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POOLS, CONFLUXES AND TRANSPORT OF AMINO ACIDS IN *STREPTOCOCCUS FAECIUM*

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SUMMARY

1. In OH^--dependent, reversed amino acid transport in *Streptococcus faecium* it is possible to distinguish the involvement of four confluxes, *i.e.*, groups of amino acids which are released in synchrony from the cell by either a *tert.*-butanol–isopropanol mixture, trichloroacetic acid, spermine, NH_2OH, or OH^-.

2. Conflux 1 (released by the solvent mixture) represents the cell's capacity for concentrative amino acid accumulation; Conflux 2 (released by OH^- or spermine without acyl–oxygen bond cleavage) originates from ion-exchanging sites which are key intermediates in reversed transport connected to the loci of Conflux 1 and probably also to sites of both cell wall and protein synthesis; Conflux 3 (released by trichloroacetic acid after a prior extraction with the solvent mixture) containing alanine, lysine, and some peptides, is of unknown relationship to the other confluxes; Conflux 4 (released by NH_2OH or OH^- with acyl–oxygen bond fission) consisting primarily of aspartic acid, but also containing some leucine, indicates the existence of an unusual, labile, aspartic acyl complex in the cell periphery. However, with regard to Conflux 4, dilute NH_4OH, which releases alanine as the amide from a labile linkage in the teichoic acids, did not release leucine amide or isoasparagine in these experiments, although it strongly promoted reversed transport.

3. The confluxes are compared with the internal and expandable amino acid pools reported in other organisms, and the implications of the heterogeneous metabolic origins and destinations of the confluxes for interpreting the physiological functions of the pools are discussed.

INTRODUCTION

In recent years it has been shown that the freely extractable amino acid pool of *Candida utilis*[1], *Escherichia coli*[2], *Neurospora crassa*[3], of rat diaphragm, and of isolated guinea-pig and rabbit lymph-node cells[4] exhibits a functional heterogeneity. Two groups of amino acids have been observed to be present among the freely extractable amino acids of these cells. The first is a pool of variable size whose composition reflects the relative concentrations of amino acids in the exocellular environment, and which

Abbreviation: TBIP, 10% *tert.*-butanol–25% isopropanol mixture.

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can be removed, or reduced in size, by osmotic shock. The second is a pool more nearly constant in size whose composition approximates that of protein and which is insensitive to osmotic shock. Further distinction can be made between the two pools on the basis of their differing affinity for amino acids and amino acid analogs and the differing kinetics observed when they incorporate radioisotopic amino acids.

At least one physiological function of the variable-size pool seems to be that of a storage reservoir[5] buffering the flow of amino acids to protein synthesis against fluctuations in the proportions and concentrations of the amino acids available in the exocellular environment. As a physiological function of the second pool, it has been suggested[1,2] that it may be that of a selective staging of amino acids prior to their activation for eventual condensation into proteins, since the relative proportions of amino acids in this pool resemble those found in proteins; however, the actual function of this pool remains largely uncertain.

Also uncertain is the cellular location of these pools and how they are integrated with other cellular components. The amino acids of the variable-size pool seem to be in an uncomplexed state within the osmotic barrier, while the constant-size pool, because of its relatively greater resistance to extraction, may be complexed with macromolecular cellular components.

Our studies on amino acid transport in Gram-positive cocci[5,6] led us to become interested in the possible heterogeneity of the freely extractable group of amino acids in these organisms.

An investigation on the response of Gram-positive cocci to alkaline environments had revealed that these microorganisms exhibit a reversal of amino acid transport when suspended in buffers possessing pH values more alkaline than 8.0: Amino acids are released from the cells to the exocellular environment under these conditions while at the same time the amino acids that are freely extractable from the cell decrease; there are differences, however, between the types of amino acids released to the environment and the types of freely extractable amino acids disappearing that indicate the existence of an intermediate amino acid–cell component complex, connected to both processes.

Further evidence for the existence of this intermediate complex was found in studies on amino acid uptake. Cells which had previously been held in an alkaline environment lacking glucose, and which had undergone the amino acid release–depletion processes, accumulated exogenously added amino acid in the presence of glucose. However, the appearance of the exogenously added amino acid in the cellular freely extractable amino acids was accompanied by the simultaneous appearance of just those amino acids which had disappeared from this group during depletion. Thus, the intermediate complex appeared to be connected with amino acid flow in both the forward and reverse directions—releasing amino acids to the cellular freely extractable amino acids in the former case and binding them from this group in the latter.

This feature of amino acid transport, common to flow in both the forward and reverse directions, suggested that both directional flows are products of the same system, one which is ordinarily responsible for the penetration of amino acids into the cell and their concentrative accumulation there as freely extractable amino acids.

Thus, the OH^--dependent reversibility of amino acid transport in Gram-positive cocci provided a different perspective for examination of amino acid penetration into

organisms. In particular it seemed that the site of action of OH^- might be the outermost of the cellular processes that are involved in the passage of amino acids from the cell's environment to its interior. Alternatively, this site might be part of the structures which enable the cell to retain concentratively accumulated amino acids in the absence of an energy source. Both possibilities, of course, might also be aspects of the same process or structure. Whatever the case, the mechanism by which OH^- affected this site should afford some clue to its nature.

As our studies progressed it became apparent that the amino acids participating in reversed amino acid transport could be grouped on the basis of synchrony in their movements to and from the external environment under the influence of osmotic pressure, trichloroacetic acid, neutral solvents, OH^-, and other agents, as well as metabolism. This report deals with the correlation and distinctions between these groupings and the functionally differing components of amino acid pools that have been described in such a wide variety of organisms as to have led to the suggestion that the functional compartmentation of amino acid pools may be a common characteristic of living cells[4].

METHODS

Chromatography and amino acid quantitation

Samples for chromatography were desalted by passage over 0.9 × 5-cm columns of Dowex-50 X8 (H^+ form, 200–400 mesh). Amino acids were removed from the column by either 2.5 N NH_4OH or, where control of the pH of the column effluent was desired, 0.1 M piperidine[7], collection ceasing when the effluent pH rose above 8.5. After taking to dryness, the amino acids were resuspended in 10% isopropanol for application to the chromatograms.

One-dimensional descending chromatography was performed on Whatman No. 1 paper using either *n*-butanol – acetic acid – water (3:1:1, v/v) or *n*-butanol – pyridine – water (1:1:1, v/v). Two-dimensional chromatography was performed by the technique of UNDERWOOD AND ROCKLAND[8], except that somewhat larger jars (27 × 14 × 28 cm) and correspondingly larger sheets of filter paper were used.

Amino acid estimations were made essentially as described by HEILMANN, BAROLLIER AND WATYKE[9]. However, cadmium acetate instead of cadmium chloride was used. The colored complexes were eluted with absolute methanol and the absorbancy of test solutions and appropriate standards determined at 540 mμ. When a mixture of amino acids was quantitated, the absorbancy of the eluted color complex was compared to that of an alanine standard.

The identification of amino acids, and the location of peptides, was facilitated by use of the polychromatic ninhydrin spray described by MOFFATT AND LYTLE[10]. *N*-Acetylhexosamines were detected by the spray described by SALTON[11], polyols by a mercuric oxide–lead acetate spray[12], and phosphate by a molybdate spray–ultraviolet light exposure procedure[13]. Amino acid hydroxamates were detected by spraying with 1% $FeCl_3$ (see ref. 14), following chromatography in *n*-butanol – acetic acid – water (11:3:4, v/v)[15].

^{18}O Analyses

Amino acid mixtures to be analyzed were fractionated into neutral, basic, and acidic fractions by electrophoresis on Whatman No. 3 paper in a Durrum-type cell

utilizing a pyridine–acetic acid–water (50:5:1195, v/v) mixture at pH 6.1 and a 4-V/cm gradient. The amino acid bands were located by ninhydrin development of narrow tracks from the paper and eluted with 10% isopropanol. The acidic fraction was resubjected to electrophoresis in a pyridine–acetic acid–water buffer (pH 4.2) and in this way resolved into aspartic and glutamic acid bands.

After quantitation of an aliquot of each sample, the various separated fractions were taken to dryness in ignition tubes. Approx. 12 times the amino acids' weight of diphenylamine and diphenylmethane[16] was added to each tube. The tubes were evacuated in the vacuum of an oil diffusion pump, flame sealed, and held overnight in an electric furnace at 265°.

When test samples of aspartic and glutamic acids were treated in this way and the evolved CO_2 measured by Van Slyke's manometric method, 100% of the theoretical yield of CO_2 expected from decarboxylation of the α-carboxyl group was obtained.

Immediately prior to mass spectrometry the sealed tubes were held in a dry ice–acetone bath and then broken in such a fashion as to admit their contents directly into a Nier-type mass spectrometer.

To estimate the dilution of the ^{18}O-enriched water (Volk Radiochemical Corp., Skokie, Ill.) portions of the samples were injected into tubes of known volume containing CO_2 at 1 atm. These tubes were flame sealed and autoclaved overnight. Thereafter they were analyzed in the same way as the amino acid samples.

The total atom fraction of ^{18}O in the CO_2 analyses was calculated from the 44/46 mass ratio by the method of ROBERTS AND UREY[17]. The enrichment of the water samples was calculated from the 46/44 mass ratios of the CO_2 with which they were equilibrated.

Cultural methods and protoplast formation

Streptococcus faecium HF8AG was grown, harvested, and washed-cell suspensions prepared as described previously[6], except that the pH of the suspending medium was adjusted to 6.8. Packed-cell volumes were determined using the cell pellet obtained by centrifugation at 15000 × *g* for 10 min; true cell volumes were determined as the inulin impermeant space of the cell pellets (inulin was measured by the anthrone method used previously[6]).

Protoplasts were prepared as described elsewhere[18] and stabilized either in 0.4 M sucrose or 0.25 M KCl, which were also 75 mM in K_2HPO_4, and adjusted to pH 6.8.

Determination of amino acid stereoisomers

Amino acid mixtures were fractionated into a neutral plus basic fraction and an acidic fraction by passage over a 2 × 8-cm column of Amberlite IR-4B previously equilibrated with 0.2 M ammonium acetate. The basic plus neutral fraction was washed from the column with water and the acidic fraction eluted with 10% formic acid.

After being taken to dryness, the basic plus neutral fraction was treated with snake venom–L-amino acid oxidase[19] (EC 1.4.3.2) (Calbiochem, Los Angeles, Calif.), while the acidic fraction was treated with a lyophilized preparation of *Clostridium perfringens*[20] to decarboxylate the L-glutamic and L-aspartic acid present.

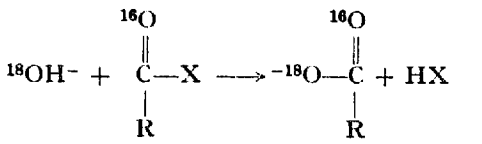
Treated samples and untreated controls were desalted by ion exchange and

chromatographed in the butanol–acetic acid–water system. The residual amino acids, and the β-alanine and γ-aminobutyric acid, were measured by the quantitative ninhydrin–cadmium method.

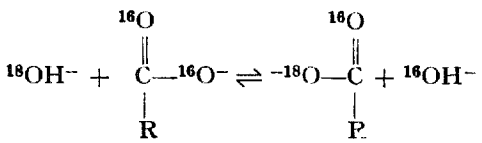
RESULTS

Labelling of amino acids released and retained by S. faecium at pH 10.5 in ^{18}O-enriched water

To estimate the extent to which the hydroxyl ion dependent, amino acid efflux from *S. faecium* was due to acyl–oxygen bond fission, cell suspensions were held at pH 10.5 in an ^{18}O-enriched buffer solution. Under these alkaline conditions, labelling of the carboxyl group of the released amino acids proceeds mainly by two reactions:

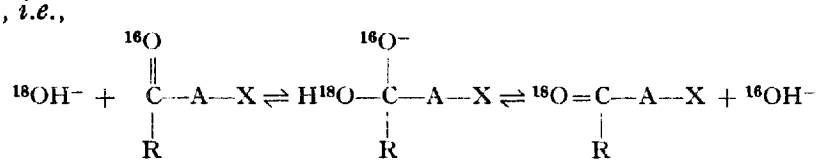


(1)



(2)

In Reaction 1, the amino acid bond to an electronegative center (X = S, O, or N), which is part of a cellular component, is hydrolysed, with the addition of ^{18}O to the carbonyl group of the amino acid. (A reversible exchange reaction of the type observed by BENDER *et al.*[21] to accompany the basic hydrolysis of carboxylic acid derivatives, *i.e.*,



(3)

would contribute to the labelling of the carboxyl groups of the released amino acids only if Reaction 3 were followed by a hydrolytic reaction, producing a result indistinguishable in our experiments from that of Reaction 1.)

Reaction 2 represents the oxygen exchange taking place between the free amino acids and the alkaline medium.

The measured carboxyl labelling of the released amino acids is the sum of Reactions 1 and 2, and to measure this labelling, packed cells were resuspended in 9.8% enriched buffer (12 mM $NaHCO_3$–6 mM K_2HPO_4 (pH 7.2)) in a ratio of packed-cell volume to buffer of 6:4. This suspension was adjusted to pH 10.5 and held at this pH for 50 min, after which it was neutralized and centrifuged. The supernatant contained the released amino acids, and the packed cells were treated with TBIP to obtain the freely extractable amino acids.

To correct for the exchange labelling resulting from Reaction 2, an equal volume of packed cells was subjected to the same release conditions in ^{16}O buffer. After neutralization and centrifugation, the supernatant containing the released amino acids was taken to dryness. The amino acids and buffer salts were then redissolved in water enriched with ^{18}O to the level calculated to exist in the extracellular medium

in the first experiment. This solution was brought to pH 10.5, held there 50 min, and then neutralized. Thereafter, these amino acids, labelled only by the exchange reactions, were treated in the same manner as the released amino acids and the TBIP-extract amino acids from the cells held in ^{18}O buffer.

After desalting by absorption on Dowex-50, followed by displacement with 0.1 N piperidine, the samples were electrophoretically separated into neutral, basic, and acidic amino acid fractions, and the isotopic enrichment of the carboxyl groups of the amino acids in each fraction was determined (the enrichment of the α-carboxyl group in the cases of aspartic and glutamic acids). The calculated labelling is shown in Table I, along with the amount of labelling in excess of exchange, *i.e.*, the labelling due to acyl–oxygen bond fission.

TABLE I

CARBOXYL LABELLING OF RELEASED AND TBIP-EXTRACT AMINO ACIDS OF *Streptococcus faecium* HELD AT pH 10.5 IN 3.8% ^{18}O WATER

Sample	*Atom per cent ^{18}O*	*Atom per cent in excess of exchange*	*Per cent of amino acid labelled*
Amino acids released in ^{16}O water and then labelled by carboxyl oxygen–water oxygen exchange at pH 10.5 in ^{18}O water	0.39	—	—
TBIP-extract amino acids from cells held at pH 10.5 in ^{18}O water:			
neutral fraction	0.41	negligible	negligible
basic fraction	0.38	negligible	negligible
acidic fraction	0.39	negligible	negligible
Amino acids released from cells held at pH 10.5 in ^{18}O water:			
neutral fraction	0.38	negligible	negligible
basic fraction	0.41	negligible	negligible
acidic fraction	0.94	0.55	15

As shown in Table I, only the acidic amino acid fraction showed labelling in excess of exchange. Consequently, the acidic fraction was resolved by electrophoresis into aspartic and glutamic acids and the primary α-labelling determined. The label was found entirely in aspartic acid, which was 35% labelled. Therefore the release of this much of the aspartic acid takes place with acyl–oxygen bond fission, a type of reaction apparently unique, by this test, to the release of this amino acid among the released amino acids. And because of the apparent lack of this type of labelling among the other released amino acids, it seems unlikely that their release—the bulk of the release—takes place as the result of the alkaline hydrolysis of phosphoanhydrides or carboxyl esters of these amino acids, bonds whose nucleophilic cleavage proceeds with acyl–oxygen fission[22–25]. Such linkages as these could be the origin of the labelled aspartate, but there are other carboxyl derivatives of this amino acid (derivatives thus far described only for aspartic and glutamic acids), such as the ε-aspartyllysines and glycosidically linked aspartic acid, which could also release labelled aspartic acid. Consideration of these is deferred until the DISCUSSION section.

It is also of interest to note that somewhat more than 80 % of the true cell volume was immediately accessible to the ^{18}O water, as revealed by a comparison between the cell pack volume permeable to the isotope and to inulin. Despite this immediate permeation of the cell, the freely extractable amino acids remained unlabelled.

Derivatization of the amino acids released and retained by S. faecium exposed to NH_2OH and NH_4OH

The finding that only aspartic acid, of the amino acids released and retained by *S. faecium* at pH 10.5, underwent acyl–oxygen bond fission prior to release was unexpected, since it was anticipated that alanine, which has been reported to exist in an alkali-labile, esteratic linkage in the cell walls of closely related species[24], would be released with acyl–oxygen bond fission. To corroborate the unique labelling of aspartic acid, nucleophiles other than the hydroxyl ion were tested for their ability to derivatize the retained freely extractable amino acids and the released amino acids. Thus, the presence of NH_2OH and NH_4OH in the exocellular medium might be expected to result in the formation of hydroxamates and amides, respectively, from these amino acids.

Buffered, washed-cell suspensions at pH 6.8 were made 50 mM in $NH_2OH \cdot HCl$. One half of the cell suspension was brought to pH 11.5 and held there for 30 min. The remainder of the NH_2OH-treated cell suspension was held at pH 6.8 during this interval, and equal volumes of cell suspension without added NH_2OH were held either at pH 6.8 or 11.5 for 30 min. A portion of each cell suspension was centrifuged prior to the start of the holding period, and the cell pellets extracted with TBIP.

The amino acid contents of the supernatants and the extracts were quantitated and chromatographed. The quantitative relationships among these samples is shown in Table II.

In the presence of NH_2OH the release normally taking place at pH 11.5 was almost completely halted. Chromatography of such amino acids as were released revealed only the presence of lysine and some basic peptides in the exocellular medium in contrast to the 13 amino acids and several basic peptides ordinarily released at pH 10.5 or 11.5. In addition, the level of freely extractable amino acids, instead of decreasing, rose—a circumstance heretofore observed only in cells metabolizing glucose.

TABLE II

EFFECT OF NH_2OH, NH_4OH, AND ELEVATED pH UPON AMINO ACID RELEASE AND DISAPPEARANCE IN *Streptococcus faecium*

Suspending medium	*Change in level of freely extractable amino acids (μg/ml packed cells)*	*Fraction of freely extractable amino acids remaining after 25 min*	*Amino acid released to the medium (μg/ml packed cells)*
pH 6.8 buffer	− 45	>0.98	28
pH 10.5 buffer	−420	0.78	360
pH 11.5 buffer	−670	0.65	480
50 mM NH_2OH	+ 36	1.02	None
pH 11.5 buffer plus 50 mM NH_2OH	+170	1.09	64
1.5 N NH_4OH (pH 11.5)	−790	0.58	630

However, although only a limited amount and a few kinds of amino acids had been released in the pH-11.5, NH_2OH-containing medium, two hydroxamate spots were chromatographically detectable both in the exocellular medium and among the freely extractable amino acids. Consequently, these samples were band streaked on chromatography paper, the chromatograms developed, and after location of the $FeCl_3$-reacting bands, they were eluted and taken to dryness. Part of the residue was dissolved in 6 N HCl and held overnight at 120°, after which the sample was returned to dryness several times, after redissolving in 10 % isopropanol, and the final solution subjected anew to chromatography to identify the hydrolyzed amino acids. Duplicate, unhydrolyzed material was simultaneously chromatographed to correct for any contamination of the hydroxamate, prior to hydrolysis, with free amino acids.

The major hydroxamate band from both the exo- and endocellular material yielded only aspartic acid on hydrolysis, while the minor hydroxamate band from the endocellular material yielded leucine. The minor hydroxamate band from the exocellular material yielded insufficient material to make identification positive, but seemed to contain alanine.

Thus, the effects observed with hydroxylamine offered corroboration to the labelling obtained with $^{18}OH^-$, indicating that an aspartic acid complex underwent this sort of fission.

Apparently, however, leucine was also present in these cells in a labile bond giving rise to leucine hydroxamate. It seems likely that this labile bond was also hydrolysed in the isotope experiment, producing [^{18}O]leucine, but in such small amounts that it was not detected.

The effects of NH_2OH, then, were two-fold. First, the hydroxyl ion dependent release of amino acids was inhibited, with the exception of the release of lysine and some basic peptides. In this first effect, the action of NH_2OH resembles that of osmotic pressure (described in a later section) which also inhibits the alkaline release of amino acids, excepting for lysine, alanine, and some basic peptides. The second effect was attack upon labile complexes of aspartic acid, leucine, and possibly alanine, producing hydroxamates. This attack was unaccompanied by release of amino acids to the medium, or by the disappearance of amino acids from the TBIP extract. Consequently the hydrolysis of these amino acid complexes is not prerequisite to the entire amino acid efflux, but is an independent component of that efflux.

Concurrently with the study of NH_2OH effects upon the amino acid efflux, the effects of NH_4OH were also examined. A portion of the cell suspension was brought to pH 11.5 with concentrated NH_4OH, making the suspension 1.5 N in NH_4OH. After 25 min the suspension was centrifuged, the supernatant neutralized, and the cell pellet extracted with TBIP. The supernatant and the extract were then examined for their amino acid content.

NH_4OH was found to produce a greater efflux than any of the other agents tested. However, no alanine amide, asparagine, isoasparagine, or glutamine were found in the exocellular medium. Instead, in addition to the amino acids regularly released, two peptides were found in the exocellular medium. Peptides with similar R_F values and showing a similar response to the polychromatic ninhydrin spray were also found in the TBIP extract of the NH_4OH-treated cells. They exhibited R_F values of 0.15 and 0.65, respectively, in the butanol–acetic acid–water solvent and yielded a golden-brown and a lemon-yellow color when developed by the polychromatic ninhydrin technique.

The peptides were resolved from the free amino acids in the same manner as the hydroxamates had been and were hydrolyzed by holding in 6 N HCl for 16 h at 120°. They were then resubjected to chromatography and an aliquot of the hydrolysate analyzed for ammonia by Nesslerization.

The hydrolysate contained lysine, aspartic and glutamic acids, alanine, proline, tyrosine, valine, and leucine. Ammonia was present in the amount of 0.5 mole per 0.15 mole of aspartic acid and 0.35 mole of glutamic acid making it possible that the side chains of these compounds were present in the unhydrolyzed peptide as the amide.

Replicate chromatograms of the hydrolysate were examined for the presence of carbohydrates, *N*-acetylhexosamines, phosphate, and ultraviolet-absorbing material. None were found. However, when sprayed with the polyol detection agent, a large spot with the R_F of glycerol was found.

Thus NH_4OH, while acting as a more powerful promoter of amino acid efflux than any of the other agents tested, did not produce the expected derivatives, particularly of aspartic acid, apparently failing to compete with OH^- at this pH. This was somewhat surprising, for while it might be expected that ammonia would not be as powerful a nucleophile as NH_2OH, which is capable of making a bifunctional attack upon an electrophilic center[26], still, under comparable conditions, the action of aqueous ammonia upon glycerol teichoic acid yields alanine amide[25], indicating that ammonia should be able to compete with OH^-.

Effect of pH and NH_4OH on the compounds extractable from S. faecium by cold and hot trichloroacetic acid

In an attempt to determine whether or not esterified alanine was liberated from teichoic acids during the OH^--dependent release, the cell crop from 5 l of growth medium was subjected to the teichoic acid extraction procedure of BADDILEY AND DAVISON[27], but the extraction yielded less than 10 mg of polyol-containing material. This low yield was possibly due to a resistance of polymerized enterococcal teichoic acids to extraction with cold trichloroacetic acid, the primary extractant in the procedure employed (SALTON[28] has noted the resistance of *Lactobacillus arabinosus* and *Staphylococcus aureus* teichoic acid to extraction with cold trichloroacetic acid).

It was found, however, that the cold trichloroacetic acid, although failing to release any appreciable amount of polymerized teichoic acid from the cells (although alcohol-soluble polyol material was released) effected a further release of amino acids even from cells previously extracted with the TBIP membrane-disrupting solvent mixture. This was unexpected since HANCOCK[29] had concluded from a comparison of the ability of a variety of organic solvents, inorganic acids and bases, boiling water, and cold trichloroacetic acid to extract the amino acid pool of *Staphylococcus aureus* that all of these agents were about equally effective. Inspection of his data shows, however, that cold trichloroacetic acid extracted 5–10 % more amino acids than any of the other extractants, except 0.1 N NaOH, and he did not report testing the ability of any of the solvents to extract further amino acid from cells extracted first with a different solvent.

To further investigate this observation, cell pellets obtained after centrifugation of TBIP-extracted cell suspensions described in some of the earlier experiments were held for 24 h in 10 % trichloroacetic acid at 4°. This suspension was then centrifuged

and the supernatant decanted. The cell pellet was extracted again with boiling 10% trichloroacetic acid for 15 min.

After five ether extractions, the trichloroacetic acid supernatants were passed through Dowex-50-H^+ columns and the effluents and distilled-water rinses were collected and pooled. The amino acids were then displaced from the columns with 0.1 N piperidine. All column effluents were subsequently taken to dryness for chromatography.

The amino acids and peptides extracted from the cell pellets by trichloroacetic acid extraction following the TBIP extraction are summarized in Table III. It is apparent that at least some of the alanine, lysine, and peptides released to the medium during transport reversal originate from a linkage with the cell resisting TBIP extraction but susceptible to extraction with trichloroacetic acid.

TABLE III

AMINO ACIDS AND PEPTIDES REMOVED FROM *Streptococcus faecium*, PREVIOUSLY EXTRACTED WITH TBIP, BY COLD AND BOILING 10% TRICHLOROACETIC ACID EMPLOYED IN SEQUENCE

Treatment of cells prior to TBIP extraction	*Extracted with cold trichloroacetic acid*	*Extracted with boiling trichloroacetic acid*
Held 25 min at pH 6.8	Lysine, basic peptides and alanine	Peptide
Held 25 min at pH 11.5	Some lysine and basic peptides	Peptide trace
Held 25 min at pH 11.5 in 1.5 N NH_4OH	None	None

Cold trichloroacetic acid also extracted phosphopolyol material from these cells, while hot trichloroacetic acid extracted phosphate-free polyol material. A loss from the cell of this polyol material during the transport reversal was suggested by visual comparison of the amounts appearing on the chromatograms of trichloroacetic acid extracts from cells at the start and at the finish of the alkaline holding period. In addition to polyol material, both trichloroacetic acid treatments extracted carbohydrate, hexosamine, phosphate-containing, and ultraviolet-absorbing materials, as might be expected from the known degradative effect of trichloroacetic acid on cells of this genus[30].

Optical activity of amino acids released and disappearing during reversed transport

Because of the involvement of D-amino acids in bacterial cell walls and with the teichoic acids, it was of interest to assess to what extent they were involved in the reversed transport process.

A portion of a washed cell suspension was centrifuged and the cell-pack TBIP extracted, while a second portion was held at pH 10.5 for 50 min and then treated in the same manner. The aqueous supernatants and the solvent extracts were passed through an Amberlite IR-4B column in the acetate form from which the acidic amino acids were eluted with 10% formic acid. The neutral column-effluent, containing the basic and neutral amino acids, and the formic acid effluent were taken to dryness and the several dried fractions redissolved in distilled water.

One-half of the neutral plus basic amino acid fraction was desalted and quantitatively chromatographed, while the second half was treated with L-amino acid

oxidase, then desalted and chromatographed. The acidic amino acid fraction was similarly treated using the clostridial decarboxylase preparation. The results of the several treatments are collected in Table IV.

It can be seen that, while D-aspartic acid disappeared from the pool more rapidly than the L-form, the released aspartate was over 80 % in the L-form. Conversely, while L-glutamic acid disappeared from the pool somewhat more rapidly than D-glutamic acid, the released glutamate was largely in the D-form. Lysine disappearing and released was completely in the L-form, but alanine disappearing and released was largely in the D-form. Released valine, leucine, tyrosine, and methionine were completely in the L-form. Released serine, threonine, proline, and histidine were either incompletely resolved or were present in such small amounts as to make quantitation difficult.

TABLE IV

OPTICAL ACTIVITIES OF RELEASED AND FREELY EXTRACTABLE AMINO ACIDS OF *Streptococcus faecium* HELD FOR 50 MIN AT pH 10.5

Amino acid	*Per cent freely extractable amino acid in L-form*		*Per cent released amino acid in L-form*
	0 min	*50 min*	*50 min**
Aspartic acid	45	60	81
Glutamic acid	51	42	25
Lysine	100	—**	100
Alanine	21	>97	24
Leucine***	—	—	100
Tyrosine***	—	—	100
Valine***	—	—	100
Methionine***	—	—	100

* Insufficient amino acid is found in the medium at zero time to quantitate.

** Lysine disappears from the freely extractable amino acids almost completely during transport reversal.

*** These amino acids are not present in the freely extractable amino acids in sufficient amount to quantitate.

In the case of aspartic and glutamic acids, it is apparent that the processes effecting the disappearance of pool amino acids and the release of amino acids to the environment exhibit differing optical specificities, the disappearance process involving both L- and D-isomers, while the release process is specific for one or the other. For alanine and lysine, both processes are specific for the same isomer, although the isomer differs between the two amino acids.

Release and disappearance of amino acids from protoplasts

Replicate portions of a washed cell suspension were suspended either in 0.4 M sucrose buffered with 75 mM K_2HPO_4 adjusted to pH 6.8 or in 75 mM K_2HPO_4 adjusted to pH 6.8. These suspensions were then exposed to lysozyme or spermine, or held at pH 10.5. The suspensions were centrifuged and the separated cells, or protoplasts, were extracted with TBIP. However, a portion of the protoplasts resulting from the action of lysozyme was not extracted, but was resuspended in 0.4 M buffered sucrose and in this medium brought to pH 10.5 in the presence and absence of glucose. After holding, they were recentrifuged, and the pellets TBIP extracted.

TABLE V

EFFECT OF 50 MIN EXPOSURE TO LYSOZYME, SPERMINE, OR OH^- UPON THE RELEASE AND DISAPPEARANCE OF AMINO ACIDS FROM *Streptococcus faecium*

Releasing agent	*Medium*	*Change in level of freely extractable amino acids (μg/ml packed cells)*	*Fraction of freely extractable amino acids remaining after 50 min*	*Amino acid released to the medium (μg/ml packed cells)*
Lysozyme (180 μg/ml)	Buffered, 0.4 M sucrose	−1800	0.49	2500
Lysozyme (180 μg/ml)	75 mM K_2HPO_4	−2900	0.09	3500
Spermine (180 μg/ml)	Buffered, 0.4 M sucrose	−300	0.90	1700
OH^- (pH 10.5)	Buffered, 0.4 M sucrose	−60	0.95	530
OH^- (pH 10.5)	75 mM K_2HPO_4	−500	0.82	330
OH^- (pH 6.8)	Buffered, 0.4 M sucrose	−50	0.95	50

TABLE VI

EFFECT OF GLUCOSE AND OH^- UPON THE RELEASE AND DISAPPEARANCE OF AMINO ACIDS FROM PROTOPLASTS OF *Streptococcus faecium*

Suspending medium	*Change in level of freely extractable amino acids (μg/ml packed cells)**	*Fraction of freely extractable amino acids remaining after 35 min*	*Amino acid released to the medium (μg/ml packed cells)*
Buffered, 0.4 M sucrose (pH 10.5)	−146	0.43	490
Buffered, 0.4 M sucrose + 2 mg glucose/ml (pH 10.5)	−40	0.78	280
Buffered, 0.4 M sucrose (pH 6.8)	−40	0.71	0

* Based on packed cell volume prior to conversion to protoplasts.

Amino acid contents of the supernatants and extracts of the cells are shown in Table V, while the same data for the protoplasts are shown in Table VI.

Lysozyme, in hypertonic medium, produces a much greater disappearance and release of amino acids than does OH^-. However, its effect, although greater than that of OH^-, is qualitatively similar. The four freely extractable amino acids decrease, while 12–13 amino acids appear in the exocellular medium, and a considerable fraction of the cellular freely extractable amino acids remains with the cells even after 50 min exposure. Lysozyme, of course, also released detectable hexosamine from the cell. In contrast, in hypotonic medium lysozyme causes a release of amino acids that are predominantly of the same species as the freely extractable amino acids—aspartic and glutamic acids, alanine, and lysine—and the cell pool is obliterated: observations that are consistent with a postulate of simple cell disruption.

Spermine, although also causing amino acid release from intact cells in hypertonic medium, caused much less pool disappearance than did lysozyme and inspection of chromatograms of the released amino acids suggested that spermine released relatively more alanine and less glutamic acid than did lysozyme.

Of considerable interest was the finding that intact cells in 0.4 M sucrose at pH 10.5 released only lysine, alanine, and some basic compounds. This was not due to the presence of a fermentable impurity in the sucrose that inhibited release, as glucose does in hypotonic media, since exactly the same phenomenon was observed with cells suspended in 0.24 M KCl, the osmotic equivalent of 0.4 M sucrose.

In protoplasts, OH^- caused both release and disappearance of amino acids chromatographically similar to that observed in intact cells, although lysine and alanine are present in the pool and in the medium in lower proportions. However, in protoplasts a disappearance of freely extractable amino acids occurred at pH 6.8 that was unaccompanied by release of amino acids to the medium.

Added glucose reduced the release of amino acids from the protoplasts at pH 10.5 and markedly depressed the disappearance of freely extractable amino acids at the same time, but the inhibition of OH^- effects by glucose was not so great in protoplasts as that observed previously in intact cells[6], where glucose completely inhibited both amino acid disappearance and release.

DISCUSSION

From the foregoing results, it is possible to distinguish at least four amino acid confluxes in *S. faecium*: groups of amino acids that move in synchrony from the cell to the environment under the influence of TBIP, trichloroacetic acid, spermine, OH^-, or NH_2OH. These are listed in Table VII.

TABLE VII

AMINO ACID CONFLUXES DISTINGUISHABLE IN *Streptococcus faecium*

Amino acid conflux	*Defining conditions*	*Component amino acids*
1	Released from cells by TBIP	Alanine, lysine, aspartic and glutamic acids
2	Released from cells by OH^-; released from cells in 0.4 M sucrose by spermine	Alanine, lysine, aspartic and glutamic acids, leucine, valine, methionine, tyrosine, threonine, proline, serine, glycine, histidine, peptides
3	Released from cells by trichloroacetic acid following extraction with *tert.*-butanol–isopropanol mixture; released from cells in 0.4 M sucrose, or 0.24 M KCl, by OH^-	Alanine, lysine, peptides
4	Released from cells by $^{18}OH^-$ with carboxyl labelling; released from cells by NH_2OH with formation of hydroxamates	Aspartic acid, leucine

In a number of aspects Confluxes 1 and 2 resemble the expandable and internal pools described by COWIE AND MCCLURE[1] in *C. utilis*, by KEMPNER AND COWIE[2] in *E. coli*, and by ZALOKAR[3] in *N. crassa*. Thus, Conflux 1 contains concentratively accumulated amino acids and its composition reflects the amino acid composition of the environment, excepting that alanine, lysine, aspartic and glutamic acids are always present[5]. It also exchanges amino acids with some cellular binding site in a manner analogous to the exchange observed in *C. utilis* between the expandable and the internal pool[2]. It differs from the expandable pool, however, in being resistant to osmotic shock.

In Conflux 2, as in the internal pool, the amino acids present are more like those found in protein (although the relative lack of aromatic and sulfur-containing amino acids and the relative abundance of basic and acidic amino acids in this conflux suggests the amino acid proportions of nucleoprotein more than of other protein species). There is a further resemblance between the relationships of Conflux 2 to Conflux 1 and the relationships of the internal to the expandable pool. As the internal pool is linked by amino acid flows with both the expandable pool and the external environment, similarly Conflux 2 is linked with Conflux 1 and with the transport of environmental amino acids: during transport reversal Conflux 2 appears while Conflux 1 simultaneously disappears, and during forward transport, along with the appearance of exogenously added amino acids, amino acids of the type that disappeared during transport reversal reappear in Conflux 1. These latter observations indicate that the binding site(s) from which Conflux 2 originates is obligatorily involved in the passage of amino acids between Conflux 1 and the environment, similar to the relationship between the environment, the expandable, and the internal pools described in *N. crassa*[3].

However, although some of the attributes that distinguish the internal pool from the expandable pool in other organisms also distinguish Conflux 1 from Conflux 2 in *S. faecium*, it seems incorrect to designate these confluxes as pools—which implies that they are a homogeneous group of amino acids correlated with respect to location, or to manner of retention by the cell, or to being precursors in some metabolic pathway— for they are demonstrably heterogeneous groups of amino acids correlated in terms of the operational conditions by which they are derived from the cell.

Considering the confluxes in turn, Conflux 1, the cellular freely extractable amino acids, seems to represent the cell's capacity for retention of uncomplexed amino acids, and these can join the conflux either from synthesis (in the case of aspartic acid and alanine), from a cellular binding site during amino acid uptake (in the case of the four amino acids always found in the conflux), or from the exocellular environment[5].

When the conflux decreases during reversed amino acid transport both isomers of aspartic and glutamic acids disappear, while only one isomer of alanine disappears (the disappearance of lysine, which occurs in only one isomeric form in this organism, perforce involves the L-form). These differences in isomer disappearance during reversed transport suggest that the processes removing aspartic and glutamic acid from the conflux are either two optically specific processes or one process with little optical specificity, while the process removing alanine is specific for the D-form; or alternatively, that the D- and L-aspartic and glutamic acids, D-alanine, and lysine of the conflux are accessible to a common, non-stereospecific removal process, while the L-alanine of the conflux is not so accessible—perhaps being located elsewhere in the cell.

Conflux 2, although composed of a greater variety of amino acids than Conflux 1, also shows stereoisomer heterogeneity, the alanine and glutamic acid of the conflux being predominantly the D-form while many of the other amino acids are the L-form. This again indicates either the involvement of multiple processes with differing stereospecificities, or differing localization of the D- and L-isomers of at least alanine and glutamic acid.

Since Conflux 2 amino acids are released by $^{18}O^-$ without carboxyl labelling, it

is unlikely that they are being released from phosphoanhydrides or carboxyl esters. The ability of spermine, a positively charged molecule without enzymic capacity, as well as the positively charged enzyme lysozyme, to produce confluxes very similar to Conflux 2, as well as the similar—but very large—conflux observed in the presence of ammonium ion, suggests that the release process is one of cation exchange in which the amino acids involved are released from negative binding sites either when they undergo amino group dissociation as the medium pH is elevated or when they are displaced by another cation. (It has previously been shown that ordinary buffer anions, other than OH^-, do not cause amino acid release[6].)

The sites from which Conflux 2 originates and the mechanism of its release from these sites are more closely involved with reversed transport than are the corresponding sites and mechanisms of Confluxes 3 and 4. For while the release of these latter confluxes can take place without an accompanying reversed transport, the release of Conflux 2 is tightly coupled to reversed transport and is the step immediately preceding the appearance of amino acids in the exocellular medium when the transport takes place. The sites from which Conflux 2 originates must be part of the structure which enables the cell to retain the amino acids of Conflux 1, even in the absence of energy-yielding metabolism. In addition, because of the association of Conflux 2 with forward transport[6], its origin and the mechanism of its release are likely to be related to transport into the cell.

A partially selective, cation-exchanging complex as the site of this conflux involved in both forward and reversed transport, could account for the ability of *S. faecium* to take up either L- or D-isomers[5], for the 35–50 % inhibition of L-isomer uptake in the presence of the D-isomer, and for the competition for uptake between amino acids of comparable base strength (alanine, glycine, serine, valine, and threonine)[31]. Such a complex could then transfer its adsorbed amino acids to the more highly stereo-selective processes of protein and cell-wall synthesis, or to the loci of Conflux 1 for accumulation and storage[5].

Considering Conflux 3, this group of amino acids seems to originate from a binding site susceptible to both the action of trichloroacetic acid and OH^-, since after release due to OH^-, this conflux is greatly reduced. IKAWA[30] has noted that following the degradation of teichoic acid in *Streptococcus faecalis* dialysable alanine and lysine appear, but the correlation of this observation with the origins of Conflux 3 is still to be established, and the origins and possible functions of this conflux are not clear at present.

In the case of Conflux 4, the defining conditions indicate the presence in the cell periphery of unique, labile leucyl and α-aspartyl acyl complexes. Possible compounds of this nature are amino acid phosphoanhydrides, carboxyl esters of nucleosides, glycosidic carboxyl esters[32], or carboxyl esters of teichoic acids. A further group of aspartyl compounds, the ε-aspartyllysines, reported to be present in cells of this genus[30], are relatively alkali stable, although ε-aminosuccinyllysine, a condensation product of the ε-aspartyllysines found in acid hydrolysates of bacterial cell walls, cleaves in alkaline solution to yield ε(β)-aspartyllysine[33]. It is not apparent why aspartyl or leucyl nucleotides by themselves should be uniquely accessible to OH^- and NH_2OH, since amino acyl nucleotides are encountered normally as intermediates in protein synthesis, and if the hydrolysis of such intermediates were occurring here, Conflux 4 would be expected to contain a much larger variety of amino acids than

it does. In the case of a glycosidic carboxyl ester, while such compounds are known in mucoproteins of animal origin, to our knowledge they have not been reported in bacteria, nor have aspartyl or leucyl esters of teichoic acid. Consequently, the nature of the labile α-amino acyl complex remains uncertain.

Comparisons of the confluxes so far observed in *S. faecium* serve to illustrate the non-identity between, and within, groups of amino acids extracted from cells by different solvents. This is obvious, for instance, when OH^- is compared with TBIP or trichloroacetic acid, but is less so when TBIP and trichloroacetic acid are compared. Employed singly, they both extract the same species of amino acids from *S. faecium*, and the amount extracted by trichloroacetic acid is perhaps only 10–15 % more than that extracted by TBIP. However, as shown when they are employed in sequence, at least part of the alanine and lysine extracted by trichloroacetic acid originates from a cellular component either not accessible to, or not affected by, TBIP.

Non-identity within groups is evident when the amino acids of Conflux 1 or 2 are examined for optical activity. It is apparent from the mixture of activities found that the amino acids in these confluxes are neither coming from, nor going to, the same areas of metabolism, but in all likelihood represent stages in the synthesis of both cell walls and proteins (and different stages in the synthesis of very different types of proteins may well be represented in the confluxes).

The assumption that the amino acids in a "pool" are committed to a common synthetic pathway is unjustified without further evidence if what is measured as the "pool" is actually an amino acid conflux based on the extractive powers of some solvent. This assumption of a common metabolic destination for "pool" amino acids has been involved in some very detailed and otherwise convincing deductions about the nature of amino acid transport and the early staging of amino acids for protein synthesis in microorganisms[32].

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