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Relation of Cell Growth and Colicin Tolerance to Vitamin B_{12} Uptake in *Escherichia coli*

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The uptake of vitamin B_{12} was measured in cells of *Escherichia coli* whose growth had been inhibited by any of a variety of treatments. In all cases, the secondary, energy-dependent phase of B_{12} uptake was depressed in proportion to the decrease in growth rate, but uptake was constant in cells growing logarithmically at different rates. The depression of B_{12} uptake activity was independent of the site of cell metabolism affected by the inhibitor or by its effect on cell viability, and was both more rapid and of greater degree than the effects on the uptake of any of the six amino acids tested. The decline was not affected by inhibitors of either cell division or proteolysis and was manifested without any apparent decrease in the surface B_{12} binding activity. Transport activity was rapidly regained upon reversal of the inhibition of protein synthesis. Prompted by this response, the uptake of B_{12} was contrasted to the apparent uptake of the E colicins, which share the same outer membrane receptor. Sensitivity to colicin E1, measured by its inhibition of proline uptake, was not affected by growth inhibition by antibiotic treatment. Finally, there was no specific depression of B_{12} uptake in cells rendered colicin tolerant either by mutation or as a consequence of phage f1 infection.

The uptake of cyanocobalamin (B_{12}) by *Escherichia coli* appears to employ a receptor protein integrated into the outer membrane of the cell envelope as an essential component (28). The binding of B_{12} to this receptor is normally followed by the energy-dependent appearance of B_{12} within the cell, where it can be employed for the terminal step of methionine biosynthesis. This receptor also serves for the cellular adsorption of the E colicins and the phage BF23 (8). Evidence for this broad specificity of binding includes the demonstration of competitive interactions of these three classes of ligands and in the protection of cells against the lethal action of the colicins or phage in the presence of B_{12}. Also, mutants selected for resistance either to the E colicins or phage BF23, carrying lesions in the *bfe* locus, lack activity and are unable to utilize, transport, or bind B_{12} (11). So far, little is known of the steps of B_{12} uptake subsequent to adsorption to the receptor.

Reliance on an outer membrane receptor as an obligatory stage of the uptake process might be manifested by some unusual characteristics distinguishing B_{12} uptake from that of other uptake systems. Prompted by the results in the accompanying paper (2) suggestive that not all colicin E receptors are equivalent, we studied the effect of growth inhibition on B_{12} uptake. A marked dependence on continued protein synthesis for function of the energy-dependent phase of uptake was observed and is described here.

Also investigated was the relation of B_{12} uptake to that of the E colicins, which share the same receptor. This study was prompted by the seeming analogy between the decline in B_{12} uptake upon inhibition of growth to the phenotype of colicin tolerance. Tolerance refers to the insensitivity of cells to the effect of certain colicins, which are still able to bind to the cell. The tolerant phenotype can be achieved by mutation at any of a number of loci (17, 18) or by nongenetic means, as a result of infection with and production of certain male-specific phages, such as f1 (26, 29). Colicin tolerance could result either from the alteration of the sensitive target or from disruption of the colicin entry process.

The evidence presented here suggests that, since B_{12} uptake can be specifically uncoupled from the receptor either by mutation or by inhibition of protein synthesis, the presence of a receptor is not sufficient for uptake to proceed. Secondly, the uptake processes for B_{12} and the colicins are distinct and can be blocked individually.

MATERIALS AND METHODS

Media. The minimal growth medium used throughout, except as specified, was based on medium A of Davis and Mingioli (5), supplemented

with glucose (0.5%), required amino acids (100 μg/ml), adenine (40 μg/ml), and thiamine (1 μg/ml). For the growth of strain K38 and its infection with phage f1, the minimal medium of Fraser and Jerrel (9) supplemented with lactate (0.5%), thiamine, and, when specified, Casamino Acids (1%) was employed. Cells were grown at 37°C with vigorous aeration. When indicated, aminoglycoside antibiotics (100 μg/ml), 5-diazouracil (2.5 μg/ml), or nalidixic acid (10 μg/ml) was added as an aqueous solution. Chloramphenicol and rifampin (final concentrations, 100 μg/ml) were added as methanolic solutions such that the final methanol concentration was 0.2%. This concentration of methanol was without effect on either the growth rate or the rate of B_{12} uptake. The protease inhibitors were added in solid form to the growth medium to provide the specified concentration.

Bacterial strains. Most of the strains employed in this study are derivatives of the *E. coli* K-12 strain KBT001 (7), derived from χ478. Strain RK4113 (*leu proC lysA metE argH bfe*$^+$ *tonA strA rif*$^+$) was used for most of the uptake studies and from it were derived strains RK4101 (as RK4113, but *rif*), RK4115 (as RK4113, but *btuA*), and RK4117 (as RK4113, but *non*). The strain RK4117 carries a point mutation in *non* and is characterized by its inability to yield mucoid colonies resistant to phage T7 (21). It was necessary to employ this strain for the isolation of colicin-tolerant mutants, since tolerant mutants have a strong tendency to acquire a mucoid phenotype upon cloning. The prototrophic strains employed for amino acid uptake studies were strain 7 from E. C. C. Lin and strain W1485, cured of F. The prototrophic F$^+$ strain K38 and wild-type phage f1 were kindly provided by R. E. Webster, Duke University.

Transport assays. Transport assays for this study were performed on cells taken directly from their growth medium to preclude changes in uptake activity that occurred during cell harvest and washing. To cells growing logarithmically in minimal growth medium, additions were made at specified times (usually designated as time 0). At indicated times thereafter, a portion of the culture was removed and mixed with the transport substrate. All transport assays were performed at room temperature (22°C).

For measurement of B_{12} uptake, a 0.5-ml portion of cells was mixed with [^{3}H]cyanocobalamin (31.7 nM: 0.14 μCi/ml, final) in a total volume of 0.7 ml. The cell density in the assay ranged from 2×10^8 to 8×10^8/ml. At 0.3, 5, and 10 min, a 0.20-ml portion of the uptake mixture was transferred to the center of a membrane filter (0.45 μm; Millipore Corp.), filtered, washed with 5 ml of medium A at 22°C, immediately removed from the filtration apparatus, and air dried. The radioactivity retained on the filter was measured in a scintillation counter with toluene-Omnifluor (New England Nuclear Corp.). All values were corrected for the binding of B_{12} to the filter in the absence of cells. The values for B_{12} uptake presented here represent the average of the 5- and 10-min uptake values less that present at 0.3 min (primarily binding to the cells) and are expressed in terms of picomoles of B_{12} accumulated over this time interval per microliter of cell water (1 μl is assumed to be equivalent to 250 μg of protein).

Isolation of colicin-tolerant mutants. Two procedures were employed and compared for the isolation of colicin-tolerant mutants. In the procedure not employing selection, cells of strain RK4113 or RK4117 growing in L broth (15) were exposed to colicin E1 or E3 at a concentration such that a 10^{-4} dilution was the lowest concentration producing a clear zone when spotted on a lawn of sensitive bacteria (25). After 10 min at 37°C, the mixture was poured onto an L agar plate and incubated overnight at 37°C. Another procedure for the isolation of tolerant mutants was developed based on the assumption that tolerant mutants would be the only survivors of colicin treatment still able to utilize B_{12}. The cell-colicin mixture from above was plated on minimal growth medium with 5×10^{-10} M B_{12} in place of methionine. Those colonies first appearing on these plates were purified, and it was shown by the criteria described below that greater than 90% of these survivors appearing earliest were tolerant.

Both the unselected and the B_{12}-selected survivors from colicin exposure were tested for their sentivitity to phage BF23 and several other colicins by streaking the strain onto a section of an L agar plate and depositing thereon a drop of the phage or colicin. In all cases described here, there was either complete clearing or no apparent effect. The presence of colicin receptor was routinely screened by the triple-layer plate test described by Davies and Reeves (4). If the B_{12} selection was not employed, approximately 80% of the survivors from colicin treatment were resistant and lacked receptor, as evidenced by their insensitivity to all the E colicins and phage BF23 and by their inability to utilize B_{12}. The remainder were tolerant as defined by their sensitivity to phage BF23 and their possession of receptor by the triple-layer technique. Similarly, all the early-appearing survivors on the B_{12} plate were tolerant by the same criteria. Colonies appearing later on these plates lacked receptor and exhibited the BtuB phenotype described earlier (11). All (of 471) tolerant mutants reponded to B_{12} in the growth medium in a manner identical to the parent strain.

Infection with phage f1. The tolerant mutants for this study were obtained in strain RK4117, whose *non* mutation prevents the appearance of mucoid cells that are resistant to the colicins and phage BF23 but which still possess normal receptor. Cells of strain K38 grown with vigorous aeration to a density of 4×10^8/ml were shaken at reduced speed for 10 min to ensure maximal piliation. Phage f1 was then added at multiplicities of 50 to 300. After 10 more min of slow shaking, 4 volumes of warm medium were added, and the aeration rate was increased. At intervals after infection, portions were assayed for B_{12} or amino acid uptake (in growth medium without Casamino Acids) as already described. Spectinomycin was added to duplicate flasks to determine the rate of decline of B_{12} uptake upon further growth inhibition. Viable counts were determined at several times, and it was shown that, under these conditions, greater than 90% of the cells were infected. Also, portions of the infected and uninfected cells were mixed with serial dilutions of colicin E1 in L broth and incubated for 4 h, after

which the turbidity increase was determined to estimate the degree of colicin insensitivity resultant from phage f1 infection.

Materials. Radioactive amino acids were obtained from New England Nuclear Corp., and [^{3}H]cyanocobalamin was obtained from Amersham/Searle Co. Most other chemicals were from the Sigma Chemical Co.

Colicin E3 was partially purified from the salt extract of mitomycin-induced cells of the colicinogenic strain CA38 through the diethylaminoethylcellulose step of the procedure of Herschman and Helinski (10). The colicin E1 preparation comprised the material from the salt wash of mitomycin-induced cells of a strain colicinogenic for Col E1 and which precipitated between 15 and 60% saturation with ammonium sulfate at 4°C. The fractions were dialyzed, lyophilized, and stored at 70°C. Portions were dissolved in colicin diluent and had activities of 10^6 to 10^8 killing units/ml, as defined by the assay similar to that of Sabet and Schnaitman (25).

RESULTS

B_{12} uptake decreased upon inhibition of growth. The uptake of B_{12} by *E. coli* consists of two steps, the binding of substrate to the receptor protein located on the outer membrane followed by the subsequent energy-dependent accumulation of substrate in the cell. The effect of the inhibition of cellular growth on these two portions of the B_{12} uptake process was investigated. Addition of chloramphenicol, erythromycin, neomycin, spectinomycin, or rifampin to cultures of strain RK4113 resulted in an inhibition of growth, as monitored by the optical density (Fig. 1A). The rate of growth inhibition was dependent on the particular antibiotic employed and, with the exception of erythromycin, was complete within 30 to 40 min. Growth of the rifampin-treated cells, not shown on this figure owing to the high absorbancy of the medium, was affected very similarly to that of spectinomycin-treated cells. Coincident with the inhibition of growth was a marked reduction in the secondary rate of B_{12} uptake (Fig. 1C), such that up to 80% of uptake activity was lost within 30 min. On the other hand, cellular binding of B_{12} as represented by the 0.3-min value declined only slowly and to a limited extent in the inhibited cultures relative to the control. In fact, subtraction of the extrapolated contribution of uptake from this value (assuming linear uptake during the first 5 min) resulted in very similar binding activities for the treated and control cultures.

The presence of these antibiotics during the assay itself had, with one exception, only slight effects (less than 10%) on the measurement of B_{12} binding or uptake. The presence of rifampin during the assay reduced B_{12} binding up to 70%

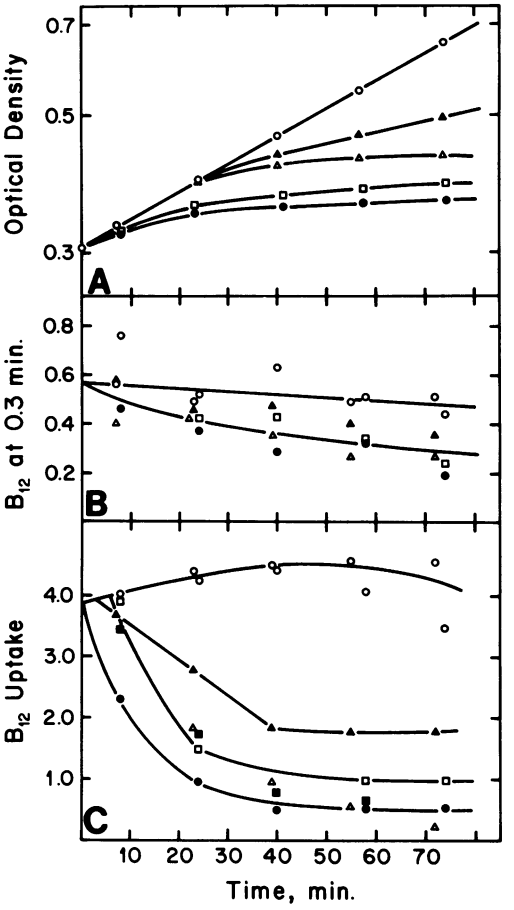

FIG. 1. *Effect of antibiotic treatment on B_{12} uptake. Cells of strain RK4113 were grown at 37°C to a density of 6 × 10^8 per ml in six flasks containing minimal growth medium. To each flask was added either nothing* (○), *chloramphenicol* (●), *erythromycin* (▲), *neomycin* (△), *spectinomycin* (□), *or rifampin* (■), *each to a final concentration of 100 μg/ml. Incubation was continued at the same temperature and aeration rate. At the indicated times, a portion of the cells were removed and mixed with [^{3}H]-cyanocobalamin at 22°C, and portions of this mixture were filtered at 0.3, 5, and 10 min, washed, and counted. The top panel (A) portrays the optical density; the middle panel (B), the amount of B_{12} (picomoles per microliter of cell water) cell associated at 0.3 min; and the bottom panel (C), the secondary rate of uptake, estimated as the average of the B_{12} retained at 5 and 10 min, less that present at 0.3 min.*

without producing a concomitant decrease in uptake. This effect of rifampin on binding occurred immediately and was observed even in a rifampin-resistant strain. No concentration of B_{12} could protect a rifampin-sensitive strain against the minimal inhibitory concentration of rifampin, and mutational loss of the B_{12} recep-

tor did not render cells resistant to rifampin. On the other hand, studies with the *rif* strain RK4101 revealed that rifampin could partially inhibit the utilization of B_{12}, suggesting some indirect or minor competition for binding or transport.

Further evidence for the independence of B_{12}-binding activity from the decline in uptake activity after growth inhibition came from studies with strain RK4115, which carries a mutation termed *btuA*. This strain lacks B_{12} uptake, although B_{12} and colicin binding remain normal (7), and hence this strain allows the measurement of B_{12}-binding activity free from the contribution of uptake. Over a period of 70 min after the addition of chloramphenicol or spectinomycin, there was no decrease in the amount of B_{12} bound per cell relative to the control. As with the parental strain, this strain bound only 30% as much B_{12} after exposure to rifampin, and this value was constant over this time period. Thus, inhibition of growth does not affect the apparent number of outer membrane B_{12} receptors, but must affect some process subsequent to binding to the receptor.

A number of other methods for the inhibition of cell growth were employed, including agents affecting different steps of cell metabolism. Any treatment that inhibited growth of the test strain, RK4113, produced a decline in B_{12} uptake. In addition, there seemed to be a relationship between the effectiveness of the treatment in the inhibition of growth and in the rate of decline of B_{12}-uptake activity. A plot of the relative rate of B_{12} uptake 30 min after the addition of an inhibitor versus the relative number of mass doublings over a 90-min period revealed a reasonably good correlation between these two parameters (Fig. 2). With the exception of rifampin, none of the treatments significantly depressed B_{12} binding over this time period. One example of this proportionality came from studies with *rif* strain RK4101, which could grow in the presence of rifampin (100 μg/ml) but at only half the growth rate as in its absence; B_{12} uptake was depressed 50% by rifampin. In summary, the inhibition of growth by any of the means tested resulted in a rapid and extensive decline in the uptake, but not the binding, of B_{12} in proportion to the decrease in mass increase.

Decrease is reversible. Regain of transport activity after relief of growth inhibition was studied in two ways. First, cells treated with chloramphenicol or spectinomycin for 30 min were washed free of the antibiotic and resuspended in warm growth medium, allowing resumption of exponential growth within 25 min.

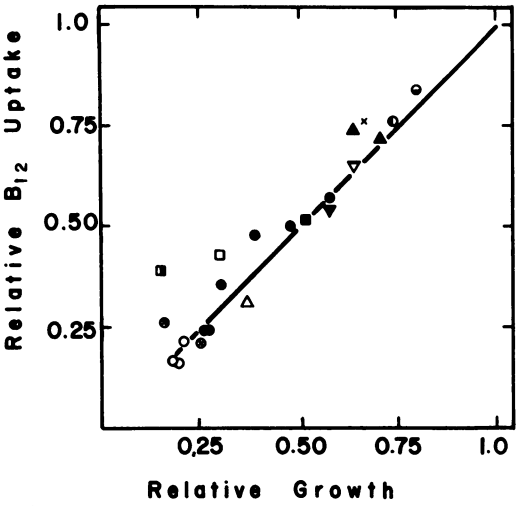

FIG. 2. *Relationship between inhibition of growth and rate of loss of B_{12} uptake. The data from experiments similar to those described to Fig. 1 with strain RK4113 are presented. Relative growth represents the numbers of doublings achieved by the inhibited culture, relative to the number of doublings in the control culture over the same 90-min period. Relative B_{12} uptake is the extrapolated secondary rate of uptake in the inhibited culture 30 min after addition of inhibitor, relative to the rate of uptake in the control culture at the same time. The inhibitors presented are: chloramphenicol (○; 100 μg/ml), spectinomycin (●; 25 to 100 μg/ml), nalidixic acid (▽; 100 μg/ml), erythromycin (▼; 100 μg/ml), neomycin (△; 100 μg/ml), p-fluorophenylalanine (▲; 100 μg/ml), rifampin (■; 100 μg/ml), L-valine (□; 100 μg/ml), starvation for required amino acids (◨), p-aminobenzamidine (◒; 5 mM), tosyl-L-lysine chloromethyl ketone (◐; 0.85 mM), 5-diazouracil (×; 2.5 μg/ml), and 5-diazouracil plus spectinomycin (⊗; 2.5 and 100 μg/ml).*

However, B_{12} uptake, which had been inhibited by 80%, began to increase within 5 min after resuspension and had reached its final value within 40 min. Cells treated with chloramphenicol regained full uptake activity, whereas spectinomycin-inhibited cells only regained about half of control activity. Restoration of activity was completely prevented by the presence of chloramphenicol, spectinomycin or rifampin in the resuspension medium.

To avoid the harvesting and washing of cells required in these experiments, a second approach employed the reversible inhibition of growth afforded by the addition of valine or by starvation for required amino acids. After the addition of valine, the growth rate declined after a 15-min lag (Fig. 3A). Coincident with the cessation of growth was a marked decline in B_{12} uptake down to 20% of the control rate.

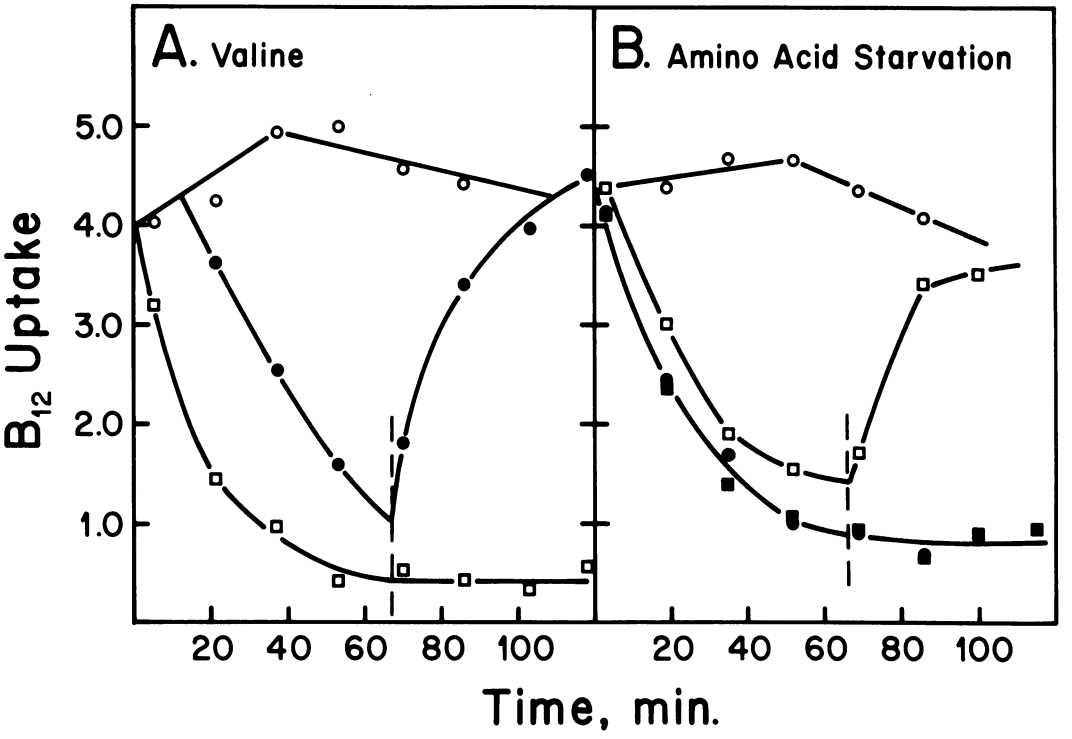

FIG. 3. *Reversal of the inhibition of growth and B_{12} uptake. (A) At zero time, one of triplicate growth flasks of strain RK4113 in minimal growth medium at 37°C received no addition (○), L-valine (●; 50 μg/ml), or L-valine plus chloramphenicol (■; 50 and 100 μg/ml, respectively). At 67 min, isoleucine (100 μg/ml) was added to each flask (-----). (B) At −130 min, 3.8 ml of cells of strain RK4113 in minimal growth medium was added to 12.2 ml of either minimal growth medium (circles) or medium A plus glucose lacking the required amino acids (squares). By zero time, the cells in the unsupplemented medium were just halting growth. At this time, spectinomycin (100 μg/ml) was added to one of each pair of flasks (filled symbols). At 66 min, the required supplements were added back to the unsupplemented culture, and logarithmic growth resumed almost immediately unless spectinomycin was present. The rate of B_{12} uptake was assayed at the indicated times as described.*

Relief of the growth inhibition by isoleucine allowed immediate resumption of growth and a rapid rise in B_{12} uptake activity back to the control level in 30 min. Simultaneous addition of valine and chloramphenicol resulted in a similar rapid loss of uptake activity, which, however, was not restored upon addition of isoleucine. Similar results were obtained upon starvation for required amino acids (Fig. 3B). Upon exhaustion of the amino acid supplement in the medium, growth stopped with the onset of a concomitant and proportionate decline in uptake activity. The rate of loss of uptake activity was independent of the presence of spectinomycin. Most of the activity was regained upon the addition of the required supplements, unless spectinomycin was also present. Thus, restoration of activity requires growth or protein synthesis and is rapid but not instantaneous.

Inhibition is specific. The growth inhibition resulting in depressed B_{12} uptake could operate through a generalized decrease in transport activity, owing to decreased energy coupling or increased permeability. The effect of growth inhibition by spectinomycin on the uptake of six amino acids was measured in the prototrophic strain 7. Assays were performed as before in that the cells were not washed prior to the assay, so that any excreted amino acids would be present in the assay mixture. The amino acids tested (arginine, glutamine, histidine, lysine, methionine, and proline) include substrates for systems that employ the protonmotive force and those that apparently use adenosine 5'-triphosphate as an energy source. In most cases, amino acid uptake activity was depressed somewhat upon spectinomycin treatment (Fig. 4). Anomalous behavior was noted for arginine uptake in that the control culture featured a decline in activity that was prevented upon inhibition of protein sythesis. The average of the ratios of the rates of uptake in

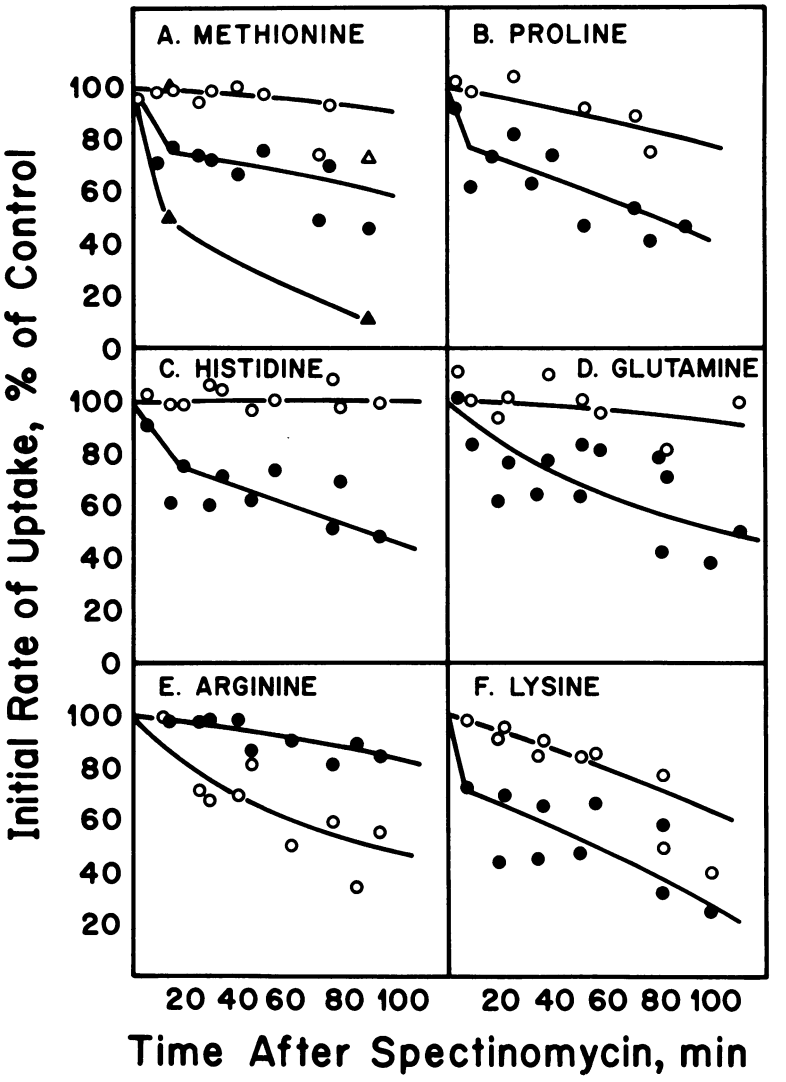

FIG. 4. *Effect of the addition of spectinomycin on amino acid uptake. Cells of the prototrophic strain 7 were grown in medium A plus glucose. At zero time, spectinomycin at a concentration of 100 μg/ml was added to one of a pair of growth flasks. At the indicated times, 200 μl of cells was mixed with labeled amino acid in a final volume of 250 μl, and 200 μl of the uptake mixture was filtered at 0.30 min and washed with 5 ml of medium A. The open symbols represent the rate of amino acid uptake in the control culture, relative to the average zero time value; filled symbols are for spectinomycin-treated cultures. Results from three experiments are presented here. (A) Methionine (2 μM; 0.2 μCi/ml), also secondary rate of uptake of B_{12} (triangles); (B) L-proline (3.68 μM; 0.2 μCi/ml); (C) L-histidine (0.65 μM, 0.4 μCi/ml); (D) L-glutamine (2 μM; 0.2 μCi/ml); (E) L-arginine (0.20 μM; 0.03 μCi/ml); (F) L-lysine (2 μM; 0.2 μCi/ml). After 90 min, the control culture went through 1.55 doublings, whereas the spectinomycin-treated culture underwent 0.41 doubling.*

the inhibited culture relative to the control at the same time was 0.77 for methionine, 0.61 for proline, 0.66 for histidine, 0.74 for glutamine, 1.51 for arginine, and 0.66 for lysine. These declines may represent in part the decreased incorporation into protein coupled with an increased excretion of the amino acids from the inhibited cells. Over the same time period with the same cells, B_{12} uptake rapidly declined to 0.16 of the control rate. Thus, the effect of the inhibition of growth on B_{12} uptake was much more rapid and extensive than that on amino acid uptake and probably reflects the requirement for protein synthesis for some process pe-

culiar to B_{12} uptake, and not for general membrane integrity or maintenance of energy coupling.

Cell division and proteolysis are not involved. It has been suggested that septation or cell division is involved in the appearance of certain phage receptors on the surface of the cell (14, 24). Thus it was possible that cell division would be necessary either for continued B_{12} uptake or for the decline after inhibition of protein synthesis. 5-Diazouracil is an effective inhibitor of cell division, acting before septation to cause filament formation, and has only minor effects on the rates of macromolecular synthesis (20). Exposure of cells of strain RK4113 to 5-diazouracil (2.5 μg/ml) slightly depressed the rate of growth, but did not significantly affect the rate of B_{12} uptake to any extent greater than that expected from the decreased rate of growth (Fig. 5). In addition, its presence did not affect the loss of uptake activity after the addition of spectinomycin. Greater than 90% of the 5-diazouracil-treated cells had formed filaments on an average of two to four cell lengths; filament formation was greatly reduced by spectinomycin. As before, B_{12}-binding activities were not altered. Similar results were obtained when cell division and deoxyribonucleic acid synthesis were inhibited with nalidixic acid. Thus, sepatation is not required either for B_{12} uptake or for its loss resultant from the inhibition of protein synthesis.

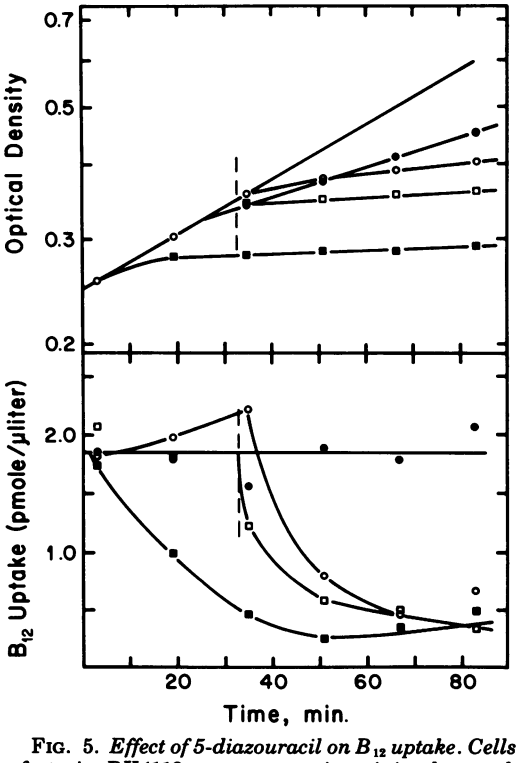

FIG. 5. *Effect of 5-diazouracil on B_{12} uptake. Cells of strain RK4113 were grown in minimal growth medium at 37°C in replicate growth flasks. The following additions were made: spectinomycin (100 μg/ml) at 33 min (○); 5-diazouracil (2.5 μg/ml) at 0 min (●); 5-diazouracil at 0 min and spectinomycin at 33 min (□); and 5-diazouracil and spectinomycin at 0 min (■). B_{12} uptake was assayed as before at the indicated times.*

Alternatively, the proteolysis of some component involved in uptake, but not binding, might be responsible for the decline, especially if this component were present in limiting amounts and had a rapid turnover rate. This proteolysis might occur either as a normal part of the uptake process or only under conditions of the inhibition of protein synthesis. The rate of B_{12} uptake and its loss after addition of spectinomycin was measured in cells exposed for several generations to the putative protease inhibitors, phenylmethyl sulfonyl fluoride (1.1 mM), L-1-tosylamide-2-phenylethyl chloromethyl ketone (1.0 mM), *p*-tosyl-L-lysine chloromethyl ketone (0.85 mM), and *p*-aminobenzamidine (0.1 to 20 mM). Several of these have been found to be effective inhibitors of proteolysis in *E. coli* in this concentration range (C. MacGregor and C. Hackett, personal communication), although chloromethyl ketones can affect cells in ways unrelated to their role as protease inhibitors, perhaps as sulfhydryl reagents (22). The rate of B_{12} uptake was not affected by exposure for 60 min to phenylmethyl sulfonyl fluoride, L-1-tosylamide-2-phenylethyl chloromethyl ketone, or *p*-aminobenzamidine (at concentrations less than 5 mM) (Fig. 6). Uptake was somewhat depressed in cells treated with *p*-tosyl-L-lysine chloromethyl ketone and greatly depressed in 20 mM *p*-aminobenzamidine, but in both cases the decrease in uptake remained proportional to the decrease in growth rate. In no case did these putative protease inhibitors prevent the decline in uptake activity after the addition of spectinomycin.

Effect of growth rate. These experiments demonstrated that, under a variety of conditions of growth inhibition, the rate of B_{12} uptake was consistently proportional to the rate of cell growth and, presumably, to that of protein synthesis. It was important to determine the rate of B_{12} uptake and its response to growth inhibition in cells growing exponentially at different growth rates. The growth rate was controlled by either the temperature of growth or the composition of the growth medium. The rate of uptake and its loss upon addition of spectinomycin were essentially identical for

cells grown at 30°C (an 84-min doubling time) or at 37°C (59 min). Similarly, no difference was observed in the behavior of cells grown at 37°C in the minimal medium supplemented with glucose (54 min), lactate (71 min), succinate (89 min), or glycerol (112 min), or in a glucose medium supplemented with all the amino acids and nucleosides (35 min). Thus, transport was independent of growth rate during exponential growth over almost a threefold range of doubling times, and thus the proportionality seen in inhibited cultures must reflect a dependence on continued protein synthesis for B_{12} uptake. It could be noted that the rate of B_{12} uptake declines in a culture approaching stationary phase, but several amino acid transport systems do likewise.

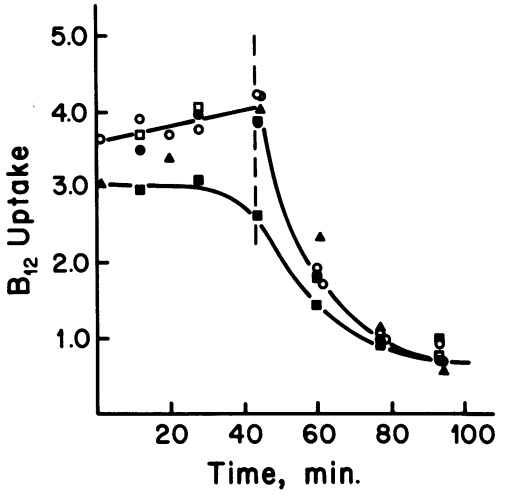

FIG. 6. *Effect of protease inhibitors on B_{12} uptake. At −26 min, replicate cultures of strain RK4113 in minimal growth medium at 37°C received, solid form, no addition* (○), *phenylmethyl sulfonyl fluoride to 1.1 mM* (●), *L-1-tosylamide-2-phenylethyl chloromethyl ketone to 1.0 mM* (□), *p-tosyl-L-lysine chloromethyl ketone to 0.85 mM* (■), *or p-aminobenzamidine to 5 mM* (▲); *at 34 min, spectinomycin was added to each culture to 100 μg/ml. During the 60 min between addition of protease inhibitor and spectinomycin, the cultures underwent 1.26, 1.18, 1.17, 0.92, and 1.06 doublings, respectively. B_{12} uptake was assayed at the indicated times.*

Relation of B_{12} uptake to colicin tolerance. This demonstration that physiological alterations could elicit a state for B_{12} uptake analogous to colicin tolerance, combined with the fact that some colicins share the B_{12} receptor, prompted studies of the relationship of B_{12} and colicin E uptake. First, B_{12} uptake properties were measured in a number of colicin-tolerant strains, and these studies allowed the development of a positive selection for tolerant mutants. Second, uptake properties were determined in cells rendered colicin tolerant by infection with phage f1. Finally, in the converse experiment, the effect of growth inhibition on the sensitivity of cells to colicin E1 was determined.

Tolerant mutants of strain RK4117 were obtained in four of the classes described by Davies and Reeves (4), namely Tol II, III, IV, and VIII. However, these assignments are tentative, since neither their genetic locations nor the entire range of sensitivities to all the typing colicins was determined. The B_{12} transport and other properties of these strains were determined (Table 1). Uptake activity was depressed in members of classes Tol II, III, and IV relative to the parent strains, but was elevated twofold

TABLE 1. *B_{12} uptake in colicin-tolerant mutants*

Tolerance class[a]	Responses to colicins					B_{12} binding[b]	B_{12} uptake[c]	Relative doubling time[d]	Uptake of:[c]	
	E1	E2	E3	K	DOC[e]				Proline	Glutamine
non$^+$ *tol*$^+$	S	S	S	S	R		2.72			
non tol$^+$	S	S	S	S	R	0.26	2.41	1.0	331	408
non Tol II	R	R	R	R	R	0.23	1.09	1.7	90	65
non Tol III	S	R	R	R	R	0.26	1.57	1.6	90	76
non Tol IV	S	R	R	S	R	0.28	1.81	1.8	58	47
non Tol VIII	R	S	S	S	S	0.29	4.81	1.0	355	428

[a] Based on the average properties of four representative members of each class. All tolerant mutants were able to utilize B_{12} and were sensitive to phage BF23.

[b] B_{12} bound in picomoles per microliter of cell water for cells poisoned with 100 mM NaF plus 10 nM NaN_3.

[c] Uptake of B_{12} and amino acids expressed in terms of picomoles per microliter of cell water per minute for the amino acids or after 6 min for B_{12}.

[d] Growth rate was determined in the minimal growth medium under conditions such that the *tol*$^+$ strain had a 100-min doubling time.

[e] Sensitivity to deoxycholate (DOC) scored by growth on MacConkey's agar medium. Abbreviations: S, Sensitive; R, resistant.

in members of the Tol VIII class. This elevation in Tol VIII has also been found with similar mutants from other parental strains. In all cases, the level of B_{12} binding, determined either with normal cells by subtraction of the extrapolated uptake component or with cells poisoned with fluoride and azide, was reasonably close to that in the parent strain. All the tolerant mutants exhibited the usual decline in B_{12} uptake after the addition of spectinomycin. It is probable that the lower B_{12} uptake in several of these tolerant classes is not specific. The strains in these classes exhibit lower growth rates, increased cell lysis, and increased segregation of nonviable offspring than the parental or Tol VIII strains. Further, mutants in the tolerant classes II, III, and IV had markedly lower rates of uptake of proline and glutamine than either the parental or Tol VIII strains. Thus, these strains demonstrate a generalized depression in uptake activity, which accounts for the depressed B_{12} uptake. Thus, as expected from the ability of tolerant strains to utilize B_{12}, there appears to be no defect or specific alteration in the B_{12} uptake system in strains rendered colicin tolerant by mutation at a variety of genetic loci.

Effect of bacteriophage f1 infection. Cells infected with phage f1 are much less sensitive to a number of colicins, while retaining sensitivity to phages employing the same receptors as colicins and possessing normal levels of colicin binding (26, 29). The effect of phage f1 infection of strain K38 on the uptake of B_{12} and several amino acids was measured. Infected cultures were 30-fold less sensitive to the lethal effect of colicin E1 than an uninfected culture. The rate of B_{12} uptake in infected cultures, which continued to grow exponentially during the course of the experiment at about half the rate of the control culture, was about half that of the control and remained constant over the period from 60 to 200 min after infection. Binding of B_{12} was not appreciably affected. In most experiments, the relative rate of B_{12} uptake was very similar (within 5%) to the relative growth rate. As with the tolerant mutants, a similar decline in uptake activity toward proline, glutamine, leucine, and methionine was observed. Finally, inhibition of growth with spectinomycin produced the characteristic further loss of B_{12} uptake activity. Thus, tolerance to colicin E1 induced by phage f1 infection, whatever its basis, is not reflected in any specific effects on B_{12} uptake or binding.

Effect of growth inhibition on colicin sensitivity. The effect of the inhibition of protein synthesis, which markedly reduces B_{12} uptake, on cellular sensitivity to colicin E1 was determined. The effect of serial dilutions of colicin E1 on the uptake of proline was measured in cells of strain W1485F$^-$ before and after treatment with various antibiotics for 30 min (Fig. 7). The rate of proline uptake was depressed in cells treated with chloramphenicol, spectinomycin, or rifampin by 26, 27, or 19%, respectively. However, the antibiotic-treated cells showed the same relative response to the presence of colicin E1 as the control cells. Thus, the access of at least this colicin to its target is not affected in parallel with the B_{12} uptake process under these conditions of growth inhibition.

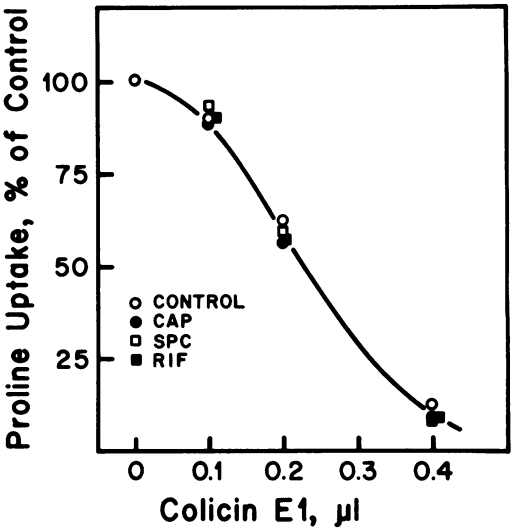

FIG. 7. *Effect of antibiotic treatment on sensitivity to colicin E1. Cells of strain 1485F$^-$ were grown in medium A plus glucose at 37°C to a density of about 7 × 10^8/ml. A 4-ml portion of the culture was mixed with 1 ml of medium and assayed immediately* (○). *Three other portions were mixed with 1 ml of medium A containing chloramphenicol* (●), *spectinomycin* (□), *or rifampin* (■), *each at a final concentration of 100 µg/ml, incubated for 30 min at 37°C, and then assayed. The assay consisted of incubating 400 µl of these cells for 10 min at 37°C with 50 µl of either colicin diluent or diluent with three serial twofold dilutions of a 1:125 dilution of the stock preparation of colicin E1. After this incubation, the mixture was returned to room temperature, and 50 µl of L-[^{14}C]proline was added (2.11 µM; 0.5 µCi/ml, final concentration). After 0.3 and 1.3 min, 200-µl portions were filtered and washed. The data from the two time points were expressed as a percentage of the control and averaged.*

DISCUSSION

Taylor et al. (27) had noted that chloramphenicol-treated cells suffered a loss of B_{12}-uptake capacity and that this loss was not an

interference of the drug with the assay. The results presented here show that any inhibitor of cell mass increase produced a proportionate decrease in B_{12} uptake, in contrast to the effects on the activity of a number of amino acid uptake systems. The pattern of response to different inhibitors, such as the lack of inhibition by inhibitors of deoxyribonucleic acid synthesis or cell division, pointed to protein synthesis as the process required for uptake activity, but proof for this is not yet available. The ready regain of uptake activity upon restoration of growth does not suggest the production of a transport inhibitor after inhibition of growth. The constancy of uptake activity of cells growing at different rates in various media indicates that the inhibition of uptake is a result of unbalanced growth.

Some of the possible explanations for the putative requirement for protein synthesis for transport derive from the involvement of outer membrane protein receptors in uptake. Growing cultures of *E. coli* and *Salmonella typhimurium* excrete complexes of phospholipid, lipopolysaccharide, and protein which appears to be derived from the outer membrane (13, 23). The rate of excretion of this complex was markedly increased upon the inhibition of protein synthesis by amino acid starvation or antibiotic treatment. It has subsequently been shown that growth inhibition can lead to alterations in surface properties, such as sensitization to actinomycin D (16) or other changes in outer membrane permeability (19) or morphology (12). Thus, the profound changes in B_{12} uptake could be merely a reflection of these general alterations on the integrity or function of the cell surface, rather than a specific alteration of the B_{12} uptake mechanism in addition to these general changes.

The decline in B_{12} uptake does not result from loss of the specific receptor by excretion. The loss of uptake activity was much more rapid (up to 80% within 30 min) than the loss of cellular lipopolysaccharide, reported to be about 20% in 30 min (23). Also, the binding activity was only slightly affected in the course of these experiments. The small decline in binding that did occur may well result from this excretion. Finally, sensitivity to colicin E1 was not seriously affected under these conditions, suggesting that there was not extensive loss of receptor from the cell surface. Certainly, a portion of the decrease in B_{12} uptake may result from the same events producing the decline in amino acid transport, such as altered permeability or energy coupling. Nevertheless, there would appear to be an additional effect of the inhibition of protein synthesis specific for the B_{12} uptake process. Probably for the cases of colicin-tolerant mutants, phage f1-infected cells, and normal cells in late log and stationary phase, the lower levels of B_{12} uptake under these conditions can be explained fully by nonspecific changes, as they are paralleled exactly by decreased amino acid uptake. It would be of great interest to determine whether the iron transport systems, which also utilize an outer membrane receptor, would show a specific decrease as a result of the inhibition of protein synthesis.

There are several explanations for a specific dependence of B_{12} uptake on protein synthesis. On the one hand, a protein that is involved in uptake is present in limiting amounts and exhibits rapid turnover. This presumptive short-lived protein could be cytoplasmic and involved in an apparent uptake by its ability to bind B_{12}. But the cells for these experiments are normally grown in the presence of methionine, such that the only enzyme known to bind B_{12} is synthesized only in repressed amounts. Another possibility is that only newly synthesized B_{12} transport proteins are at the proper site to promote uptake, and that upon the inhibition of protein synthesis the existing transport systems become inoperative owing to degradation or change in location. The results presented here do not allow a choice between these possibilities.

No conditions, save those leading to cell disruption, eliminated B_{12} uptake, such that about 20% of the control activity remained after complete inhibition of growth. This residual activity is dependent on the function of the *bfe*-coded receptor and is similar to the full activity in its sensitivity to inhibition by dinitrophenol. Presumably, this residual activity employs the same mechanism as the system in growing cells.

No evidence was obtained here for the utilization by the E colicins of the B_{12} uptake systems subsequent to the step of receptor binding. There was no specific alteration in the uptake of B_{12} in cells rendered tolerant to colicin either by mutation at a variety of loci or as a result of phage f1 infection. Several of the tolerant strains, and the phage-infected cells, did exhibit lower rates of B_{12} uptake, often in proportion to the decreased relative growth rate. However, in all of these cases and in contrast to the case with the inhibition of protein synthesis, the uptake of amino acids was depressed to at least an equivalent degree. Also, strains mutant at *btuA* have normal receptor properties but are defective in some step of uptake subsequent to binding. This strain was fully sensi-

tive to the E colicins and phage BF23. Strains mutant at *tonB* are also defective in B_{12} uptake, although normally sensitive to the E colicins and phage BF23 (1). Finally, growth inhibition, which drastically depressed B_{12} uptake, did not affect the sensitivity of cells to colicin E1, indicating further the independence of the uptake mechanisms for B_{12} and the E colicins.

It was of interest to find an evolutionary basis for the existence of phage or colicin receptors on cell surfaces as an accidental function secondary to their involvement in some transport process of use to the cell. The same question can be applied to the subsequent step of colicin entry, as to whether this also takes advantage of a normal cell process. It would appear from this work that the subsequent steps for the entry of the E colicins do not utilize the B_{12} uptake mechanisms.

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