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Direct Determination of the Properties of Peptide Transport Systems in *Escherichia coli*, Using a Fluorescent-Labeling Procedure

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A direct study of peptide uptake by *Escherichia coli* was made using a fluorescent procedure. After incubation with the bacteria, peptides remaining in the medium were dansylated, separated chromatographically, and quantitated from their fluorescent intensities and/or from their incorporated radioactivity when tritiated dansyl derivatives were prepared. Peptide uptake was apparently not regulated and proceeded continuously until complete, with the absorbed peptides undergoing rapid intracellular hydrolysis and the excess amino acid residues leaving the cell. Thus, peptide uptake and amino acid exodus occur concurrently. However, peptidase-resistant substrates, e.g. triornithine and glycylsarcosine, which can be similarly estimated in cell extracts, were accumulated about 1,000-fold. The influence of amino acid composition and chain length on rates of transport was assessed. Different strains of *E. coli* showed variability in their rates of di- and oligopeptide transport. With respect to energy coupling, both the di- and oligopeptide permeases behaved like shock-sensitive transport systems.

The transport of substances by bacteria is usually studied in the following way. A radioactively labeled substrate is incubated with a suspension of bacteria for an appropriate time, the bacteria are collected on a membrane filter and washed, and the radioactivity retained within the cells is then determined. Use of this procedure has provided great insight into mechanisms of bacterial transport. Nevertheless, the method has a number of drawbacks. For example: (i) a radioactively labeled substrate is essential; (ii) the transport of only one substrate (say ^{3}H, ^{14}C, or ^{32}P labeled), or at best two differently labeled substrates, can be studied at one time; (iii) it is difficult to study uptake and exodus concurrently. The first point presents a fundamental difficulty for studies of peptide transport, since only about half a dozen radioactively labeled dipeptides, and no oligopeptides, have ever been commercially available, and presently none are being produced. A few investigators have themselves synthesized particular labeled peptides, but for general studies of peptide uptake a number of indirect methods have had to be used that do not require labeled substrates. These include using amino acid auxotrophs to monitor peptide-dependent growth (9, 16, 18-20, 22), specific enzyme synthesis (4), or protein synthesis (21).

The present paper describes a direct approach in which peptide transport by *Escherichia coli* is studied by monitoring the disappearance of the substrate(s) from the incubation medium. This is done by preparing dansyl derivatives of the substrate(s) that remains in the medium, separating the derivatives by two-dimensional thin-layer chromatography, and then directly quantitating the intensities of the fluorescent spots. Substrates accumulated intracellularly are similarly detected in cell extracts. Use of the method for studying peptide transport illustrates how it can be used to monitor the simultaneous uptake of many different substrates and to investigate concurrent exodus and uptake of different substrates.

MATERIALS AND METHODS

Materials. Lysine peptides were synthesized as described previously (16). Other peptides and amino acids were purchased from British Drug Houses (Poole, Dorset, U.K.) and Sigma (London) Ltd. Dansyl chloride (dimethylamino naphthalene sulfonyl chloride) was obtained from the British Drug Houses, and polyamide sheets (25 cm^2) were bought from British Drug Houses and from Pierce and Warriner (UK) Ltd. (Chester). Sodium D-lactate, succinate, neomycin sulfate, and carbonylcyanide chlorophenylhydrazone (CCCP) were from Sigma (London) Ltd. [^{3}H]dansyl chloride (specific activity, 8.3 Ci/μmol) was supplied as a solution in benzene by the Radiochemical Centre, Amersham. Before use, the benzene was removed in vacuo, and the dansyl chloride was redissolved in

acetone. Membrane filters (type HA, 13-mm and 25-mm diameter, 0.45 μm pore size) were obtained from Oxoid Ltd. (London) or from Millipore (London), and the corresponding Swinnex filter holders came from Millipore; 45-cm filters were purchased from Oxoid Ltd.

Growth of bacteria. The strains of *E. coli* W used were the lysine auxotroph M2626 *lys* (previously designated M-26-26 [9]), its spontaneous wild-type revertant M2627 *lys*$^+$ (previously referred to as strain M-26-26.R [16]), and their respective oligopeptide transport-deficient strains M2628 *lys opt* and M2629 *lys*$^+$ *opt* (previously referred to as strains M-26-26.TOR and M-26-26.R TOR, respectively [16]). The transport deficiency was established by resistance to triornithine (10, 16) and additionally, for the auxotroph, by failure to utilize trilysine but ability to grow on dilysine. The following strains of *E. coli* K-12 were obtained from Y. Anraku (15): strain 7 (wild type), NR70 (*unc*), DF364 (*pgi*), and DF360 (*pgi unc*), as were also *E. coli* ML308-225 and its derivative DL-54 (*unc*). Strain W was grown in minimal medium A of Davis and Mingioli (8), and all other strains were grown in medium M-56 (26). Media were routinely supplemented with 0.5% (wt/vol) glucose; 1% (wt/vol) lactate was used in certain experiments; and 0.5% (wt/vol) fructose was used for strains DF360 and DF364. Thiamine (10 μg/ml) and amino acids (0.1 mM) were added as required, and neomycin (20 μg/ml) was added to cultures of NR70. Growth was carried out at 37°C, with shaking, in a water bath. *unc* mutants were checked for inability to utilize succinate as carbon source using liquid media and minimal salts-agar; strain NR70 was additionally checked as being resistant to neomycin.

Transport assays. In early studies, exponential-phase bacteria, at 10^9 bacteria per ml, were harvested by centrifuging at about 8,000 × *g* for 15 min at room temperature (22°C), washed twice using equal volumes of 20 mM potassium phosphate buffer (pH 7.2) at 37°C, and suspended in 50 mM potassium phosphate (pH 7.2) containing 0.4 mM NH_4Cl at 37°C to 1.5 × 10^9 bacteria per ml (i.e., an absorbance of 0.75 at 660 nm in a Bausch & Lomb Spectronic 20 spectrophotometer). In more recent experiments, the bacteria were harvested by membrane filtration using either a 25-mm Swinnex system or, for larger volumes, a 45-mm filter attached to a water vacuum pump. In either case, bacteria were washed with approximately equal volumes of buffer as described above, the filter was added to appropriate medium (as above), and the bacteria were resuspended using a Vortex mixer. In routine uptake studies, 1.0-ml samples of the suspension were added to aqueous solutions (1.3 ml; 37°C), containing glucose (final concentration, 20 mM) plus any other supplements, and incubated with aeration for 10 min at 37°C. Transport was initiated by adding 0.2-ml aqueous peptide solutions as indicated in Results. Samples (0.3 ml) were removed from the incubation mixture at appropriate intervals using a 1-ml syringe, and were immediately freed from bacteria by expelling the suspension from the syringe through a membrane filter (0.45-μm pore size), held in a Swinnex filter holder (13-mm diameter), into a small vial. This procedure takes 10 to 15 s for each sample.

For studies into the effects of anaerobic conditions, samples of the resuspended bacteria were bubbled with nitrogen gas for 10 min; the incubation media and peptide solutions were treated similarly. The bacterial suspension was then added to the incubation mixture and treated with nitrogen for a further 10 min before adding the peptide solution. During subsequent incubation, suspensions were maintained under an atmosphere of nitrogen. Control samples were bubbled with air in an exactly analogous manner.

For experiments testing the effects of arsenate, one cannot use phosphate buffer. Therefore, after the bacteria were harvested, they were washed and then suspended in 200 mM tris(hydroxymethyl)aminomethane buffer (pH 7.2) containing 2 mM potassium phosphate and 0.2 mM NH_4Cl. However, tris(hydroxymethyl)aminomethane itself produces an intense fluorescent derivative on polyamide sheets (see below), and we therefore tested other volatile buffers, of which *N*-ethylmorpholine proved particularly suitable. Thus, bacteria were washed and suspended in 200 mM *N*-ethylmorpholine-hydrochloride buffer (pH 7.5) containing potassium phosphate and NH_4Cl, as described above.

For determination of intracellular pool contents, normal transport assay conditions were used. Then 2 ml of bacterial incubation mixture was passed through a 25-mm filter, and the cells were immediately washed twice on the filter with 2 ml of appropriate suspension buffer (37°C, 20 mM). The filter was immediately added to a tube containing 2 ml of boiling water, 0.05 ml of toluene was added, the tube was blended in a Vortex mixer to resuspend the bacteria, and the suspension was boiled for 15 min. After cooling, the extract was filtered using a Swinnex system, and the filtrate was made up to 2 ml.

Dansylation for fluorescence determination. The procedure is based upon that described by Hartley (11). Portions of 50 μl of the filtered incubation media were added to Durham tubes (30 by 5 mm) together with an internal standard, usually 5 μl of 1 mM ornithine, and the solutions were dried in vacuo; for intracellular extracts, five separate 100-μl samples were dried sequentially. $NaHCO_3$ (200 mM; 10 μl) was added to each sample, which was again dried in vacuo. Water (10 μl) and a solution of dansyl chloride in acetone (2.5 mg/ml; 100 μl) were then added. The pH of this solution should be about 9.5. The tubes were sealed with silicone rubber stoppers and incubated at 45°C for 1 h. The dansylated mixtures were taken to dryness, and the residues were redissolved in 10 μl of aqueous pyridine (1:1, vol/vol). Samples (5 μl) were spotted onto polyamide sheets, which were developed in the following solvent systems (23) in the order given, each being run for about 45 min: first dimension, water–formic acid (98.5:1.5, vol/vol); second dimension, acetic acid–toluene (10:90, vol/vol); second dimension, methanol–butyl acetate–acetic acid (40:60:2, vol/vol/vol), and, finally, in the first dimension again using solvent 1. A standard mixture of dansyl amino acids and peptides can be run on the reverse side of the sheet to facilitate identification of materials in the test samples (11). After chromatography, the concentrations of peptides and amino acids in the incubation media and cell extracts were estimated by comparing

the fluorescent intensities of the spots on the chromatograms with the fluorescent intensities of known standards of the same substances. Intensities reported here were estimated visually and were found to be reliably accurate to ±5%. Recently (unpublished data) a recording spectrofluorimeter (Fluoripoint model FP 101) equipped with an automatic thin-layer chromatography plate scanner (model FP 115; Baird Atomic Ltd., Braintree, Essex) has been used to confirm the accuracy of the routine visual determinations. The fluorescence of amino acids was usually more intense than the fluorescence of equimolar peptides. The intensity of the internal standard provides a check upon recoveries and efficiency of dansylation. A different internal standard, usually diaminopimelic acid, was used if a transport substrate, e.g. lysyl- or ornithyl-homopeptides or Ala-Trp, moved to a position similar to ornithine on the chromatogram, or when intracellular extracts that contain ornithine were studied. A permanent record of each chromatogram was obtained by photographing it under UV light using Micro-Neg Pan film type B 35 mm (Ilford) and a Kodak Wratten no. 3 filter. Polyamide sheets were reused after being cleaned for 3 h in acetone–water–0.88 sp. gr. ammonia (35% NH_3) (50:46:4, vol/vol/vol).

Dansylation of radioactive detection. All steps up to the addition of the dansyl chloride were the same as described above. The quantity of tritiated dansyl chloride used permitted incorporation of about 1×10^6 to 2×10^6 cpm into each peptide and/or amino acid spot on the chromatogram, while the total dansyl chloride added allowed visual detection of the samples. Thus, [^{3}H]dansyl chloride (1 nmol) was dissolved in 20 μl of an acetone solution of unlabeled dansyl chloride (2.5 mg/ml), and 10 μl was used to label filtered samples as described above for the fluorescent method. Chromatograms were prepared as usual and were then scanned, using a Panax thin-layer scanner RTLS-1A, with a tritium counting efficiency of about 1%. The radiolabeled derivatives were quantitated from comparison of their incorporated counts with the counts present in known amounts of standards prepared by the same procedure. Because the radioactive spots also fluoresced, we could locate their positions before scanning, which obviated having to scan the entire plate.

RESULTS

In our early experiments using the dansyl procedure, we incubated bacteria with a single peptide substrate and monitored transport by assaying substrate in the incubation medium after thin-layer chromatography. However, when we observed the great capacity of the polyamide sheets to separate amino acids and peptides, it led us to try to obtain more information from each incubation and chromatographic separation. *E. coli* has separate dipeptide and oligopeptide permeases, and no evidence for their mutual competition during transport is normally detectable using indirect auxotrophic growth assays (16); it appeared possible, therefore, that we could study uptake of di- and oligopeptides at the same time. With strain M2627 *lys*$^+$ we found, using dansylation, that mutual inhibition of di- and oligopeptides is minimal, and for a variety of di- and oligopeptides identical uptake was observed when they were studied alone or as pairs of di- and oligopeptides. Therefore, we have on occasion studied the transport of a di- and an oligopeptide together. For example, Fig. 1A to D illustrates the disappearance of Ala-Ala and the slower disappearance of Gly-Gly-Gly from the medium, as a result of their concurrent transport. The concomitant appearance in the medium of alanine and glycine, resulting from intracellular hydrolysis and amino acid exodus, is also shown. In Fig. 1E to H, the relatively slower uptake of Pro-Gly-Gly and Ala-Trp is shown, together with concurrent exodus of their constituent amino acids. Strain M2626 *lys* gave exactly the same results, except that an additional spot, corresponding to diaminopimelic acid (which is excreted by this strain as a consequence of its mutation in diaminopimelic acid decarboxylase), was also detected on chromatograms. When strains M2628 *lys opt* and M2629 *lys*$^+$ *opt* were used, the tripeptides did not disappear from the medium, although similar results were found for the dipeptides.

Influence of chain length and amino acid composition on peptide uptake. The rate at which a peptide disappears from the medium provides a direct measure of its rate of uptake by *E. coli*. Therefore, using strain M2627 *lys*$^+$ we monitored rates of uptake for several series of homologous peptides to determine the influence of amino acid side chains and overall chain length on rate of transport. Table 1 shows that for all oligopeptide series tested, rates of uptake decreased with increasing chain length. For dipeptides, which use a separate system, rates were usually faster than for the corresponding tripeptides, but this was not invariably the case. Results for the homologous peptides (Table 1) were determined from three or four samples taken during 10-min incubation periods, throughout which constant rates of uptake occurred. Additionally, for Gly-Gly, Gly-Gly-Gly, Ala-Ala, and Ala-Ala-Ala rates were also determined by incubating each peptide alone and taking samples every 15 s up to 10 min, and by using various initial peptide concentrations in the range 50 to 400 μM; the same values for the rates of uptake were obtained under all these conditions. It should be noted that the apparent peptide transport affinity constants are usually in the range 1 to 10 μM (7, 21), and although this parameter has not been determined for all the peptide substrates used here the initial concentrations in all these assays were considerably

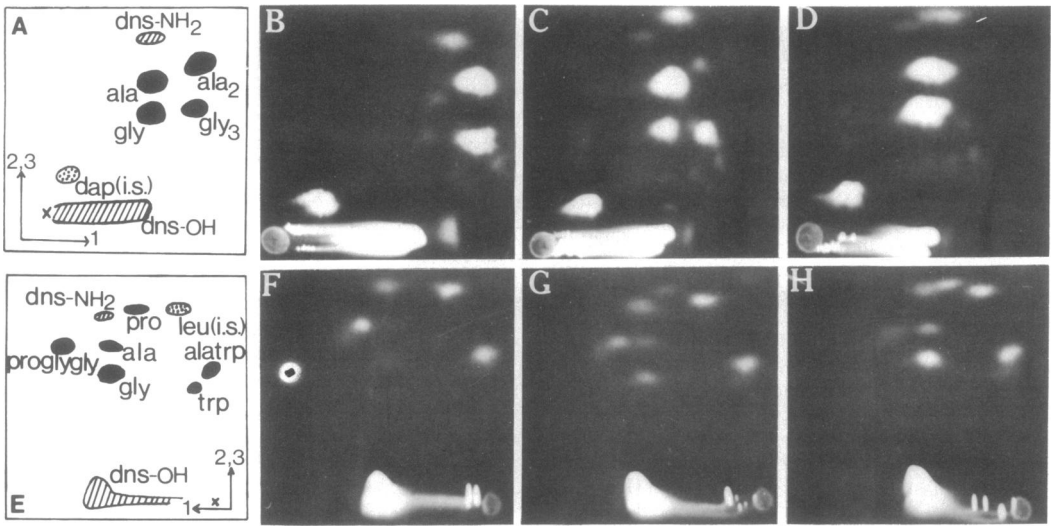

FIG. 1. *Peptide uptake and concurrent amino acid exodus by strain M2627 lys⁺. Each peptide was supplied at 80 μM. Periodically samples of the incubation media were dansylated and chromatographed in solvents 1, 2, and 3, and the thin-layer plates were photographed under UV light. (A-D) Uptake of Ala_2 and Gly_3; B, C, and D are 0, 2, and 20 min of incubation, respectively. (E-H) Uptake of Pro-Gly-Gly and Ala-Trp; F, G, and H are 0, 10, and 20 min of incubation, respectively.*

above this range; since linear uptake rates were also observed, it seems likely that the rates observed represent V_{max}s, although this remains to be shown rigorously. The above four peptides were also examined using the radioactive detection procedure (Materials and Methods). Rates of transport determined by this method were 19, 21, 30, and 42 nmol/min per mg of protein for Gly-Gly, Gly-Gly-Gly, Ala-Ala, and Ala-Ala-Ala, respectively, in good agreement with those in Table 1 based on measurement of fluorescent intensities.

The influence of amino acid side chains is also clearly shown by the results in Table 1. For example, uptake of an alanyl peptide was consistently more rapid than the uptake of the analogous glycyl peptide. Although side-chain effects are apparent, it appears to us to be premature to try to rationalize the influence of this structural parameter until a wider, more systematic, selection of peptides has been studied.

Evidence for a stereochemical requirement for oligopeptide transport is shown by failure of *E. coli* to take in D-Ala-D-Ala-D-Ala. Indirect evidence for this feature had been obtained previously from growth response tests (3, 17).

Peptide mixtures and competition for permeases. Having determined rates of uptake for different di- and oligopeptides studied individually, we investigated their transport from solutions containing mixtures. With such mixtures it was readily apparent that different dipeptides competed with each other and, likewise, that oligopeptides mutually inhibited each

TABLE 1. *Influence of amino acid composition on peptide uptake*

Peptide	Rate of uptake[a] (nmol/min per mg of protein)	
	Range	Avg
Diglycine	12–21	15
Triglycine	14–22	21
Tetraglycine	16–19	18
Pentaglycine	2–3	2
Hexaglycine	1	1
Dialanine	29–42	32
Trialanine	38–51	39
Tetraalanine	24–27	26
Pentaalanine	23–25	24
Dilysine	14–15	15
Trilysine	18–24	21
Tetralysine	7	7
Diornithine	11–15	13
Triornithine	6–7	7
Divaline	12–22	17
Trivaline	9–10	9
Diphenylalanine	21–22	22
Alanyltryptophan	11	11
Glutamylalanine	7–12	10
Trileucine	7	7
Triserine	18–28	23
Trithreonine	8–9	9
Prolyldiglycine	12	12
D-Ala-D-Ala-D-Ala	ND	ND

[a] Cells of strain M2627 *lys⁺* were prepared as described for Fig. 1, and uptake was initiated by adding peptide (80 to 200 μM). Samples were assayed periodically up to 15 min, during which time constant uptake rates occurred. Results are the range and the average of two to five experiments. ND, Not detectable.

other's uptake. Some measured rates of uptake from analysis of the chromatograms are shown in Fig. 2. For example, after 20 min of incubation triglycine was completely transported, but its uptake was about 50% inhibited by an equal amount of triserine and over 90% inhibited when triserine and trilysine were both present (Fig. 2A). From these and other results, the competitive abilities of these particular di- and oligopeptides can be ranked thus: $Ala_2 > Val_2 \approx Phe_2 > Gly_2$; $Ala_3 > Ser_2 > Lys_3 > Gly_3$.

Energetics of peptide transport. Unstarved cells were used in the experiments described here. However, for comparison we carried out some studies with cells starved by prolonged incubation with 2,4-dinitrophenol (DNP) as described by Berger (5). Comparable results were found with the starved and unstarved cells, although stimulation of transport by added carbon source was greater in the former case, in agreement with the report by Cowell (7) for diglycine transport.

Effects of inhibitors on peptide uptake. Using suspensions of M2627 *lys*$^+$ in the range of 0.1 to 1.5 turbidity (absorbance at 660 nm), and with Ala-Ala-Ala, Val-Val-Val, Lys-Lys, and Met-Leu as substrates, the concentrations at which various inhibitors produced their maximum degree of inhibition were determined. These were 25 mM KCN, 30 mM KH_2ASO_4, 3 mM DNP, and 20 μM CCCP. These were therefore used at these concentrations in the experiments presented below. The pH was checked before and after each incubation, and, if a change occurred, the buffer concentration was increased as required.

Arsenate drastically depletes intracellular ATP in *E. coli* (13), thereby specifically inhibiting the transport of those substrates (like glutamine) that are coupled to ATP availability, while having little effect on substrates (like proline) that utilize the energized membrane state (5, 6). Uptake of di- and oligopeptides, for which separate transport systems exist in *E. coli*, was generally decreased more than 80% by this inhibitor, with glucose, lactate, or succinate (not shown) as energy sources (Table 2). These results agree with the report of Cowell (7) on inhibition of diglycine uptake. Uncouplers of oxidative phosphorylation, such as DNP and CCCP, dissipate the energized membrane state and therefore inhibit transport of substrates like proline that use this state directly as a source of energy (5, 6). These reagents had relatively little effect on peptide uptake when glucose was used as an energy source (i.e., substrate-level phosphorylation), but greater inhibition was noted with lactate (i.e., ATP via oxidative phosphorylation) (Table 2); these results are therefore in

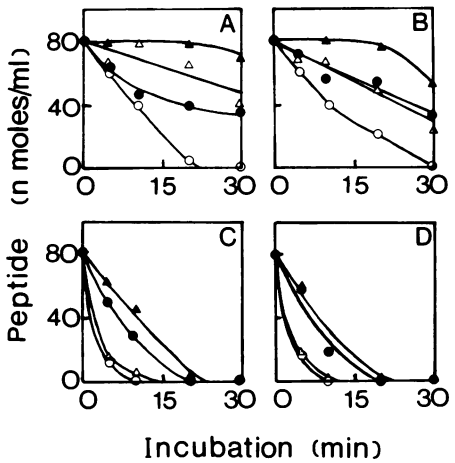

FIG. 2. *Competition during peptide uptake from mixtures by strain M2627 lys*$^+$. *All peptides were supplied at 80 μM, and the amounts remaining in the incubation media were determined at the times indicated. (A) Gly_3 uptake: alone* (○); *+Ser_3* (●); *+Lys_3* (△); *+Ser_3 + Lys_3* (▲). *(B) Gly_2 uptake: alone* (○); *+Val_2* (●); *+Phe_2* (△); *+Val_2 + Phe_2* (▲). *(C) Ala_3 uptake: alone* (○); *+Ser_3* (●); *+Lys_3* (△); *+Ser_3 + Lys_3* (▲). *(D) Ala_2 uptake: alone* (○); *+Val_2* (●); *+Phe_2* (△); *+Val_2 + Phe_2* (▲).

accord with those from the arsenate study, which suggests an ATP dependence. DNP was more inhibitory than CCCP, perhaps indicating it has additional effects; e.g. Berger and Heppel (6) reported that DNP could decrease intracellular ATP by about 40%. Anaerobic incubation produced only a slight inhibition of di- and oligopeptide uptake (Table 2), although, interestingly, it caused the specific exodus of valine and alanine, the identities of which were confirmed using an automatic amino acid analyzer. Results with cyanide were more variable and inhibition was generally greater than expected for an ATP-dependent transport system (Table 2). At this time we have no explanation for these differences. We also noted that incubation with cyanide caused massive, selective exodus of an unknown pool constituent, labeled (a) in Fig. 3.

Other strains of *E. coli* in addition to strain W were also studied to determine the generality of the above observations on the ATP requirement for di- and oligopeptide transport (Table 2). For all peptides studied, rates of uptake were generally slower in these other strains than in strain W, and oligopeptide uptake was decreased relatively more than dipeptide uptake. This was not caused by the different growth media used, for similar rates were found with M2627 *lys*$^+$ grown as usual in medium A (8) or in medium M-56 (26), used routinely for growth of the other strains. In spite of the rate differences, the gen-

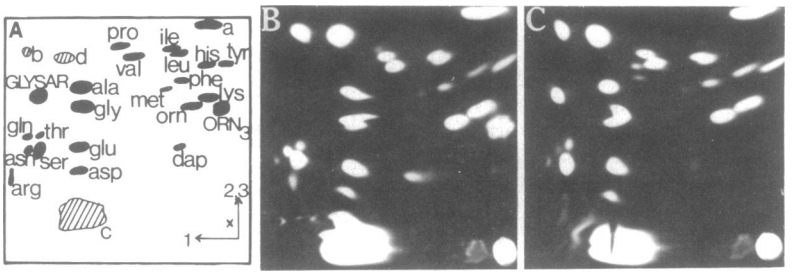

FIG. 3. *Accumulation of intact peptides by strain M2627 lys⁺. Pools were extracted after 20 min of incubation with (B) 500 μM* Orn_3 *or (C) 200 μM Gly-Sar. In addition to indicated pool components, (a) is the major unknown pool constituent, (b) is an unknown by-product of dansyl reaction, (c) is dansyl-OH, and (d) is dansyl-*NH_2.

TABLE 2. *Effects of energy source and inhibitors on peptide uptake*

Strain[a]	Transport substrate	Energy source added[a]	Control uptake[a] (nmol/min per mg of protein)	Uptake relative to control (%)					
				−Energy source	+Arsenate[b] (30 mM)	+DNP (3 mM)	+CCCP (20 μM)	+KCN (20 μM)	$+N_2$[c]
M2627 (G)	Met-Leu	G	28	20	<5	50	80	20	80
	Ala_3	G	39	25	10	45	85	25	75
M2627 (L)	Met-Leu	G	25		<5			60	
	Ala_3	G	38	15	<5			25	
	Ala_2	G	32	15	<5			55	
	Lys_2	G	13					60	
	Lys_3	G	15					60	
	Met-Leu	L	13			30	45	30	
	Ala_3	L	19		<5	30	50	30	
	Ala_2	L	17		<5	20		30	
	Val_3	L	5		<5	5		5	
ML308-225 *unc*⁺ (G)	Ala_3	G	22	30	<5	25			90
	Ala_2	G	23	25	<5	30			90
	Gly_3	G	3		<5	20			
	Gly_2	G	7		<5	20			
	Lys_3	G	8						60
	Lys_2	G	15						50
DL-54 *unc* (G)	Ala_3	G	21		<5	55			
	Ala_2	G	23	25	<5	55			
	Gly_3	G	3	25	<5	75	90		80
	Gly_2	G	8		<5	75	85		80
	Lys_3	G	4			35			60
	Lys_2	G	10			25			60
7 (G)	Ala_3	G	18		5	40			
	Ala_2	G	22		5	35			
NR70 (G)	Ala_3	G	14		10	95			
	Ala_2	G	16		10	95			
DF364 (F)	Ala_3	F	7		<5	65	85		85
	Ala_2	F	18		<5	70	90		80
DF360 (F)	Ala_3	F	5		<5	65	85		85
	Ala_2	F	17		<5	65	85		90

[a] Cells grown in glucose (G), lactate (L), or fructose (F) were suspended and preincubated with or without (control) the designated inhibitor for 10 min at 37°C; an energy source—20 mM glucose (G), D-lactate (L), or fructose (F)—was added as indicated, and incubation was continued for 10 min more. Uptake was then initiated by addition of 100 μM peptide and monitored for 15 min.

[b] Cells were washed and then resuspended in tris(hydroxymethyl)aminomethane in place of phosphate buffer; this procedure itself decreased uptake about 10%.

[c] Cells were grown aerobically but then washed, suspended, and incubated in N_2-gassed solutions.

eral effects of inhibitors and the conclusion regarding the ATP requirement of transport are both in agreement with those found in strain W. Strain DL-54 (*unc*), which cannot synthesize ATP via oxidative phosphorylation, and its parent ML308-225 were used by Cowell (7) in studies on the energetics of diglycine accumulation. In these two strains (and the other pairs), rates of uptake of various di- and oligopeptides were closely similar (Table 2). Arsenate abolished transport in all cases. DNP decreased uptake about 75% in the parent strain ML308-225 but to a lesser extent in the mutant DL-54. The possibility that the coupling of phosphate bond energy to transport may be less effective in the absence of the proton-motive force is suggested by (i) the marked effects of DNP, (ii) the inhibition by anaerobiosis (which varies for neutral and charged peptides), and (iii) the lower uptake rates seen with strain NR70 (which is not only deficient in the Ca^{2+} Mg^{2+} adenosine triphosphatase but is normally unable to maintain a proton gradient) compared with its parent strain 7. This defect is presumably responsible for the general exodus of pool constituents that is uniquely observed with strain NR70 during transport assays.

Cell extracts. By dansylating cell extracts it should be possible to determine accumulated substrates and thus obtain results that are complementary to those obtained by assaying incubation media. However, examination of cell extracts for accumulation of intact peptides is unlikely to be useful normally because of their rapid intracellular cleavage. The observed exodus of cleaved amino acid residues supports this idea. Nevertheless, unambiguous evidence for a progressive accumulation of two peptidase-resistant peptides, triornithine (Orn_3) and glycylsarcosine (Gly-Sar), by strain M2627 *lys*$^+$ was found by examining extracts (Fig. 3). When arsenate was added to the incubations (as for Table 2) no accumulated Gly-Sar or Orn_3 was detectable in cell extracts, indicating that this accumulation is energized via phosphate-bond energy. Calculations based on the amount of Orn_3 that disappears from the medium and on the amount present in extracts indicate that it is accumulated about 1,000-fold relative to an external concentration of about 50 μM, supporting the hypothesis that its bactericidal effect stems from the intact peptide acting at high concentration on an intracellular target (1, 2, 10).

While monitoring incubation media for uptake of peptides containing aspartic acid (Asp-Ala, Asp-Gly, Gly-Asp) and glutamic acid (Glu-Gly, Glu-Ala), and with triserine, virtually no exodus of Asp, Glu, and Ser, respectively, had been observed, although the peptides were taken up from the medium and the other amino acid residues underwent exodus. Therefore, for all these peptides we examined pool extracts, but found that the small increases in pool levels of free Asp, Glu, or Ser plus the amounts in the media did not match the total peptide uptake. It is clear that these residues are rapidly metabolized, no doubt in part to derivatives devoid of amino groups and unable to yield dansyl derivatives.

The relative concentrations of the amino acid pool constituents in various strains of growing *E. coli* were very similar, although variations in certain components (Glu, Asp, Ala) were noted. A considerable number of unknown minor components were detectable, but only one major unidentified component was present (Fig. 3, a). For routine examination of pools the dansyl procedure has many advantages over an autoanalyzer using ninhydrin (12). It is cheaper, much faster, and about 50 to 100 times more sensitive. It is emphasized that the black and white plates (Fig. 1 and 3) are at best a poor reproduction of the original chromatograms, on which different fluorescent intensities are apparent and several derivatives, e.g. Tyr, His, and Pro, have characteristic colors.

DISCUSSION

The influence of strain differences and the effects of chain length and amino acid composition on peptide uptake by *E. coli*, which have been studied here by use of a fluorescent assay, could not be assessed previously from growth response tests (19, 20, 22). Thus, although glycyl- and lysyl-homopeptides have different rates of uptake and these decrease with increase in chain length (Table 1), all these peptides support virtually equal auxotrophic growth rates (9, 16). Only Smith et al. (25), using radioactive peptides, have previously shown that oligopeptide uptake rates are inversely related to the number of residues in a peptide.

Cowell's conclusion (7) that uptake of diglycine is energized by ATP has been confirmed and extended to a variety of dipeptides and to oligopeptides, which are transported by a separate system.

When *E. coli* is incubated with a peptide, the hydrolyzed amino acid constituents appear in the incubation medium. This might be interpreted as evidence for external cleavage. However, the following observations persuade us that this actually results from a sequential process of peptide uptake, intracellular hydrolysis, and amino acid exodus: (i) direct tests have repeatedly failed to reveal extracellular or periplasmic peptidases (24); (ii) the characteristics of peptide

transport and its distinction from amino acid uptake are only compatible with the absence of external peptide hydrolysis (9, 16–22); (iii) no peptide fragments were detected in incubation media by fluorescent labeling; (iv) by the dansyl method, *opt* strains show very little oligopeptide uptake and cleavage, and di- and oligopeptides do not compete (to explain these results on the basis of external cleavage requires both di- and oligopeptidases to be present); (v) on incubation with the dipeptides Asp-X, X-Asp, and Glu-X, only the X residues are labeled in the medium; (vi) on incubation with peptides containing valine or threonine (but not with the free amino acids), proline and glycine, respectively, appear in the medium in addition to the amino acid residues of the peptides (unpublished data); (vii) disappearance of all peptides and appearance of released amino acid residues is markedly inhibited by arsenate and, to a lesser extent, by uncouplers and anaerobiosis (Table 2); (viii) peptide uptake and appearance of amino acid residues in the medium are indistinguishable in the parent and the mutant strain 9202 of *E. coli* K-10 that apparently lacks a membrane-bound peptidase (14) (unpublished data). Peptide transport, intracellular hydrolysis with consequent metabolic activity, and amino acid exodus allow a unifying explanation of these results, whereas any explanation invoking external peptidases requires involvement of a diversity of further reactions.

The observation that a peptide continues to be absorbed while its previously accumulated amino acid residues are undergoing simultaneous exodus seems to indicate that *E. coli* has no specific mechanism for regulating peptide transport. Alternatively, and nutritionally more efficient than inhibiting peptide uptake, is exodus of only those specific amino acids which, as a consequence of peptide transport, are tending towards excess in the pool. For *E. coli* utilizing a heterogeneous mixture of peptides in the mammalian gut, a balanced uptake of amino acids presumably occurs, making exodus unnecessary. However, in a typical growth test in which 10 ml of medium containing a defined peptide (100 μM) is inoculated with 10^8 bacteria (about 0.022 mg of protein) that divide hourly, calculation based on typical rates of peptide uptake and amino acid exodus indicates that after about 4 h the auxotroph will be reabsorbing the amino acid residues. We have confirmed this conclusion for strain M2626 *lys* utilizing trilysine (unpublished data).

It is apparent that, in a standard transport assay based on accumulation of radioactively labeled peptides, the accompanying amino acid exodus would not be detected. Ignorance of this feature could well invalidate conclusions which are not based on initial rates of uptake. The frequent observation of selective amino acid exodus resulting, for example, from anaerobiosis convinces us that it is prudent to carry out media analysis of the type described here when performing any transport studies that rely solely on determination of the movement of radioisotopes or proton fluxes.

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