation conditions, i.e., the cells were deprived of the carbon source and did not synthesize protein. Therefore, the amino acid under study was not incorporated to a great extent into protein or other TCA-insoluble material and it accumulated in the cell. This type of experiment allows direct measurement of the initial rate of uptake.

(b) The cells were growing normally and uptake was studied as incorporation of radioactive amino acid into the proteins of the growing bacteria, either by using very low concentrations of amino acid (therefore making the uptake by the permease the limiting step in the incorporation of the radioactive amino acid into protein), or with excess amino acid and addition of inhibitors or competitors. This type of experiment permits study of the permease activity under physiological conditions.

(c) A mutant for the aromatic permease was isolated and its properties were studied. Methods for the isolation of mutants were examined.

(d) The growth rates of the wild type and of a permease mutant, in the presence of specific inhibitory analogs, were compared. The results confirm the existence of various permeases with different specificities.

### I. THE HISTIDINE PERMEASE

#### MEASUREMENT OF INITIAL RATE OF HISTIDINE UPTAKE IN STARVED CELLS

The histidine permease was assayed as uptake of $C^{14}$-amino acid by cells which had been starved for glucose.[1] Figure 1 shows a time curve for the uptake of histidine. The initial rate of uptake was proportional to the concentration of bacteria in the range used for all experiments and it was linear for at least 5 minutes. After the first 5 minutes the rate decreased until it reached zero at 40

[1] Cells were starved by preincubation in the absence of glucose for 90 minutes, at 25°C. During the preincubation the rate of uptake was found to vary. It decreased to approximately 50% of its initial value during the first 10 minutes and then slowly increased until it reached a constant value after 90 minutes of preincubation and remained unaltered for at least 100 more minutes. When starvation was done at 37°C. the rate remained constant only for a short period (about 10 minutes) and the initial rate was linear for a very short time, rendering accurate measurements impossible. For these reasons a preincubation of 90 minutes and the temperature of 25°C. were chosen as convenient for all assays. All the radioactivity accumulated could be extracted by boiling water and was shown chromatographically to be unmetabolized histidine.
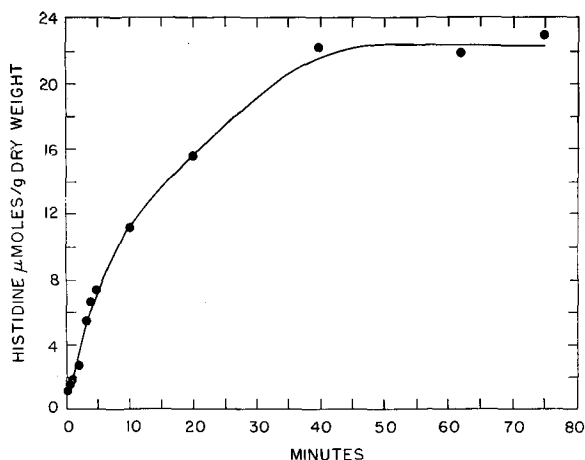
FIG. 1. Time curve for the uptake of histidine in starved cells. Histidine concentration, $3 \times 10^{-4}$ $M$; temperature, 25°C. Cells had been starved by preincubation in the absence of glucose for 90 minutes, at 25°C. The internal concentration at the plateau corresponds to 19 times the external concentration.

minutes and net uptake ceased. At 0° there was no active uptake and equilibrium was reached when the internal concentration equalled the external concentration (e.g., about 0.6 μmole per gram dry weight in Fig. 1). Cells inactivated by heating at 100° for 10 minutes did not accumulate significant amounts of radioactivity (e.g., about 0.2 μmole per gram dry weight in Fig. 1).

It was technically impossible to measure initial rates in the presence of glucose because when protein synthesis is proceeding normally, the size of the amino acid pool is extremely small and is equilibrated in a matter of seconds, i.e., the time required for its formation is too small to allow accurate measurement. Protein synthesis could be inhibited with chloramphenicol, but it was found that histidine uptake per se was inhibited by preincubation with chloramphenicol. It was therefore preferred to stop protein synthesis by eliminating glucose from the assay medium. This had also the effect of increasing the size of the pool and, therefore, of allowing measurement of the initial rate (see section on effect of carbon source).

MEASUREMENT OF AFFINITY CONSTANTS

The $Km$ for histidine uptake was calculated from the measurement of the initial rates (Fig. 2). A striking feature of this

curve is the sharp break occurring around $2 \times 10^{-5}$ $M$ histidine. By extrapolating the two branches of the curve, two $Km$ values are obtained: $1.7 \times 10^{-7}$ $M$ and $1.1 \times 10^{-4}$ $M$. The break in the $Km$ curve is due to two systems being involved in histidine uptake: a highly specific histidine permease ($Km$: $1.7 \times 10^{-7}$ $M$) and a nonspecific aromatic permease (for phenylalanine, tyrosine, and tryptophan) with low affinity for histidine. Further evidence for these two permeases has been obtained through inhibition studies and is presented in the next section.

INHIBITION OF THE UPTAKE OF HISTIDINE IN STARVED CELLS

The existence of these two permeases, a specific histidine permease and an aromatic permease, is confirmed by the following competition studies.

All the naturally occurring α-amino acids (tested at $10^{-3}$ $M$) and a number of histidine analogs[2] (tested at 10–150-fold excess over the histidine concentration) were assayed

[2] The following analogs were assayed: D,L-1,2,4 triazole-3-alanine; L-histidinol; D,L-α-methylhistidine; L-1-methylhistidine; L-3-methylhistidine; L-histidine hydroxamate; L-carnosine; 3-pyrazolealanine; imidazole propionic acid; and 2-thiazolealanine.
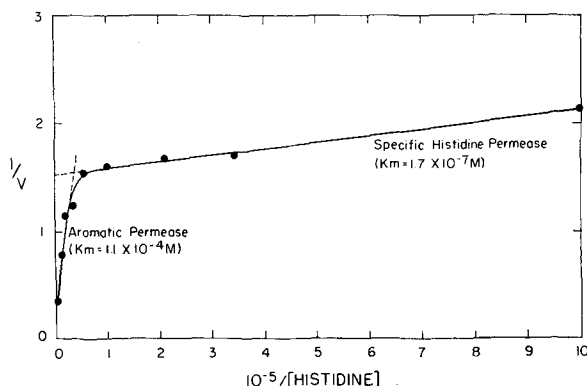
FIG. 2. Lineweaver-Burk plot for histidine uptake in starved cells. Accurate $Km$ values were obtained from additional experiments (not shown in this figure) in which numerous histidine concentrations were chosen in the two separate sections of the $Km$ plot. No correction was made for the contribution of the other permease to the activity that was being assayed. At the concentration of $4 \times 10^{-6}$ $M$ only the specific histidine permease is active; at the concentration of $3 \times 10^{-4}$ $M$, 70% of the rate of uptake is due to the aromatic permease, and the remaining 30% is due to the saturated histidine permease.

as inhibitors of uptake at two different histidine concentrations, representing each of the two branches of the $Km$ curve (Fig. 2): $4 \times 10^{-6}$ $M$ and $3 \times 10^{-4}$ $M$. At the low histidine concentration, none of the amino acids or of the analogs gave any significant inhibition of uptake. At $3 \times 10^{-4}$ $M$ histidine, however, tryptophan, phenylalanine, tyrosine, and leucine,[3] among the amino acids, and the analogs 1-methylhistidine, 3-pyrazolealanine, and 2-thiazolealanine, inhibited the uptake by 70%. This represents a complete inhibition of uptake of histidine by the aromatic permease (see legend to Fig. 2).

The inhibition by aromatic amino acids[3] was investigated further. Each of the three

[3] Leucine was a much less effective inhibitor than the aromatic amino acids. When present at one-tenth the histidine concentration it did not inhibit at all. The leucine used in this experiment was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. The absorption spectrum in the ultraviolet region obtained on a 0.1 $M$ leucine solution showed a peak in the tyrosine region (274 m$\mu$) which corresponded to a tyrosine concentration of $3 \times 10^{-4}$ $M$. The presence of phenylalanine could not be demonstrated unequivocally by the spectrum, but is considered probable. Such contaminations can account for the apparent competition for uptake between leucine and histidine.

aromatic amino acids still caused about 70% inhibition at concentrations as low as one-tenth the concentration of histidine. Tryptophan and phenylalanine gave approximately 50% inhibition at one-hundredth the histidine concentration.

The fact that tyrosine, tryptophan, and phenylalanine all inhibited the low affinity histidine permease ($Km = 10^{-4}$ $M$) with $Ki$ values in the $10^{-6}$–$10^{-7}$ $M$ range supported the suggestion of one permease for the aromatic amino acids which worked poorly on histidine. A study of the uptake of aromatic amino acids by the aromatic permease is presented in later sections.

CONTROL OF THE HISTIDINE PERMEASE

The control of the histidine permease was investigated. Cells derepressed for the histidine biosynthetic enzymes had a normal level of permease; addition of histidine to the growth medium did not induce an increased level nor repress it. It was therefore concluded that the gene(s) for the histidine permease is not located within the histidine operon (Ames and Hartman, 1963). The possibility of the permease being regulated with the histidine degradative pathway could not be investigated, as the strain of *Salmonella* used does not degrade histidine.

## II. THE AROMATIC PERMEASE

### Uptake of the Aromatic Amino Acids in Starved Cells

In starved cells aromatic amino acids are only taken up by the aromatic permease. The uptake was measured in the same way as described for histidine.[4] The measured initial rates were not linear because of the rapidity of the reaction; the rates obtained by extrapolation of the first two points (30 seconds and 1 minute after the addition of radioactive amino acid) were assumed to represent initial rates. No assay was made on the nature of the accumulated radioactivity: this strain of *S. typhimurium* does not utilize any of the aromatic amino acids either as a carbon or as a nitrogen source. Boiled cells took up a negligible amount of labeled amino acid in the range of concentrations used.

The uptake of any one of the aromatic amino acids was inhibited by the others added in all possible combinations. Competition for the uptake of tryptophan by either phenylalanine or tyrosine was measured and the data were plotted according to the method of Dixon and Webb (1958). The $Km$ for tryptophan obtained in this way was $5 \times 10^{-7} M$. The $Ki$ for phenylalanine and for tyrosine, as competitive inhibitors of the tryptophan uptake, were $7 \times 10^{-7} M$ and $2 \times 10^{-6} M$, respectively.

1-Methylhistidine and 3-pyrazolealanine, which inhibited the uptake of histidine by the aromatic permease, also inhibited (at a 3000-fold excess) the uptake of tyrosine. 3-Methylhistidine and histidinol (at a 3000-fold excess), which were ineffective on histidine uptake, were ineffective on tyrosine uptake also.

Several other amino acid analogs were assayed as inhibitors of the uptake of tryptophan. *p*-Fluorophenylalanine (FPA), 5-methyltryptophan, $\beta$-2-thienylalanine, $\beta$-3-furylalanine, and azaserine[5] were good inhibitors.

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[4] The cells were starved by preincubation for 30 minutes only. The initial rate of uptake remained constant between 0 and 145 minutes of starvation.

[5] L-Azaserine gave 90% inhibition at 200-fold excess and 40% inhibition at 66-fold excess. The

## III. ASSAY OF PERMEASE ACTIVITY IN GROWING CELLS

### Measurement of the Permeases by Protein Incorporation Assay

The following method was devised to study initial rates of uptake under physiological conditions, i.e., in normally growing cells.

Addition of a radioactive amino acid to exponentially growing cells results in an immediate uptake of the amino acid into the cell and its incorporation into protein. By filtering samples of an incorporating culture on a Millipore filter one obtains an assay of the total quantity of amino acid taken up by the cells. This will include both the amino acid present in the pool and the amino acid incorporated into the protein. Addition of 5 % TCA to the sample followed by heating for 10 minutes at 90° causes a release of the amino acid present in the pool and bound to s-RNA; the remaining TCA-precipitable radioactivity represents amino acid incorporated into protein (see *Methods*).

The earliest sample taken after addition of the amino acid, namely after 15 seconds, always represents a completely equilibrated pool, i.e., the final pool size has been reached and remains constant thereafter (Fig. 3). Therefore, no initial rates of uptake into the pool can be measured in growing cells under these conditions for the amino acids studied.

However, by decreasing considerably the concentration of the radioactive amino acid in the medium, the permeases become unsaturated and therefore limiting for the process of incorporation of the $C^{14}$-amino acid into protein. Thus, determination of the rate of incorporation into protein gives an indirect assay of the permease activity. Of course the growth rate remains normal, because the biosynthetic pathway supplies

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analog of azaserine, L - diazo - oxonorleucine (DON), at 120-fold in excess of tryptophan, did not give any inhibition. This agrees with the observation that the inhibition of growth by azaserine can be reversed by any of the three aromatic amino acids (Kaplan *et al.*, 1959), while inhibition by DON cannot (Maxwell and Nickel, 1957).
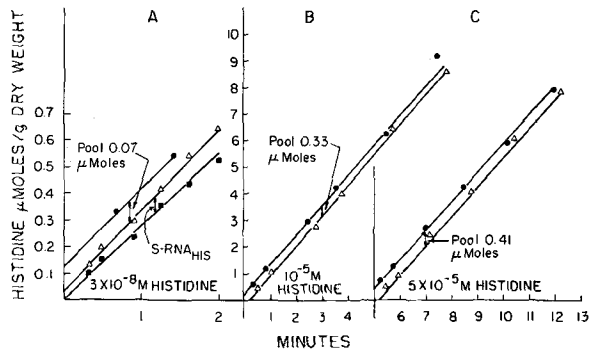
FIG. 3. Histidine incorporation into protein at three different histidine concentrations. In curve A the rate of incorporation is 0.3 $\mu$mole per gram dry weight per minute. In both curves B and C the rate is 1.0 $\mu$mole per gram dry weight per minute. ($\bullet$) Total uptake; ($\triangle$) cold TCA samples; ($\blacksquare$) hot TCA samples. For experimental details see *Methods*.
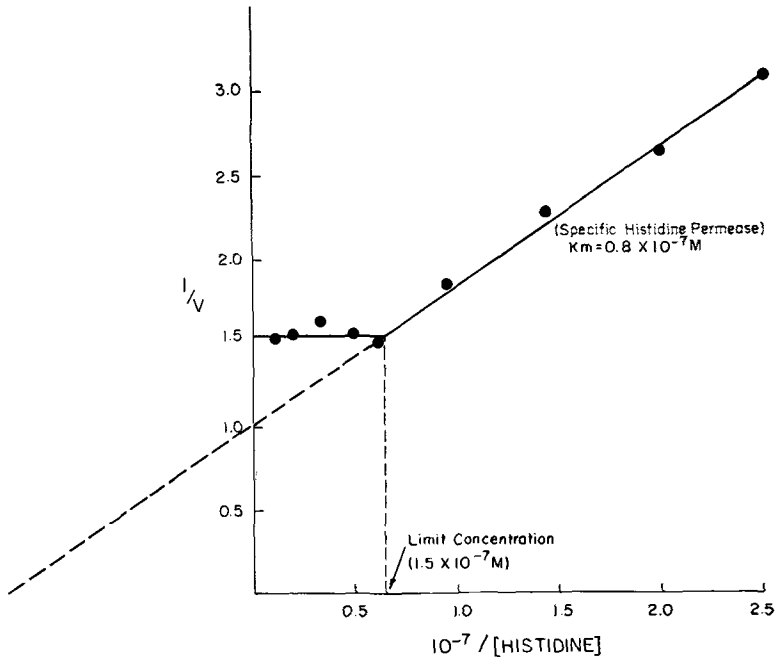


FIG. 4. Lineweaver-Burk plot for histidine uptake, measured by protein incorporation. Maximal rate of histidine incorporation at these low bacterial densities is 0.66 $\mu$mole per gram per minute (see details of this experiment in *Methods*). The incorporation at high bacterial density (e.g., $4 \times 10^8$ cells per milliliter) is 1.0 $\mu$mole per gram per minute. This discrepancy has been found consistently and is also true for phenylalanine (Fig. 5). At low bacterial density the composition of cellular proteins might be different.

that part of the amino acid requirement which does not come from the outside. It is necessary to decrease the bacterial concentration to relatively low values (about $1 \times 10^7$ cells per milliliter) in order not to change the concentration of the substrate during the assay, and to have a sufficiently high specific activity for the labeled amino acid to allow detection of very small amounts.

MEASUREMENT OF AFFINITY CONSTANTS

By the use of this method, which can be applied to the study of other amino acids,
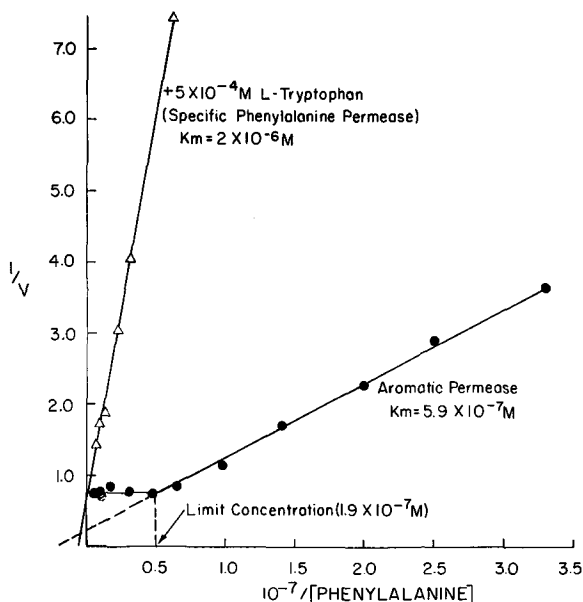
FIG. 5. Lineweaver-Burk plot for phenylalanine uptake, measured by protein incorporation. In the absence of tryptophan (●) the curve corresponds to the aromatic permease. In the presence of $5 \times 10^{-4}$ $M$ tryptophan (△) the curve obtained is due to the specific phenylalanine permease. No correction for the activity of the phenylalanine permease is made on the aromatic permease curve; such correction would be very small. The maximal rate of phenylalanine incorporation at these low bacterial densities is 1.3 $\mu$moles per gram per minute (see details of this experiment in *Methods*). The incorporation at high bacterial density (e.g., $4 \times 10^8$ cells per milliliter) is 1.6 (see legend to Fig. 4).

the $Km$ values were measured for the uptake of histidine and of phenylalanine (Figs. 4 and 5, respectively). The observed values of $0.8 \times 10^{-7}$ $M$ and $5.9 \times 10^{-7}$ $M$, respectively, are in good agreement with those obtained in starved cells (i.e., $1.7 \times 10^{-7}$ $M$ for the histidine permease, and $7 \times 10^{-7}$ $M$ for the uptake of phenylalanine by the aromatic permease).

The histidine $Km$ curve (Fig. 4) does not show the break due to the activity of the aromatic permease (as starved cells do). This is due to the fact that once the permease activity is no longer limiting for the protein incorporation, any further increase in its rate will not appear as an increase in the rate of protein incorporation: when concentrations of histidine are reached which involve transport by the aromatic permease, the rate of protein incorporation has already reached saturation.

The phenylalanine data in Fig. 5 show a peculiarity which did not appear in similar experiments with starved cells. Addition of a great excess of tryptophan (500–5000-fold) gives a maximal, but limited, inhibition of uptake, thus suggesting the existence of a second permease, specific for phenylalanine, and with a $Km$ of about $2 \times 10^{-6}$ $M$ and a $V_{max}$ of 1.5.

Both the histidine and phenylalanine (aromatic permease) $Km$ curves level off at high substrate concentration and give an apparent $V_{max}$ which is smaller than the $V_{max}$ obtained by extrapolation. This is also due to the saturation of the protein synthesizing system.

INHIBITION OF UPTAKE IN GROWING CELLS

The presence of a specific phenylalanine permease (distinct from the aromatic permease) and of specific tryptophan and tyrosine permeases as well, is confirmed by experiments, in growing cells, involving competition between aromatic amino acids and use of inhibitory analogs with saturating levels of substrate.

TABLE I

COMPETITION BETWEEN AROMATIC AMINO ACIDS MEASURED BY INCORPORATION INTO PROTEIN

| Additions | Rate of incorporation of labeled amino acid[a] ($\mu$moles/g. dry weight/min.) | | | | | |
|---|---|---|---|---|---|---|
| | C14-phenyl-alanine (5 × 10⁻⁶ M) | % of Control | C14-tyrosine (5 × 10⁻⁶ M) | % of Control | C14-tryptophan (5 × 10⁻⁶ M) | % of Control |
| None | 1.6 | 100 | 1.4 | 100 | 0.57 | 100 |
| Tryptophan[b] (10⁻⁴ M) | | | 1.1 | 79 | | |
| Phenylalanine[b] (10⁻⁴ M) | | | | | 0.53 | 93 |
| Tyrosine (10⁻⁴ M) | 1.2 | 75 | | | | |

[a] The rates were assayed within 2 minutes of the simultaneous addition of the labeled amino acid and the competing substrate.

[b] Higher concentrations gave essentially the same inhibition.

TABLE II

INTERACTIONS BETWEEN AROMATIC AMINO ACIDS AND p-FLUOROPHENYLALANINE MEASURED BY PROTEIN INCORPORATION

| Additions | Rate of incorporation of labeled amino acid[a] ($\mu$moles/g. dry weight/min.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C14-phenyl-alanine (5 × 10⁻⁶ M) | % of Control | C14-tyrosine (5 × 10⁻⁶ M) | % of Control | C14-trypto-phan (5 × 10⁻⁶ M) | % of Control | C14-FPA (2 × 10⁻⁵ M) | % of Control |
| None | 1.6 | 100 | 1.4 | 100 | 0.57 | 100 | 1.5 | 100 |
| Phenylalanine (10⁻³ M) | | | 0.22[c] | 16 | | | | |
| Tryptophan (5 × 10⁻⁴ M) | | | | | | | 0.19 | 12 |
| FPA (2 × 10⁻³ M)[b] | 0.14 | 9 | 0.15 | 11 | 0.46 | 81 | | |

[a] Rates were assayed within 2 minutes of the simultaneous addition of the labeled amino acid and the competing substrate.

[b] Rates are corrected for the inhibition of growth caused by addition of 2 × 10⁻³ M FPA, i.e., the rate of valine incorporation decreased to 80% within 3 minutes of the addition of 2 × 10⁻³ M FPA.

[c] Tyrosine appears to be transported poorly by the phenylalanine permease, in fact, it inhibits 58% of C14-phenylalanine incorporation when added at 60-fold excess.

From the data illustrated in Table I, the following conclusions are reached:

(a) There is a specific phenylalanine permease. In the presence of sufficient tyrosine to inhibit the aromatic permease completely, the specific permease takes up 75% of the phenylalanine incorporated into protein (at the phenylalanine concentration used).

(b) Tyrosine is similarly transported by a specific permease, which accounts for 80% of the uptake, when the aromatic permease is inhibited.

(c) The tryptophan required for proteins can be almost completely supplied by a specific tryptophan permease, as only a 7% inhibition is obtained by adding an excess of phenylalanine.

Specific permeases have to be postulated here because these data cannot be explained in terms of one single permease transporting all three aromatic amino acids.[6] If this were true, complete inhibition should have been obtained in each of the competition experiments of Table I, as is obtained in starved cells.

[6] The possibility that the competition between aromatic amino acids occurs at the level of protein synthesis, rather than at the permease level, has been discarded because of the properties of mutant AZA-3.
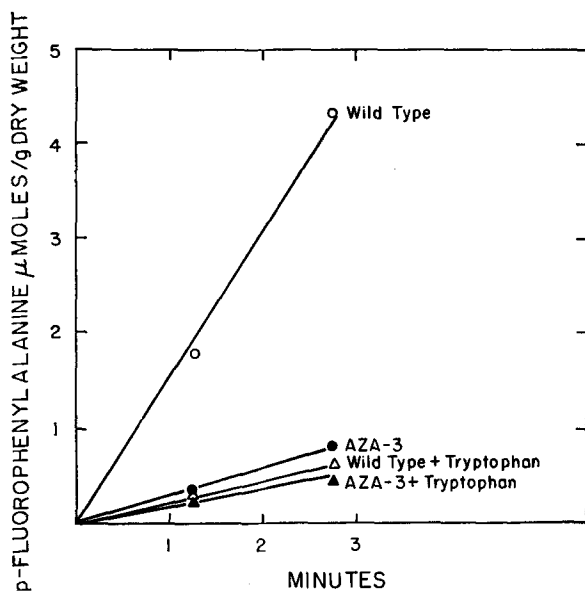
FIG. 6. Rate of $C^{14}$-FPA incorporation into protein. Open symbols: wild type; closed symbols: AZA-3; (O) $2 \times 10^{-5} M$ $C^{14}$-FPA; ($\triangle$) $2 \times 10^{-5} M$ $C^{14}$-FPA $+ 5 \times 10^{-4} M$ tryptophan. The rate of FPA incorporation in the wild type, in the absence of tryptophan, is 1.5 $\mu$moles per gram per minute, and is maximal, as expected, because it completely substitutes for phenylalanine.

From Table II the following conclusions are reached:

(a) Phenylalanine can also be transported poorly by the tyrosine permease as it inhibits $C^{14}$-tyrosine uptake when present in great excess (second line). This inhibition is considerably bigger than the one obtained with corresponding amounts of tryptophan.[7]

(b) The analog p-fluorophenylalanine (FPA) is transported by the aromatic permease as its uptake (assayed by incorporation of $C^{14}$-FPA) is inhibited by tryptophan (last column, third line; see also following section).

(c) FPA appears to be transported also by the phenylalanine and tyrosine permeases as at $2 \times 10^{-3} M$ it inhibits 90% of $C^{14}$-phenylalanine and $C^{14}$-tyrosine uptake, while it only inhibits 20% of $C^{14}$-tryptophan incorporation (fourth line). By competition with phenylalanine a Ki for FPA of $9 \times 10^{-5} M$ for the phenylalanine permease was

[7] The alternative explanation, that phenylalanine only inhibits the tyrosine permease, without being transported, is less likely in view of the results with FPA, which are described later.

obtained. p-Fluorophenylalanine completely inhibited the uptake of all three aromatic amino acids in starved cells.

RATE OF UPTAKE OF $C^{14}$-FPA AS AN ASSAY FOR A DEFECTIVE PERMEASE

About 90% of $C^{14}$-FPA is transported by the aromatic permease (when added at $2 \times 10^{-5} M$; Table II). The remaining 10% is transported by the phenylalanine permease. Therefore a defective aromatic permease would appear as a greatly decreased incorporation of FPA.

The amount of FPA transported by the aromatic permease can be determined by using tryptophan as a competitor in the incorporation of labeled FPA into proteins. p-Fluorophenylalanine is known to be an analog of phenylalanine and to completely substitute for it in proteins (Munier and Cohen, 1959). Addition of tryptophan, in 25-fold excess, to a culture incorporating radioactive FPA gives a maximum inhibition of 88% when FPA is at the concentration of $2 \times 10^{-5} M$ (Fig. 6, open symbols). Therefore 88% of the FPA incorporated into

protein, when the FPA concentration is $2 \times 10^{-5}$ $M$, is transported by the aromatic permease; the remaining 12% (completely inhibited by the addition of small amounts of phenylalanine) is transported by the specific phenylalanine permease and is not saturated in respect to FPA concentration. At higher concentrations, the rate of transport of FPA by the phenylalanine permease will increase further. Higher concentrations of FPA cannot be used because nonspecific adsorption of FPA, to the cells or to the Millipore filters, then becomes large and interferes with the assay.

This interrelationship between FPA and tryptophan is confirmed by growth experiments in later sections.

## IV. ISOLATION OF MUTANTS

The isolation of permease mutants was undertaken once some understanding of the complexities of the permeation systems had been obtained. One type of mutant for the aromatic permease has been isolated, but no mutants have so far been isolated for any of the specific permeases. A number of mutants, initially thought to be permease mutants, were isolated, but on further investigation turned out to have normal permeases. For this reason the following methods for isolation of mutants are presented in some detail here, and some criteria for the recognition of permease mutants will be discussed later.

Two methods were used for the isolation of permease mutants: (a) resistance to analogs transported by specific permeases (Schwartz et al., 1959) and (b) penicillin selection (Lubin et al., 1960).

### RESISTANCE TO INHIBITORY ANALOGS

Ultraviolet-irradiated cells were plated on minimal medium containing an inhibitory amino acid analog. Resistant colonies were isolated and tested for a defective permease, which would exclude the analog from the cell. Two analogs were used: azaserine and FPA.

(i) The mutant AZA-3 was isolated on azaserine, which is transported by the aromatic permease. AZA-3, besides being resistant to azaserine, is also resistant to low concentrations of 5-methyltryptophan

and FPA, but not to high ones. AZA-3 was shown to be missing the aromatic permease (see later sections).

(ii) A class of mutants resistant to FPA was isolated. Among these, those that were also resistant to 5-methyltryptophan were examined. All of these appeared to be aromatic permease mutants because starved cells showed decreased uptake of each of the three aromatic amino acids and also because they showed decreased incorporation of phenylalanine and FPA into protein. Nevertheless, these are not permease mutants; in fact, the one with the lowest incorporation rate, chosen to represent this class, was found to excrete phenylalanine, and this excretion would account for all the results. The most sensitive way of showing this excretion was by the inhibitory effect obtained when supernatant from the mutant culture was added to the wild type incorporating $C^{14}$-phenylalanine. The usual criterion for excretion, namely the appearance of a halo on the isolation plates (Adelberg, 1958), was not evident with these mutants.

Another class of colonies, resistant to FPA plus tyrosine (the tyrosine inhibits the aromatic permease, so that FPA is taken up only by the specific phenylalanine permease), was isolated. As predicted for mutants for the specific phenylalanine permease, they showed no resistance to FPA in the absence of tyrosine. Nevertheless, none had a decreased rate of incorporation of FPA. Their resistance, therefore, was not due to a permease defect.[8]

### THE PENICILLIN SELECTION

The technique, as described by Lubin et al. (1960), involves ultraviolet irradiation of an auxotroph for the amino acid under study, and exposure to penicillin in the presence of very small amounts of the amino acid. This allows survival of permeaseless mutants which grow slowly, or not at all,

[8] The behavior of these mutants can be explained in the following terms: a specific cell component, initially sensitive to FPA, has become resistant to FPA supplied in small amounts (i.e., in the presence of tyrosine). Large amounts of FPA are still inhibitory, either to the same cell component or to a different one.
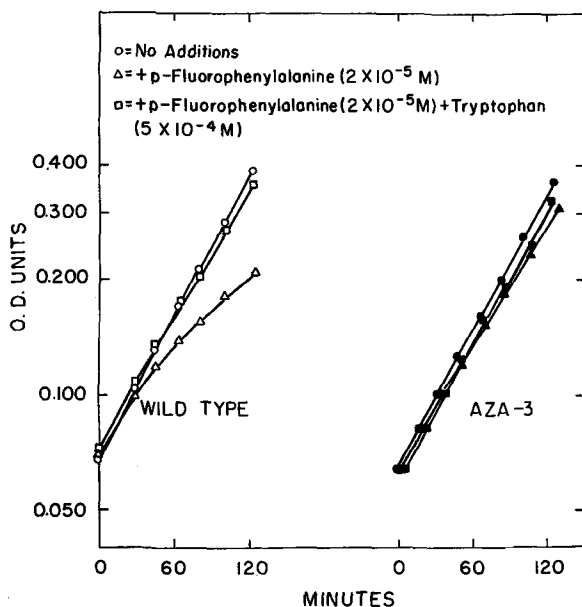
Fig. 7. Inhibition of growth by FPA at low concentration ($2 \times 10^{-5} M$), with ($\square$) or without ($\triangle$) tryptophan. Control (no FPA): ($\bigcirc$). Open symbols: wild type; closed symbols: AZA-3. The additions were made to a culture growing exponentially in medium E.

on small concentrations of the supplement, but are able to grow on high concentrations. This method was used on a histidine-requiring mutant and on a phenylalanine-requiring mutant. No permeaseless mutant was isolated in either case. Up to three successive cycles of penicillin treatment were used in some instances. It is not known whether more recycling would have yielded mutants (Lubin, 1962).

## V. MUTANT AZA-3

### INCORPORATION RATE OF $p$-FLUOROPHENYL-ALANINE IN AZA-3

The following results show that AZA-3 has a defective aromatic permease. A striking decrease in the rate of incorporation of $C^{14}$-FPA in the protein of mutant AZA-3 was observed (Fig. 6): at $2 \times 10^{-5} M$ FPA, the incorporation rate was only 19 % of the rate of the wild type; this is about the same level as the one obtained in the wild type upon addition of tryptophan. The incorporation rate of phenylalanine was 80 % of the wild type control; the incorporation rate of tyrosine was 80 % of control; and the tryptophan incorporation was unaffected.

The decreased incorporation of FPA by AZA-3 could be due to excretion of some compound competing with FPA for uptake and/or incorporation. This possibility was discarded by assaying the effect of the supernatant from an AZA-3 culture when added to wild type cultures incorporating each of the aromatic amino acids and FPA; no decrease in any incorporation rate was noticed.

### EFFECT OF FPA ON THE GROWTH OF WILD TYPE AND OF MUTANT AZA-3

The scheme of permeases presented in the previous section is confirmed also by the results of growth experiments presented in this section. They are as follows:

(1) FPA is efficiently transported by the aromatic permease at low external concentrations ($2 \times 10^{-5} M$). Figure 7 shows that sufficient analog is transported to considerably inhibit growth of the wild type. Such inhibition can be completely reversed by the unrelated amino acid tryptophan, confirming the existence of a competition between FPA and tryptophan at the level of the aromatic permease.
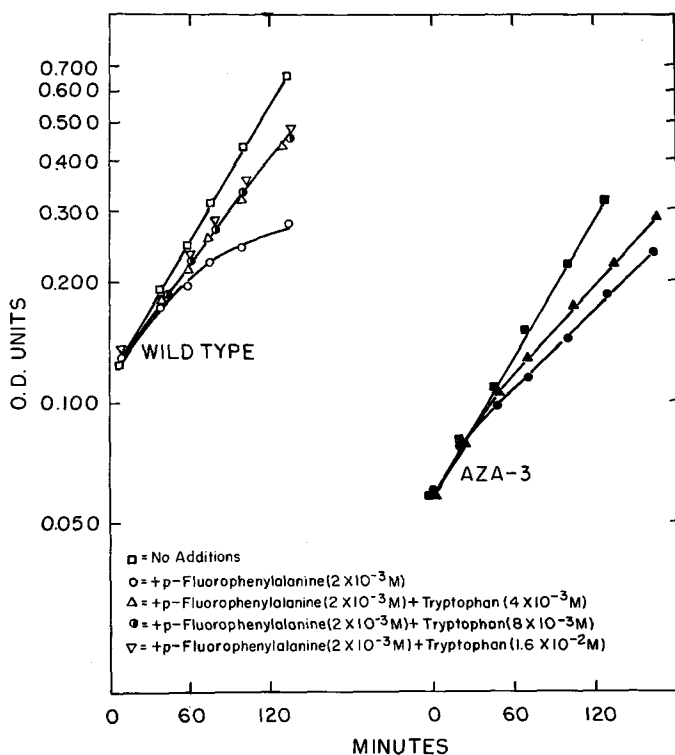
Fig. 8. Inhibition of growth by FPA at high concentration $(2 \times 10^{-3} M)$, with or without tryptophan. Open symbols: wild type; closed symbols: AZA-3. The small improvement of growth obtained by addition of tryptophan to the AZA-3 culture inhibited by FPA is due to the residual activity of the aromatic permease. The resulting growth rate corresponds to that of the wild type in the presence of the same concentrations of FPA and of tryptophan.

(2) Higher concentrations of FPA involve transport by the phenylalanine permease. Thus the inhibition caused in the wild type by $2 \times 10^{-3} M$ FPA can be only partially reversed by an excess of tryptophan (Fig. 8).

(3) In contrast to the wild type, the mutant AZA-3, which has a defective aromatic permease, is resistant to $2 \times 10^{-5} M$ FPA (Fig. 7); in fact, at this concentration only small amounts of FPA are transported into the cell through the phenylalanine permease (Table II). However, AZA-3 is inhibited by higher concentrations, which saturate the phenylalanine permease ($2 \times 10^{-3} M$ FPA, Fig. 8), though less than the wild type. This inhibition, as expected, is not reversed by tryptophan.

Also fitting in the scheme are the following facts: (a) AZA-3 is resistant to azaserine, on which it was isolated [azaserine appears to be transported only by the aromatic permease. Any of the aromatic amino acids will completely reverse its inhibition (Kaplan et al., 1959)]. (b) AZA-3 is also resistant to 5-methyltryptophan at $10^{-5} M$, but not to higher concentrations (e.g., $6 \times 10^{-5} M$; presumably 5-methyltryptophan is transported by a second permease at higher concentrations, possibly the specific tryptophan permease).

EFFECT OF OTHER AROMATIC ANALOGS ON GROWTH AND RATES OF INCORPORATION

Other analogs can be transported by the specific phenylalanine permease. The following compounds gave an inhibition of growth in the wild type which could be reversed either partially, or not at all, by addition of tryptophan: β-2-thienylalanine, β-3-thienylalanine, β-3-furylalanine, o-fluorophenyl-

## TABLE III

EFFECT OF AROMATIC ANALOGS ON PHENYLALA-
NINE INCORPORATION IN WILD TYPE AND IN
AZA-3

| Additions | Rate of $C^{14}$-phenylalanine incorporation ($5 \times 10^{-6}$ $M$)[a] ($\mu$moles/g. dry weight/min.) | |
| --- | --- | --- |
| | AZA-3 | Wild type + tryptophan ($1.3 \times 10^{-3}$ $M$) |
| None | 1.2[b] | |
| 5-Methyltryptophan ($10^{-3}$ $M$) | 1.0 | |
| $\beta$-2-Thienylalanine ($9 \times 10^{-4}$ $M$) | 0.05 | 0.1 |
| $\beta$-3-Thienylalanine ($9 \times 10^{-4}$ $M$) | 0.03 | 0.07 |
| $\beta$-3-Furylalanine ($1.3 \times 10^{-3}$ $M$) | 0.03 | 0.09 |
| $o$-Fluorophenylalanine ($9 \times 10^{-4}$ $M$) | 0.03 | 0.07 |
| $m$-Fluorophenylalanine ($9 \times 10^{-4}$ $M$) | 0.05 | 0.07 |

[a] The rates were assayed within 2 minutes of the simultaneous addition of $C^{14}$-phenylalanine and the analog.

[b] A wild type culture gives under these conditions (no additions, no tryptophan) a rate of 1.6.

alanine, and $m$-fluorophenylalanine. These compounds were also efficient inhibitors of $C^{14}$-phenylalanine incorporation in a culture of AZA-3; complete inhibition was obtained with a 200-fold excess over the phenylalanine concentration (Table III). Tryptophan had no effect on the inhibition. The same extent of inhibition was obtained when wild type in the presence of tryptophan was used as the test organism; in the absence of tryptophan the residual rates were 2 to 4 times larger than in its presence. These results show that the analogs are only partially transported by the aromatic permease, as AZA-3 (missing the aromatic permease) or wild type plus tryptophan (i.e., with an inhibited aromatic permease) are still inhibited by them. It is probable that the analogs are transported to a large extent by the phenylalanine permease. As a comparison, 5-methyltryptophan does not inhibit phenylalanine incorporation in AZA-3.

## VI. EFFECT OF GLUCOSE ON UPTAKE

Of considerable interest is the effect of glucose on uptake in general. The conclusion reached from the following experiments is that glucose increases the initial rate of uptake and decreases the size of the pool both for histidine and the aromatic amino acids. Glucose has been found to have a similar effect on the $\beta$-galactoside permease (Kepes, 1960), on glutamic acid accumulation in Lactobacillus arabinosus (Holden and Holman, 1959), on the galactose permease (Horecker et al., 1960), and, incidentally, on amino acid transport in kidney slices (Segal et al., 1962). The mechanism of such a phenomenon is unknown.

Whenever glucose is used in an experiment, the starved cells are incubated with chloramphenicol (50 $\gamma$ per milliliter) for 10 minutes before adding the radioactive amino acid in order to stop protein synthesis. Figure 9 shows the effect of: (a) glucose when added at the beginning of uptake, and (b) the addition of glucose when uptake has reached equilibrium. The initial rate is unchanged or increased by the presence of glucose, but the size of the pool is considerably depressed; addition of glucose to a suspension that has been exposed to histidine for 35 minutes causes an active and very rapid expulsion of most of the accumulated radioactivity. Qualitatively this same result is obtained in a culture treated exactly in the same way, but with the omission of chloramphenicol. In this case protein synthesis occurs upon addition of glucose and renders quantitative measurements more difficult.

The decrease in pool size was studied as a function of glucose concentration. Figure 10 shows that at concentrations below $3 \times 10^{-4}$ $M$ the inhibition decreases, being almost nonexistent at a concentration of $7 \times 10^{-5}$ $M$. The inhibition reached at $3 \times 10^{-4}$ $M$ is maximal and further increases in concentration are without effect.

### DISCUSSION

The uptake, by bacterial cells, of materials present in the external medium is achieved through specific systems with enzymatic properties. These systems are here referred
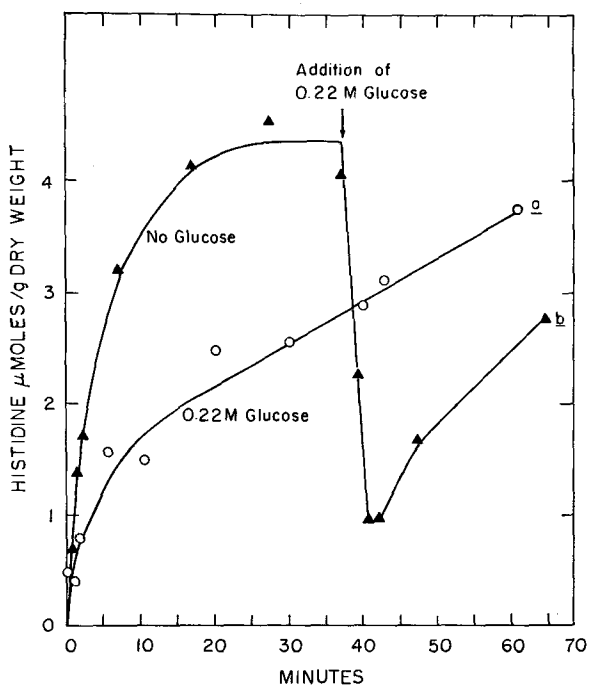
FIG. 9. Effect of glucose on uptake of histidine in starved cells. Cells had been incubated with chloramphenicol (50 $\gamma$ per milliliter) for 10 minutes prior to addition of histidine and after 90 minutes of starvation. Medium E-C. Temperature: 25°C. Under these conditions chloramphenicol causes an inhibition of the initial rate of uptake which increases with time of preincubation, being 30% at 20 minutes and approximately 70% at 60 minutes.
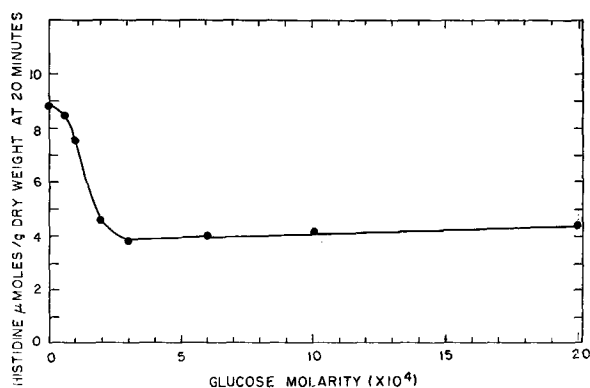


FIG. 10. Inhibition of pool formation by glucose. Cells were assayed 20 minutes after the simultaneous addition of histidine and of glucose. Cells were pretreated as described in Fig. 9.

to as *permeases*.[9] For general reviews on amino acid transport in bacteria, see Holden

[9] The term *permease* has been defined by Rickenberg *et al.* (1956) and does not refer to any specific mechanism of action.

(1962), which covers also transport in organisms other than bacteria, and Kepes and Cohen (1962). Extensive studies on the kinetics of uptake of some amino acids and of pool formation in *E. coli* have been pre-

sented by a group working at the Carnegie Institution of Washington (reviewed by Britten and McClure, 1962). The technique of Millipore filtration, used throughout this work, was also originally introduced by this group. Transport of some amino acids and of β-galactosides in *E. coli* has been studied by a group at the Pasteur Institute in Paris (Cohen and Rickenberg, 1956; Rickenberg *et al.*, 1956; Kepes, 1960). Both these groups have presented kinetic models which are attempting to explain the mechanism of uptake. A few attempts have been made to correlate known biochemical functions with transport mechanisms in various bacteria (Holden, 1959a,b; Rogers and Yu, 1962, 1963; Zabin *et al.*, 1962; Englesberg *et al.*, 1961). However, very little is yet known about the actual mechanism of action of these systems.

The bacterial cell (as compared to mammalian cells or tissue slices) is particularly suited for this type of investigation, mainly because of its capability to give specific mutants. A mutant missing a definite permease with well-known properties is essential for a biochemical study of transport mechanisms. As the assay for a permease depends on the integrity of the cell, a permeaseless mutant offers a control for a study in a cell-free system where no assay is available. This approach has been used by Nikaido (1962) in the very interesting study on the stimulation of P³² incorporation in phospholipids in the galactoside permease system; this system has the additional advantage of being inducible, thus offering another means of comparison. The aromatic permease mutant, AZA-3, is currently being used in this way.

Mutants for some amino acid permeases have already been isolated in *E. coli* (Schwartz *et al.*, 1959; Lubin *et al.*, 1960). One of these, a mutant unable to transport proline, has been characterized (Kessel and Lubin, 1962) and shown to fit the description of a specific proline-permease mutant.

The characteristics of the uptake of histidine, tryptophan, phenylalanine, and tyrosine by *S. typhimurium* has been presented in the text; only some points of interest will be discussed here.

Bacteria have two possible sources of amino acids for protein synthesis: an external supply brought in by the permeases and the biosynthetic pathway. The specific permeases have very low affinity constants ($Km$ values about $10^{-7}$ $M$) and even very small concentrations of external amino acid will increase the size of the pool, thus inhibiting the biosynthetic pathway, and supply the full requirement of the cell for the amino acid. In fact, the histidine concentration in the pool of an exponentially growing culture in minimal medium (1.5 × $10^{-5}$ $M$, see *Methods*), is of the same order of magnitude as the $Ki$ value found for the feedback inhibition of the first enzyme of histidine biosynthesis [at pH 7.5, $Ki = 5 ×$ $10^{-5}$ $M$ (Martin, 1963)], i.e., at this concentration the feedback control mechanism gives the most sensitive response to changes in concentration.

As one lowers the external concentration of the amino acid, a point is reached at which the permease can no longer bring in enough amino acid to satisfy the protein requirement. The remainder of the requirement is then supplied by the biosynthetic pathway, as the feedback inhibition is relieved. I would like to call this critical point the *limit concentration*, i.e., the minimum external concentration required by the permease in order to supply the cell with enough amino acid to carry on protein synthesis without utilizing any of the biosynthetic amino acid. The limit concentrations of phenylalanine and histidine, for *Salmonella* growing in minimal medium, are 1.9 × $10^{-7}$ $M$ and 1.5 × $10^{-7}$ $M$, respectively. Their determination is shown in Figs. 4 and 5, respectively. The limit concentration for a permease is a biological parameter of more significance than the $Km$, because it expresses the actual efficiency of the uptake with respect to growth. Such low limit concentrations indicate a high efficiency. This is desirable from the point of view of the cellular economy, since the energy involved in the uptake of one molecule of amino acid is probably much smaller than that required for the biosynthesis of the same molecule from glucose.

While it is easy to see the significance of the specific permeases, it is more puzzling to

understand the existence of the aromatic permease. Its broad specificity allows it to work on any amino acid having some aromatic character as well as on histidine and, surprisingly, azaserine [the uptake of azaserine by a phenylalanine transport system was independently suggested by Brock and Brock (1961)]. One reasonable hypothesis would be that the aromatic permease has the function of transporting amino acids in large scale and for purposes other than supplying amino acids for protein synthesis (e.g., supplying the amino acid as carbon or nitrogen sources). Two facts support this hypothesis. The first is that large amounts of amino acids are transported by the aromatic permease alone in starved cells, where no protein synthesis occurs and the specific permeases for the aromatic amino acids seem to be inactive. The specific permeases seem therefore to have a stringent energy requirement. The histidine permease itself is much less active under conditions of carbon starvation. The second fact is that in *E. coli*, which degrades (transaminates) the aromatic amino acids, the rate of degradation is dependent upon the rate of entry into the cell (Moyed and Friedman, 1959), and entry, in this case, is mediated by the aromatic permease (this is deduced from the description that Moyed and Friedman give of the permeation system; they actually suggest one common transport mechanism for the three aromatic amino acids).

With the knowledge of the existence of these various permeases and of their properties, a search for a permeaseless mutant was started. One type of mutant with a defective aromatic permease was obtained by isolation on azaserine. A number of other mutants were isolated and initially believed to be specific permease mutants. Further investigation showed this not to be true. As the isolation methods giving these false permease mutants have been used by other workers, some necessary precautions in their use are discussed here:

(a) The false permease mutant might be an excretor of the amino acids. A good example is a class of mutants isolated as resistant to FPA. They were at first thought to be phenylalanine permease mutants on the basis of a decreased uptake of $C^{14}$-phenylalanine by starved cells and a decreased rate of incorporation of $C^{14}$-phenylalanine and $C^{14}$-FPA into protein. The mutants were later found to excrete phenylalanine; resistance in this case is due to exclusion of the analog by competition with the excess phenylalanine produced, and the decreased uptake of $C^{14}$-phenylalanine results from dilution of the hot material by the excreted cold material. Furthermore, these mutants did not show the halo that was expected to be present around excreting colonies, on the isolation plate. The appearance of a halo would indicate that the excretion product of the mutant protects surrounding bacteria from the inhibition by the analog, thus allowing them to grow. Evidently the appearance of a halo (Aldelberg, 1958) is not as sensitive a test for excretors as the one introduced here, namely the assay of uptake in the wild type in the presence of supernatant fluid from the mutant culture.

Most, or all, of the mutants isolated as resistant to FPA alone are probably excretor mutants; some of the mutants reported in the literature could actually fall in this category (Schwartz *et al.*, 1959). Lubin *et al.* (1960) devised a method for the isolation of permease mutants which involves selection, in the presence of penicillin, for auxotrophs which do not grow on very small amounts of the required amino acid but grow normally on large amounts. Mutants isolated by this technique could also be excretor mutants, rather than permease mutants, if they excreted material capable of competing with the uptake of the small amounts of amino acid supplied during the penicillin treatment.

It should be mentioned that excretor mutants might be permease mutants if permeases were involved also in the retention of substrates inside the cell, or in catalyzing an exit reaction. There is no conclusive evidence, as yet, for any such activity of permeases.

(b) Resistance to an analog at the level of protein incorporation. If the analog used is incorporated into protein (as FPA is), mutation in the corresponding s-RNA or activating

enzyme would produce resistance (and, therefore, selection) and a decreased rate of incorporation of the analog into protein. This type of mutant would be indistinguishable from a permease mutant if the growth rate of the organism did not change (the mutation would not necessarily affect the incorporation of the physiological substrate) and if the uptake were assayed by protein incorporation only. The chloramphenicol or starved cell assays for permease, however, would distinguish them. One such activating enzyme mutant has actually been isolated by Neidhardt and Fangman (1963).

(c) The analog, or the amino acid used in a penicillin selection, is degraded by the mutant. Although no mutants have been isolated with these properties, they could certainly be encountered and mistaken for permease mutants if the uptake, as measured by protein incorporation, appears to be decreased.

After having checked through all the above possibilities, none of the mutants isolated (about 50) qualifies as a specific permease mutant (for histidine or for phenylalanine, which were the amino acids studied in this case). Specific permease mutants for these amino acids might be very rare or may be lethal. Further attempts to isolate such mutants are being made.

## ACKNOWLEDGMENTS

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