On the Incorporation of 5′,5′,5′-Trifluoroleucine into Proteins of *E. coli**

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Leucine-requiring mutants of *E. coli* continue to grow exponentially if 5′,5′,5′-trifluoroleucine is added to the medium. No inhibition is observed until the concentration of trifluoroleucine exceeds that of leucine by several fold. When the trifluoroleucine-to-leucine ratio in the medium is 2, at least one half of the leucine in the bacterial proteins is replaced by trifluoroleucine. In a modified chemostat *E. coli* can be adapted to grow at normal rates on a medium containing only trifluoroleucine, and under these conditions leucine in the proteins is entirely replaced by trifluoroleucine. This adaptation does not appear to result from selection of mutants or induction of enzymes. In contrast, the wild type of *E. coli B* does not incorporate trifluoroleucine added to the medium. Individual organisms in a culture grown on trifluoroleucine have some three times the volume and protein content of organisms grown on leucine. The deoxyribonucleic acid and ribonucleic acid content of such cells is unchanged.

Exponential growth is abolished and protein synthesis inhibited if amino acid auxotrophs are inoculated into media in which the required amino acid is replaced by an analog, *e.g.*, phenylalanine and tyrosine by one of the fluorophenylalanines (Munier and Cohen, 1956, 1959a,b) or by thienylalanine (Pardee, 1958; Munier and Cohen, 1959b), tryptophane by azatryptophane (Pardee *et al.*, 1956), serine by azaserine (Pardee, 1958; Pardee and Prestidge, 1958), methionine by norleucine (Munier and Cohen, 1959b; Cowie *et al.*, 1959), and leucine by methallylglycine (Dittmer, 1948). In general, some increase in cell mass (linear growth) is found, but only small amounts of the analogs are incorporated into the bacterial proteins. More extensive incorporation of an analog is observed in the case of selenomethionine, which can satisfy the methionine but not the cysteine requirements of a methionine auxotroph (Cohen and Cowie, 1957; Cowie and Cohen, 1957).

In the experiments reported here it is found that the leucine analog, 5′,5′,5′-trifluoroleucine (trifluoroleucine), can substitute for leucine in the proteins of a leucine-requiring mutant of *E. coli B*. This leucine auxotroph can be adapted by an apparently nongenetic mechanism to grow on trifluoroleucine alone. After adaptation, cell division and exponential growth occur with undiminished rates in the presence of this analog. Some properties of the trifluoroleucine-containing organisms are discussed.

EXPERIMENTAL

*Synthesis of 5′,5′,5′-Trifluoro-*DL*-leucine.*

Ethyl-trifluoromethyl butyrate.—Commercial trifluoromethyl crotonic acid, 100 g, is diluted with 200 ml absolute ethanol and hydrogenated at 3 atm. pressure in the presence of 2 g of Adams catalyst. After the theoretical amount of hydrogen is consumed, the liquid is filtered from the catalyst, 400 ml of absolute ethanol and 100 ml of concentrated sulfuric acid are added, and the mixture is refluxed for 24 hours. After extraction of the sulfuric acid with water, the ester is distilled; b.p. = 123–125°; yield: 100 g (82%).

α - Oximino - 5′,5′,5′ - trifluoroisocaproic acid.—Fifty-five grams of sodium hydride (52% in oil) is suspended in 500 ml of absolute ether, and 100 g of ethyl-trifluoroisovalerate and 122 g of diethyl oxalate are added. The reaction mixture is refluxed with stirring for 36 hours, at which time all the sodium hydride has reacted. Then 1 kg of ice and 65 ml of concentrated sulfuric acid are added, the ether layer is separated, and the ether is evaporated. To the residue are added 250 ml of 85% formic acid and 250 ml of concentrated hydrochloric acid; the mixture is stirred for 24 hours and the oily layer is discarded. The aqueous layer is distilled until 500 ml of distillate has been collected. To the residue are added 250 ml of water and a warm solution of 65 g hydroxylamine in 125 ml of water. After 24 hours in the refrigerator 90 g of crude oxime is collected by filtration, dissolved in 125 ml dioxane, treated with charcoal, and warmed with 625 ml of water. On cooling 55 g of oxime is obtained. From the mother liquors a second crop of 10 g is recovered. Melting point 171°; yield: 56%.

*5′,5′,5′-Trifluoro-*DL*-leucine.*—The oxime, 65 g, is suspended in ethanol–water–concentrated ammonium hydroxide (10:10:1) and hydrogenated with 1 g of Adams catalyst and hydrogen until the theoretical quantity of hydrogen has been taken up. Fresh catalyst is added during the hydrogenation as required. After removal of the catalyst and evaporation of the alcohol, the product is recrystallized from water with charcoal. Yield: 39 g (64%).

Anal. Calcd. for $C_6H_{10}O_2NF_3$ (185): C, 38.9; H, 5.4; F, 30.8. Found: C, 39.2; H, 5.4; F, 32.1.

Culture Conditions

Growth media.—All experiments are carried out in a medium containing 10.5 g of disodium hydrogen phosphate, 4.0 g of potassium dihydrogen phosphate, 12.0 g of ammonium chloride, 1.5 ml of 0.4 M magnesium sulfate, 0.6 ml of 0.4 M calcium chloride, and 24 ml of glycerol per liter. DL-Leucine and/or DL-trifluoroleucine are added as indicated. The medium is sterilized by autoclaving for 40 minutes at 130°. A 15-ml portion of medium is inoculated with 0.1 ml of a suspension of about 10^7 organisms per ml. The inocula are prepared from colonies grown from a single cell on agar plates. The cultures are incubated at 37° with shaking in 125-ml Erlenmeyer flasks to which colorimeter tubes are attached by means of $^{19}/_{38}$ ground joints. Side-arms protected by a cotton plug permit access of air. After inversion of the flasks the optical density of the suspension at 650 mμ is determined.

Strains.—Several leucine-requiring strains of *E. coli* were tested for independence of leucine in a leucine-free medium. It was found that the rate of back-mutation was rather high, and growth was frequently

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TABLE I
DEADAPTATION AFTER TRANSFER FROM A TRIFLUOROLEUCINE TO A LEUCINE MEDIUM

	Time After Transfer (min.)	No. of Colonies on Leucine Plates	Relative No. of Colonies on Plates Containing			
			Leucine	Trifluoroleucine	Trifluoroleucine / Leucine = 100	Trifluoroleucine / Leucine = 10
Exp. A	0	96	1	0.87	0.86	0.94
	30	135	1	0.84	0.97	0.93
	60	213	1	0.014	0.45	0.94
	120	360	1	0.00003	0.094	0.80
Exp. B	0	30	1	0.73	0.93	1.1
	15	73	1	0.9	0.77	0.95
	30	67	1	0.5	0.30	1.0
	60	116	1	0	0.03	0.05
	120	260	1	0	0	0.1

TABLE II
DEADAPTATION AFTER TRANSFER FROM TRIFLUOROLEUCINE TO A TRIFLUOROLEUCINE AND LEUCINE (2:1) MEDIUM

Time After Transfer (min.)	No. of Colonies on Leucine Plates	Relative No. of Colonies on Plates Containing			
		Leucine	Trifluoroleucine	Trifluoroleucine / Leucine = 100	Trifluoroleucine / Leucine = 10
0	69	1	1.05	0.84	0.61
30	122	1	1.01	0.96	0.89
75	260	1	0.69	1.10	0.94
105	360	1	0.066	0.58	0.73
140	680	1	0.0001	0.35	0.84

observed after prolonged periods of incubation in the glycerol-salt medium. Strain B 615 F[1] was selected, since no growth occurs on the minimal medium for at least 96 hours of incubation.

Adaptation to trifluoroleucine.—The organisms are inoculated into the growth tube of a chemostat (Novick and Szilard, 1950a) modified by including a mixing flask between the pump and the growth tube. This flask is filled with medium containing equimolar concentrations of leucine and trifluoroleucine. The reservoir is filled with medium containing only trifluoroleucine at a molar concentration equal to the sum of the molar concentrations of trifluoroleucine and leucine in the mixing flask. For every volume of medium pumped into the mixing flask the concentration of leucine in the medium entering the growth tube decreases by a factor of e while the sum of the molar concentrations of trifluoroleucine and leucine remains constant. Fractions are collected from the chemostat at suitable intervals. Each fraction is tested for growth on the proper media.

Deadaptation.—Aliquots of cultures growing exponentially in media containing trifluoroleucine are centrifuged, washed, and inoculated into media containing leucine or trifluoroleucine and leucine (2:1) respectively. The organisms are then tested at suitable time intervals for their ability to grow on agar plates containing either leucine, trifluoroleucine, or a mixture of the two compounds in ratios of 1:10 and 1:100 (Tables I and II). Other aliquots are incubated after transfer to or after dilution with a leucine-containing medium (Fig. 1 and 2). Similarly a culture grown on leucine was incubated after dilution with a trifluoroleucine containing medium (Fig. 1).

Analyses.—Batches of medium containing leucine, trifluoroleucine, or both are inoculated and incubated at 37° for 16 hours. The organisms are harvested by centrifugation for 20 minutes in the cold at 6000 × g and washed twice with glycerol-salt medium and once with demineralized water. After addition of a cold 10% trichloracetic acid solution the precipitate is centrifuged and extracted with ethanol, ethanol-ether, and twice with hot 5% trichloracetic acid solution. The protein content is obtained from the weight of the extracted residue. After refluxing in 6 N HCl for 24–28 hours, the hydrolysate is chromatographed on paper with a solvent containing isopropanol–acetic acid–water (70:20:10) (Roberts *et al.*, 1955). The area containing valine, isoleucine, leucine, and trifluoroleucine is identified by comparison with a standard chromatogram and eluted with a few milliliters of water. The eluate is evaporated to dryness and the residue dissolved in water. This procedure is repeated twice. The final solution is brought to neutrality by the addition of ammonium hydroxide and chromatographed for 20 hours in n-butanol saturated with water. In this solvent valine, leucine, isoleucine, and trifluoroleucine are cleanly separated from each other; the R_F values are 0.47, 0.51, 0.57, and 0.69 respectively. Other aliquots of the bacterial proteins are analyzed in an amino acid analyzer according to a modified Stein and Moore procedure (Moore and Stein, 1954; Hamilton, 1958) (Table III). Nucleic acids are determined in the trichloracetic acid extracts. The orcinol method (Dische, 1955) is used for ribose and the diphenylamine reaction (Dische, 1955) for deoxyribose. Photomicrographs are made of the organisms at a total photographic magnification of some 600-fold. Measurements of width and length are used to determine cell volumes. These data are given in Table IV.

RESULTS

A leucine-less strain of *E. coli B* (B-615-F) grows in

[1] We would like to thank Dr. E. Vollmayer, Department of Bacteriology, Harvard Medical School, for these mutants.

TABLE III
RELATIVE AMINO ACID CONCENTRATIONS OF *E. coli* PROTEINS

Amino Acid	Organisms Grown on Leucine	Organisms Grown on Trifluoro-leucine–Leucine (2:1)	Organisms Grown on Trifluoro-leucine
Glycine	0.91	0.91	0.94
Glutamic	1.1	1.0	0.99
Alanine	1.0	1.0	1.0
Valine	0.015	0.014	0.017
Trifluoro-leucine	0	0.41	0.81
Isoleucine	0.42	0.42	0.55
Leucine	0.70	0.37	0
Lysine	0.67	0.64	0.70
Histidine	0.18	0.13	0.18

TABLE IV
COMPOSITION OF *E. coli*
Values determined in aliquots of cultures removed during exponential growth.

	Grown on Leucine	Grown on Trifluoro-leucine	Ratio
Volume[a]	0.55 μ^3	1.5 μ^3	2.7
Cell weight (mg)	2.8×10^{-13}	8×10^{-13}	2.8
Protein per cell (mg)	2×10^{-13}	6×10^{-13}	3
Total RNA per cell (mg)	2.6×10^{-14}	3.4×10^{-14}	1.3
Total DNA per cell (mg)	1×10^{-14}	1.1×10^{-14}	1.1

[a] Determined from photomicrographs.

the presence of leucine as shown in Figure 3. The final optical density of such cultures, reached after about 20 hours of incubation, is plotted against the leucine concentration. Leucine is the limiting component in the medium below a concentration of some 10^{-3} M. While all experiments are carried out with DL-leucine and DL-trifluoroleucine, experiments with L-leucine indicate that the D isomers are not used. If both leucine and trifluoroleucine are present in the medium the final number of organisms depends on the ratio of the two compounds, providing the sum of their concentrations is growth limiting. The addition of small amounts of trifluoroleucine to a medium containing a limiting concentration of leucine enhances growth; if the amount of trifluoroleucine exceeds that of leucine several fold, inhibition of growth is observed (Fig. 4). The greatest optical density is obtained if the concentration of trifluoroleucine is about twice that of leucine. These experiments suggest that the increased mass of organisms is due to incorporation of trifluoroleucine into the bacterial proteins in addition to leucine, thus increasing the total amount of protein available. The presence of trifluoroleucine in the bacterial proteins is actually demonstrated by an analysis of the fluorine content, and trifluoroleucine itself is identified in the protein hydrolysate by paper chromatography and by quantitative amino acid analysis. The analyses show that the relative proportions of a number of amino acids are not appreciably altered if the leucine auxotroph is grown on a mixture of trifluoroleucine and leucine (2:1) instead of leucine alone (Table III). The sum of the molar quantities of leucine and trifluoroleucine in the former case is nearly equal to the total amount of leucine in the latter. In contrast to the

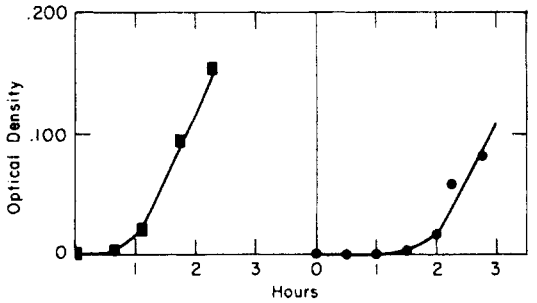

FIG. 1.—Growth of organisms after transfer at zero time from ■ a leucine to a trifluoroleucine and leucine (2:1) medium and from ● a trifluoroleucine to a leucine medium.

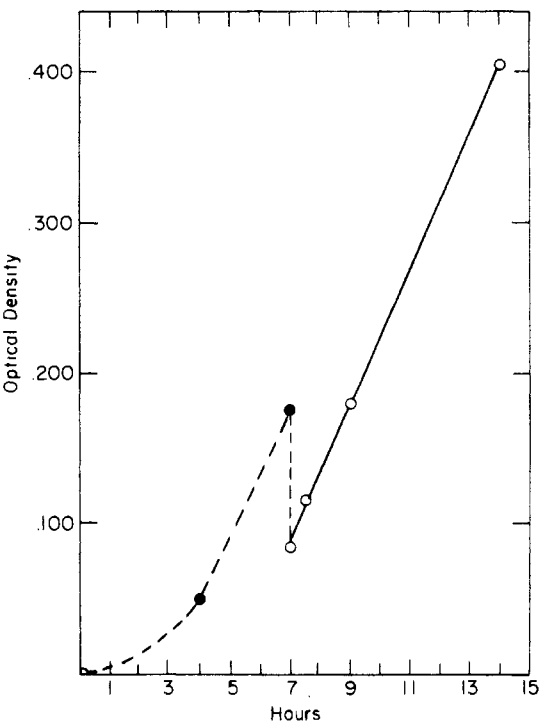

FIG. 2.—Growth of organisms ● on a trifluoroleucine medium and ○ after dilution with 2 volumes of leucine medium at 7 hours.

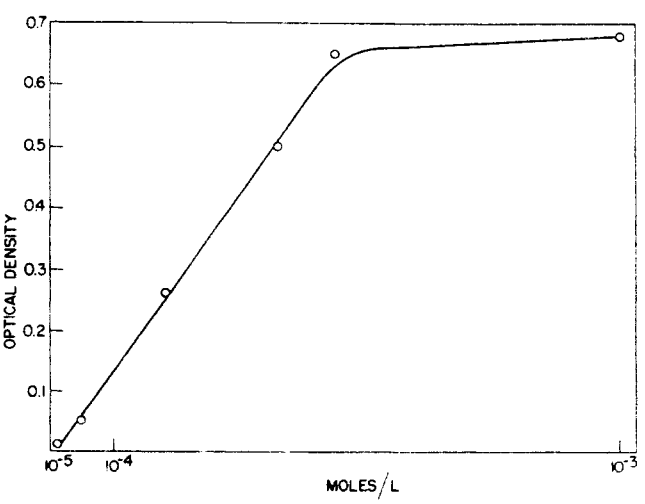

FIG. 3.—Maximal optical density (after 20 hours of incubation) as a function of the leucine concentration.

incorporation of trifluoroleucine into the bacterial proteins of the leucine auxotroph, the wild type of *E. coli B* incorporates less than 0.04% of fluorine if grown at a concentration of 5×10^{-4} M trifluoroleucine in the

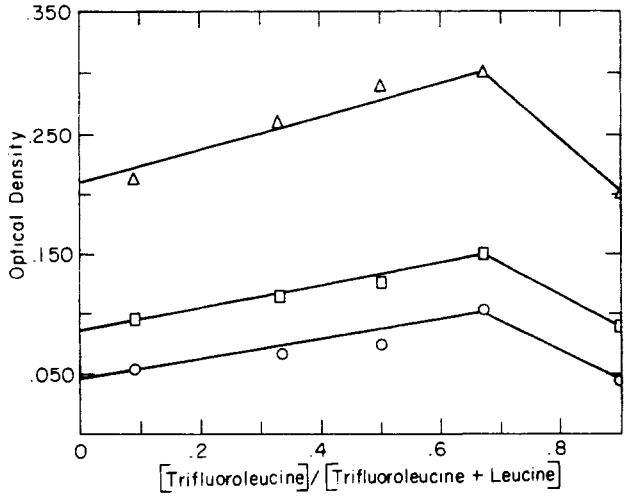

FIG. 4.—Maximal optical density as a function of the trifluoroleucine concentrations of media containing: ○, 5×10^{-5} M leucine; □, 10^{-4} M leucine; and △, 2×10^{-4} M leucine.

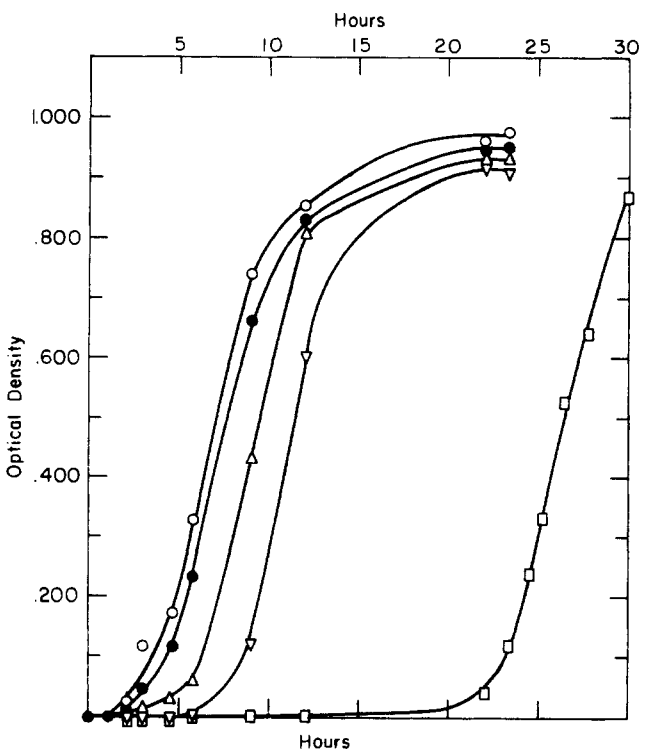

FIG. 5.—Growth of wild-type *E. coli B* on: △, glycerol-salt medium; ●, 5×10^{-4} M leucine; ○, 5×10^{-4} M trifluoroleucine and 5×10^{-4} M leucine; ▽, 5×10^{-4} M trifluoroleucine; □, 5×10^{-3} M trifluoroleucine.

medium. The growth rate of the wild type is little affected by the presence of this amount of trifluoroleucine, either alone or in addition to equal quantities of leucine. At much higher concentrations of trifluoroleucine (5×10^{-3} M and 5×10^{-2} M) prolonged lag periods (20 and 50 hours) are observed (Fig. 5).

After preliminary experiments have shown that good growth is obtained if an organism grown in the presence of a given ratio of trifluoroleucine to leucine is inoculated into a medium with twice this ratio it appears possible to adapt the organism to grow on a medium containing only trifluoroleucine. A chemostat is constructed which permits a continuous increase in the ratio of the concentrations of trifluoroleucine to leucine in the growth tube. Samples of organisms are collected at

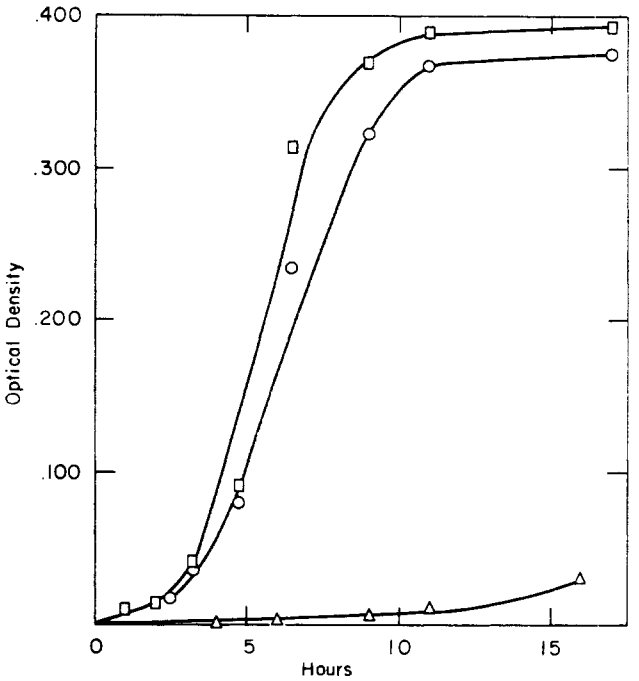

FIG. 6.—Growth of adapted organisms on: △, glycerol-salt medium; □, 5×10^{-4} M trifluoroleucine; ○, 5×10^{-4} M leucine.

intervals and tested for their ability to grow on minimal medium and media containing trifluoroleucine, leucine, or a mixture of trifluoroleucine and leucine corresponding to the composition of the medium in the growth tube at the time the fraction is taken. All samples grow on leucine and on the proper trifluoroleucine and leucine mixture. However, only those organisms grow on trifluoroleucine alone which are taken from the chemostat after the trifluoroleucine-to-leucine ratio exceeds 1000. Such organisms continue to grow on trifluoroleucine after repeated inoculations into new media containing only trifluoroleucine. The growth rate and lag period of these organisms are nearly identical in media containing either leucine or trifluoroleucine (Fig. 6).

If it is assumed that leucine is replaced in the *E. coli* mutant by trifluoroleucine without other changes in the composition of the protein, it can be calculated from the reported leucine content of 7.9% (Luria, 1960) that the trifluoroleucine content of these proteins will be 11.1%. This value corresponds to a fluorine content of 3.4%, which agrees reasonably well with the fluorine analysis of 3.8%.[2]

Amino acid analyses of organisms grown on trifluoroleucine show that the relative proportions of a number of amino acids are quite similar to those of organisms grown on leucine (Table III). Leucine itself is absent from the protein of organisms grown on trifluoroleucine and this protein contains a quantity of trifluoroleucine about equimolar to that of leucine.

Microscopic measurements of organisms adapted to grow on trifluoroleucine show that such organisms are larger by a factor of approximately 3 than normal organisms. A similar ratio is observed for the weight and the protein content (Table IV). In contrast, the DNA and RNA contents of organisms grown on trifluoroleucine or leucine are nearly identical. If the auxotroph is grown on a mixture of trifluoroleucine and leucine (2:1), the same increase in size is observed as for the organisms grown on trifluoroleucine.

[2] Clark Microanalytical Laboratory.

If a culture grown on leucine is transferred to a medium containing trifluoroleucine and leucine in a ratio of 2:1, an increase in the turbidity is observed within the first hour of incubation without a corresponding increase in cell numbers (Fig. 1). Contrariwise, if a culture grown on trifluoroleucine is transferred to a medium containing leucine initially an increase of the number of organisms is observed without a change of turbidity (Fig. 1, Table I). It appears, therefore, that on transfer from a trifluoroleucine to a leucine medium cell division takes place before appreciable protein synthesis sets in, while on transfer from a leucine to a trifluoroleucine medium protein synthesis sets in without concomitant cell division in accordance with the characteristic protein contents observed for the two types of cells. Upon transfer from a trifluoroleucine medium to a medium containing trifluoroleucine and leucine (2:1), in which no change of size of the organisms occurs, cell number and turbidity of the culture increase in a parallel fashion (Fig. 2, Table II). Apparently more protein is required to carry out the necessary metabolic functions in a cell in which trifluoroleucine replaces leucine. The mechanism of this requirement is at present not understood.

It does not appear likely that the incorporation of trifluoroleucine into the proteins of a leucine auxotroph and its adaptation to growth on trifluoroleucine involves a genetic change, since, when the rate of deadaptation of organisms grown on trifluoroleucine is measured after transfer to a medium containing leucine, it is found that in the leucine medium the ability to grow on trifluoroleucine is reduced to one hundredth or less in 1 hour, while in the same period of time the number of organisms increases only 2–3 fold (Table I). After 2 hours no organisms at all grow on agar plates containing trifluoroleucine. The number of deadapted organisms able to grow on media with trifluoroleucine-to-leucine ratios greater than 2 is similarly reduced. On transfer of adapted organisms to a medium containing trifluoroleucine and leucine (2:1) a similar deadaptation is observed (Table II). These results contrast with the finding that the concentration of an inducible enzyme in a growing culture of *E. coli* remains constant after removal of the inducer (Novick, 1958). Additional support for a nongenetic mechanism of adaptation is found in the fact that inoculation of as much as 10^9 unadapted organisms on agar plates containing trifluoroleucine and prolonged incubation does not result in any colonies (Novick and Szilard, 1950b). Furthermore, neither a particularly long lag period nor any change of the growth rate of the adapted organisms upon transfer to a leucine medium can be detected (Fig. 6). The mechanism of adaptation and deadaptation is at present not clear.

Discussion

Most amino acid analogs, such as the fluorophenylalanines, thienylalanine, and azatryptophane, are incorporated to only a small extent into proteins, and exponential growth does not occur in their presence. In contrast, trifluoroleucine can substitute completely for leucine and exponential growth is observed in adapted organisms. It should be pointed out in this context that leucine presumably has no other function in living cells than to serve as a protein constituent, while the analogs of tyrosine, tryptophane, and serine can interfere with a variety of metabolic reactions other than protein synthesis and the inhibition of these reactions may be responsible for the growth-inhibiting effects of these substances. As a case in point the analog selenomethionine (Cohen and Cowie, 1957; Cowie and Cohen, 1957) may be cited. This material, like trifluoroleucine, is incorporated into bacterial proteins, completely replacing methionine, but it is not metabolized. Therefore, in the presence of selenomethionine the microorganisms require a medium containing cysteine for growth.

It follows from the fact that the leucine auxotroph of *E. coli B* can be adapted to grow on trifluoroleucine that all enzymes and other proteins containing trifluoroleucine in place of leucine are functioning in an approximately normal manner. The increased protein content of each cell may be due to a lesser efficiency of trifluoroleucine-containing enzymes. It seems unlikely that trifluoroleucine interferes with the control mechanisms involved in protein synthesis, since the nucleic acid content is largely unchanged and since similar effects are not observed with the wild type, which has normal size if grown in the presence of trifluoroleucine at 5×10^{-4} M and 5×10^{-3} M.

The replacement of one of the methyl groups of leucine by a trifluoromethyl group results in an amino acid which is sufficiently similar to leucine that its incorporation into bacterial proteins in place of leucine proceeds with little difficulty. The similarity is perhaps due to the fact that the trifluoromethyl group has about the same molecular volume as the methyl group (Brockway, 1937). However, the trifluoromethyl group is more electrophilic (Brockway, 1937; Walborsky *et al.*, 1955) than the methyl group, and an additional asymmetric carbon atom at position 4 is present in trifluoroleucine. From a comparison of growth efficiency, it may be concluded that the L isomer of trifluoroleucine is used irrespective of the configuration at carbon atom 4.

If it is assumed that the enzymes which transfer activated amino acids to *t*-RNA are those components of the protein-synthesizing system which are specific for individual amino acids, the replacement of leucine by trifluoroleucine indicates that the specificity of these enzymes is not absolute. A similar lack of specificity is seen with analogs of other amino acids. Although the relative innocuousness of the replacement of leucine by trifluoroleucine is unexpected, the limits of specificity of the protein-synthesizing components of the cell remain to be determined.

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Fractionation and Partial Characterization of the Proteolytic Enzymes of Stem Bromelain*

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By modification of the procedure of Murachi and Neurath five proteolytically active components have been separated from crude stem bromelain by chromatography on Bio-Rex 70 at *p*H 6.10. Zone electrophoresis on Sephadex G-75 also gave five proteolytically active components. These components had similar absorbancy at 280 mμ and similar specific activities on casein at *p*H 7.0 and in clotting milk. They differed in chromatographic properties on Bio-Rex 70, electrophoretic properties on Sephadex G-75 and cellulose acetate, absorbancy at 260 and 292 mμ, stability to heat, inhibition by iodoacetamide, activity on α-benzoyl-L-argininamide, and variation in activity on casein at different *p*H values.

The presence of proteolytic activity in the juice of the pineapple fruit has long been known (Chittenden, 1892), and some of the properties of this activity have been described (Chittenden, 1892; Caldwell, 1905; Willstätter *et al.*, 1926; Maschmann, 1934; Bergmann and Fraenkel-Conrat, 1937; Berger and Asenjo, 1939; Bergmann *et al.*, 1937; Greenberg and Winnick, 1940; Balls *et al.*, 1941). A number of studies have indicated that there are at least two proteolytic enzymes in the juice of the fruit (Chittenden, 1894; Caldwell, 1905; Bergmann *et al.*, 1937). Heinicke and Gortner (1957) reported that the juice of the stem of the pineapple plant also contains proteolytic activity. On the basis of its action on different proteins at several *p*H values they concluded that stem bromelain is a mixture of four proteases. Ota *et al.* (1961) have reported work on the fractionation of bromelain. Murachi and Neurath (1960) separated two proteolytically active fractions from stem bromelain by ion exchange column chromatography on Duolite CS101 and reported some properties of these two fractions.

The purpose of the present study, which was well under way at the time the publication of Murachi and Neurath appeared, was to separate the several proteolytic enzymes of stem bromelain and to characterize these components partially. It is anticipated that the ready availability and commercial usefulness of stem bromelain will stimulate a lot of basic research on this group of enzymes. It will be shown in this report that stem bromelain can be fractionated, by a slight modification of the procedure of Murachi and Neurath, into five components which have proteolytic activity. Some of the properties of these components will be presented.

Materials and Methods

"Bromelain" No. 15 from the Dole Corporation, Honolulu, Hawaii was used.[1] The yellowish-brown powder was readily soluble in water and dilute salt solutions, had a specific activity against casein at *p*H 7.0 of 0.77, and contained 3.20% water and 51.5% protein on a moisture-free basis by the biuret method (Layne, 1957).

Amberlite IRC-50 (XE-64, 200–400 mesh, lot No. 124) was obtained from Rohm and Haas Co., Philadelphia, Pa. Bio-Rex 70 (200–325 mesh, control No. DS 2572 B-1065) was obtained from Bio-Rad Laboratories, Richmond, Calif. Bio-Rex 70 is prepared by grinding, sizing, and purifying standard-grade Duolite CS101 resin. DEAE-cellulose[2] was obtained from Eastman Kodak Company, Rochester, N. Y. CM-cellulose was prepared in this laboratory from Whatman coarse-grade, ashless cellulose (carefully sieved dry to give 100-mesh size) by the method of Peterson and Sober (1956). It had a titratable carboxyl content of 0.41 meq per g. Before use, it was washed with cysteine (1.25×10^{-2} M) to remove all traces of monochloroacetic acid. Sephadex G-75 (lot No. To 6386) was obtained from Pharmacia, Uppsala, Sweden. It was screened to obtain 100–150 mesh size (dry) for use. Purified potato starch (lot No. 91662) was from J. T. Baker Chemical Co., Phillipsburg, N. J. Hydrolyzed starch (lot No. 159) for gel electrophoresis was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

L-Cysteine hydrochloride, "Hammersten-quality" casein, ninhydrin, hydrindantin, *N*-ethyl maleimide, and iodoacetamide were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Versene was from Eastman Organic Chemicals, Rochester, N. Y. α-Benzoyl-L-argininamide hydrochloride monohydrate was obtained from Mann Research Laboratories, Inc., N. Y. Carnation instant nonfat dry milk was used in assaying milk-clotting activity. Deionized water was used throughout.

Proteolytic activity was measured by the method

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[2] The following abbreviations are used: DEAE-cellulose, diethylaminoethyl cellulose; CM-cellulose, carboxymethylcellulose; BAA, α-benzoyl-L-argininamide.