INCORPORATION OF *p*-FLUOROPHENYLALANINE INTO PROTEINS OF *LACTOBACILLUS ARABINOSUS**

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The concept that the phenotypic expression of an organism may be correlated with the identity of its constituent proteins, has been in the literature for decades[1]. Any alteration in the content of any one amino acid in a protein molecule might accordingly be related to a detectable alteration in the organism. A number of attempts to alter constitution of total proteins by control of the diet or medium have been recorded[2-9]. Some of the claims of successful alteration of this kind have been refuted[10-14]. The reported findings fail to meet the stated experimental objective for one or two main reasons: (1) only inadequately precise methods of amino acid assay were available for use and (2) a change in amino acid composition of an entire organism or tissue may be explained simply as a shift in proportion of proteins rather than as an alteration in composition of one or more proteins.

Accordingly, the use of amino acids containing groups not found naturally becomes of interest. To the extent that it might be established that such an unnatural amino acid is incorporated into protein[15], compelling evidence of the alterability of protein synthesis would be at hand. A substance which early received similar study is found in the work of DYER[16]. DYER synthesized the now highly useful methionine antagonist, ethionine, and fed it to rats. She discovered thereby an early antimetabolite. Evidence for incorporation of ethionine into protein has been presented[28]. Parenthetically, it may be noted that the sulfur of ethionine is metabolically available[17].

The suggestion of incorporation of selenocystine into protein has been offered[18]. Selenocystine may however be looked upon as a natural amino acid. The examples given indeed indicate that there is not an *a priori* qualitative distinction between natural amino acids and unnatural amino acids. The deciding factors may be merely the identities of the amino acids in the environment and the opportunity for the organism to adjust to them.

Results most easily interpreted as incorporation of an unnatural or unusual amino acid were reported with *p*-fluorophenylalanine (FPA) in 1951[15]. This appears to

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be the first recorded instance of the incorporation of an unnatural amino acid into the protein of an organism[19]. An earlier, doubtful possibility of the incorporation of D-alanine[20] has been shown to involve an amino acid which is definitely natural in its D form[21]. More conclusive evidence of incorporation of FPA into proteins of *L. arabinosus* was presented in 1954 by way of partial hydrolysis followed by blocking N-terminal and free amino acids with phenylisothiocyanate and total hydrolysis of the peptides remaining[22]. These experiments and subsequent experiments are described in detail in this paper. MUNIER AND COHN[23] have recently reported the incorporation of *p*-fluorophenylalanine, and of thienylalanine, into the proteins of *Escherichia coli*.

Besides the unlikelihood of its natural occurrence, *p*-fluorophenylalanine was selected for the present study because of the small halogen atom and the consequent similarity of FPA to the aromatic amino acids phenylalanine and tyrosine, because of the unlikelihood of transfer of aryl fluoride to another amino acid[24], and because of the presumed feasibility of selectively following fluorine by elemental analysis, in contrast to determining altered ratios of natural amino acids in proteins. In practice the high R_F of FPA in the solvents employed proved to be more useful in tracing it; in a developing solvent of *tert.*-butyl alcohol–methyl ethyl ketone–water (4:4:1.5), FPA ran ahead of all amino acids in protein hydrolyzates.

METHODS

Organisms

Lactobacillus arabinosus (ATCC 8014) was maintained as previously described, as were phe$^+$ tyr$^+$, tyr$^+$, and the FPA-tolerant strain[15]. *L. arabinosus* Wright was able to synthesize its tryptophan; it was obtained from Dr. L. D. WRIGHT[25]. It did not lose its ability to synthesize tryptophan when the stock culture was transferred.

Culture of organisms

The basal medium of KUIKEN *et al.*[26] was used with the proper amounts of PA and FPA added. For plate assays, washed agar was added (1$^3/_4$%). The medium was autoclaved for 15–20 min at 15 lb. pressure and incubated at 36–37°. Growth was measured in 10 ml cultures in optically matched Pyrex tubes (11 × 150 mm) with a Model 11 Coleman Universal spectrophotometer, at 575 mμ.

Preparation of cytolyzate

When growth had ceased, the cells were collected by centrifugation in a Sharples continuous centrifuge. The cells were suspended in physiological saline overnight. After centrifuging, the cells were resuspended in saline and centrifuged again. The cells were cytolyzed by grinding with sand and ether; the ether was decanted and extracted several times with water. The aqueous extracts were combined with the cytolyzate.

Blocking of free amino acids

Nitrogen was determined in the cytolyzate by micro-Kjeldahl, and phenylisothiocyanate (PTC) was added[27] on the basis that one-half of the nitrogen was present as free amino groups with an additional nineteen-fold excess. An equal volume of pyridine was added to an aliquot of the cytolyzate and the mixture was made alkaline to bromothymol blue with NaOH. The PTC was added and the reaction kept alkaline by the periodic addition of NaOH while the reaction was incubated at 37°. When the consumption of alkali ceased, the excess pyridine and PTC were removed by extraction with five volumes of benzene. The remaining mixture was hydrolyzed with an equal volume of conc. HCl.

Paper chromatography

Paper chromatograms were run on Whatman No. 1 filter paper in sheets of 7$^1/_2$ × 9$^1/_4$ inches in chambers which were 5-lb. chemical-reagent bottles with screw caps. A cylinder was made of the paper by stapling the two opposite edges together without the edges touching. For optimum

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separation of FPA and PA the solvent of *tert.*-butyl alcohol–methyl ethyl ketone–water (4:4:1.5 by volume) was run to the top of the paper 3 times with the cylinder removed from the chamber and dried between each ascent to the top. For quantitative results the paper chromatogram was dipped into 0.2% ninhydrin in acetone and allowed to dry in air at room temperature. Estimation was made by visual comparison of color intensities of unknown samples with standards.

Inhibition assay

The zone of inhibition caused by FPA on *L. arabinosus* phe$^+$ tyr$^+$ was compared with the area of inhibition of the unknown samples. The FPA was added to the agar plate by placing the filter paper disk cut from a chromatogram onto the previously inoculated and solidified medium. Phenylalanine and tyrosine were omitted from the medium for this organism. The paper disk containing the FPA was cut on the basis of the location of FPA on a companion chromatogram which had been treated with ninhydrin. The area of inhibition was related to the amount of L-FPA*.

RESULTS

The R_F values of phenylalanine, *p*-chlorophenylalanine, and FPA are given in Table I. It is seen that each of the halophenylalanines travels ahead of phenylalanine, which is the fastest of the amino acids from protein hydrolyzates, in this solvent mixture. Of the two unnatural amino acids, the chloro analog exhibits the higher R_F.

In Fig. 1 are presented the results of a standard assay of FPA by inhibition analysis. A fresh standard was prepared for each estimation. In each case, the plot tended to be linear in the range in which values had meaning in the experiments.

TABLE I

R_F VALUES OF PHENYLALANINE, *p*-CHLOROPHENYLALANINE, AND *p*-FLUOROPHENYLALANINE IN *tert.*-BUTYL ALCOHOL–METHYL ETHYL KETONE–WATER (4:4:1.5)

Amino acid	R_F
Phenylalanine	0.60
p-Chlorophenylalanine	0.84
p-Fluorophenylalanine	0.76

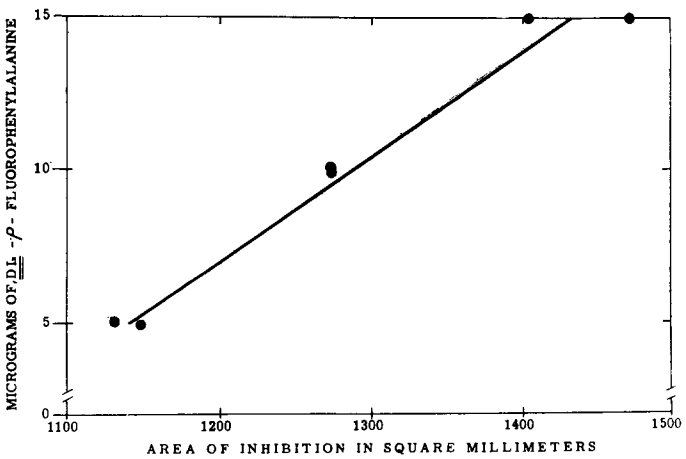

Fig. 1. Plot of area of inhibition in disk assay *vs.* concentration of DL-*p*-fluorophenylalanine.

* Other experiments with known amounts of free FPA mixed with a portion of the cytolyzate of organisms grown in the absence of FPA showed that 85–90% of the free FPA could be blocked by this method.

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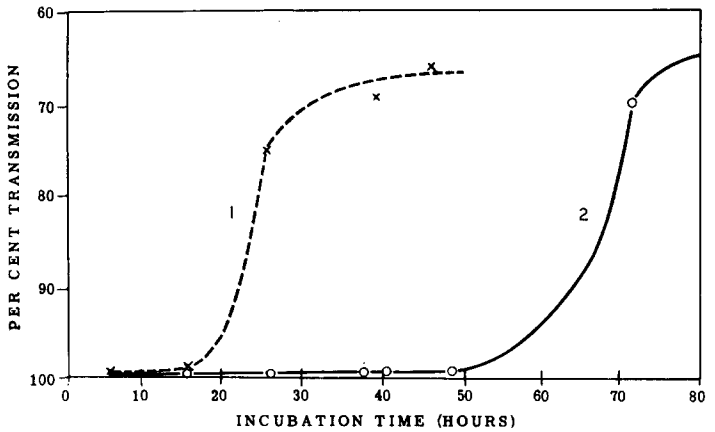

Fig. 2. *L. arabinosus* grown in medium containing a 10:1 ratio of *p*-fluorophenylalanine to phenylalanine. Typical time-growth relationship of *L. arabinosus* on culturing (solid curve) and subculturing (dotted curve) in the presence of *p*-fluorophenylalanine. 0.08 μmoles phenylalanine per tube, 0.8 μmoles *p*-fluorophenylalanine per tube.

In Fig. 2 are presented the results of subculturing parental strain in the presence of an inhibitory proportion of FPA (10 FPA:1 PA on a DL molar basis). It is clear that the parental strain grows only after an extended lag period. Once the surviving adjusted cells are reinoculated into medium containing FPA at the parentally inhibitory level, initiation of growth is as rapid as in the controls. This evidence substantiates the conclusion, based on the phenomenon of haphazard appearance of FPA tolerance[15], that the tolerant strain is a naturally selected mutant. These results are for FPA-tolerance. The phenomenon of stimulation by subinhibitory ratios of FPA has exhibited no time lag in any of the numerous trials. No evidence is at hand that stimulation of the parental strain involves mutation, nor is there any evidence for the puzzling interpretation by others that the organism develops a requirement for the analog in these experiments[28]. On the contrary, the early evidence was interpretable as direct utilization of FPA by the parental strain[15].

Table II reveals trends which have been observed also in a number of other experiments. The FPA-tolerant strain is virtually unaffected by growth in subinhibitory concentrations of FPA. The parental strain however demonstrates stimulation. The difference between these two was larger in other trials. The other three strains of *L. arabinosus* suffered marked inhibition at this level. The sensitivity to inhibition by FPA by three of the strains also explains why one of these, phe$^+$ tyr$^+$, was selected for use in inhibition assays.

Table III represents typical stimulation due to FPA at limiting concentrations of PA. This type of result was obtained repeatedly.

Tables III and IV present the results of large scale stimulation experiments. In a typical one of these, 30 l of medium containing 39.6 mg DL-PA (phenylalanine) and 98.6 mg DL-FPA, was harvested after 48 h of incubation of *L. arabinosus* at 37°. The cells were cytolyzed; to the final volume of 300 ml an equal volume of pyridine and 50 g of PTC was added. The reaction was considered to be complete when consumption of alkali had stopped; the solvent was then removed by distillation under reduced pressure. The residue was resuspended in 100 ml of distilled water which

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TABLE II

GROWTH RESPONSE OF VARIOUS STRAINS OF *Lactobacillus arabinosus* TO *p*-FLUOROPHENYLALANINE*

Strain	*FPA/PA molar ratio*	*Incubation time (hours)*						
		7	*16*	*24*	*37*	*40*	*48*	*55*
Parental	0	91	62	59	56	—	—	—
	2.5	92	69	53	49	44	44	—
Tyr+	0	93	57	47	40	34	31	—
	2.5	—	—	95	94	83	71	65
Phe+ tyr+	0	92	42	21	18	16	—	—
	2.5	—	—	91	88	72	61	54
Wright	0	92	80	63	61	—	—	—
	2.5	93	86	81	80	81	80	77
FPA-Tolerant	0	98	86	66	60	60	—	—
	2.5	97	83	63	58	56	—	—

PA = phenylalanine, FPA = fluorophenylalanine

* Values are given in per cent transmission, and are averages of quadruplicate 10-ml cultures; DL-PA present is at level of 0.08 μmole per tube.

TABLE III

GROWTH RESPONSE OF *L. arabinosus* TO FPA UNDER LIMITING CONDITIONS

*Micromoles of DL-FPA/tube**	*Incubation time in hours*		
	24	*31*	*48*
0	56	54	53
0.20	50	46	45

PA = phenylalanine, FPA = fluorophenylalanine

* Each tube contains 0.08 μmole of DL-PA. Values are per cent transmission; averages of duplicate 10-ml cultures.

TABLE IV

BIOLOGICAL ASSAY FOR TOTAL AND AMINO-BOUND FPA IN ALIQUOTS OF *L. arabinosus* CYTOLYZATE SUBJECTED TO PARTIAL HYDROLYSIS, PTC TREATMENT, AND TOTAL HYDROLYSIS

Culturist	*Assayist*	*Total FPA/20 μl*	*Amino-bound FPA/20 μl*
RSB	RSB	10 μg*	8 μg*
RSB	JEJ	15 (16.2, 15.5, 14.5)	10 (10.6, 10.6, 10.6, 9.8)
MM	JEJ	15 (15.1, 15.6)	11 (12.0, 11.2, 10.6)

* By paper-chromatographic methods, 12 μg FPA/20 μl were found for the total FPA and 6 μg FPA/20 μl for the amino-bound FPA. FPA = *p*-fluorophenylalanine, PTC = phenylisothiocyanate.

was then removed under reduced pressure. The residue was hydrolyzed by suspending in 200 ml of distilled water and 200 ml of conc. HCl and refluxing for 24 h. After the acid was removed under reduced pressure, the residue was again suspended in distilled water and taken to dryness again. The residue was extracted with 250 ml of diethyl ether and 200 ml of distilled water. The ether layer was separated and washed with two 50-ml portions of distilled water. The ether washings were added

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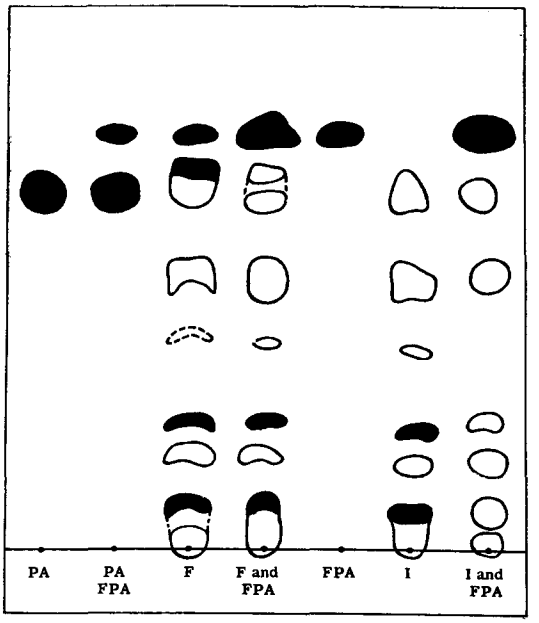

Fig. 3. Chromatographic analysis of hydrolyzate of *L. arabinosus*. Left to right. Phenylalanine; Phenylalanine and *p*-fluorophenylalanine; Sample F, (see text); Sample F and *p*-fluorophenylalanine; *p*-fluorophenylalanine; Sample I (see text); Sample I and added *p*-fluorophenylalanine. The dotted lines, the solid lines, and the filled in areas represent the increasing intensity of ninhydrin reacting areas.

to the residue remaining in the aqueous layer of the extraction procedure. Two and one-half g of activated Norite A[29] was added to the aqueous solution and the mixture was shaken mechanically for 4 h. The filtered charcoal was eluted by stirring with 250 ml of 20% aqueous acid containing 5% phenol. After the charcoal was filtered, the filtrate was extracted with 300 ml of diethyl ether to remove the excess phenol and the ether extract was washed with 100 ml of distilled water which was combined with the filtrate and taken to dryness under reduced pressure. The residue was suspended in 50 ml of distilled water, centrifuged, the supernatant was poured off, and the residue was resuspended in distilled water and centrifuged again. The supernatants were combined and concentrated under reduced pressure. The precipitate which formed was centrifuged, washed with a small quantity of hot distilled water, the supernatant was combined with the concentrated solution and the pH was adjusted to 6 with 0.2 *N* NaOH. On the chromatograms in Figs. 3 and 4 this is sample F.

Two controls were employed to determine the efficiency of the PTC treatment for the removal of free FPA and to make certain the FPA could be detected if present in the protein hydrolyzate. For these controls, *L. arabinosus* cells grown on a complete synthetic medium without FPA were used and the procedure was the same as used in the previous experiment. In control G, 8 mg of DL-FPA was added to the cytolyzate before the treatment with PTC in order to see if the treatment blocked the free amino acids. In control sample H, 8 mg of DL-FPA was added after PTC treatment in order to ascertain if FPA, which was known to be present, could be detected. Control I did not contain any FPA.

The results are shown in the chromatograms in Figs. 3 and 4. In Fig. 3, the chromatogram shows the following: (i) PA and FPA are readily separated, (ii) sample F contains a ninhydrin spot which has the same R_F as FPA and is intensified

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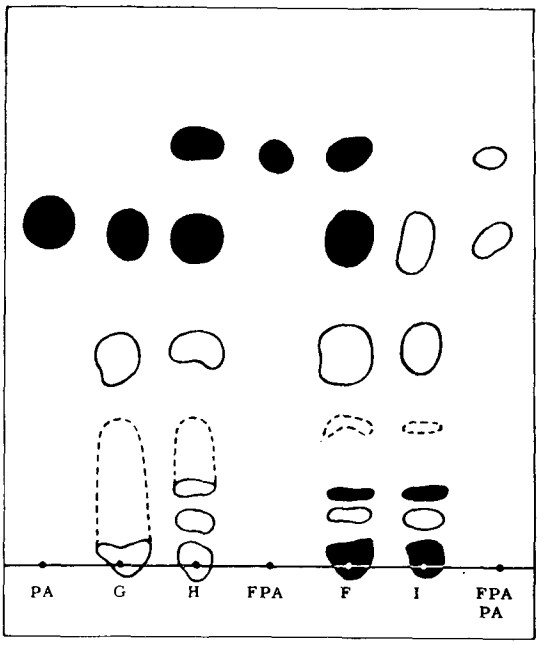

Fig. 4. Chromatographic separation of hydrolyzates of samples F, G, H, and I. Left to right. Phenylalanine; Sample G (see text); Sample H (see text); *p*-Fluorophenylalanine; Sample F (see text); Sample I (see text); *p*-Fluorophenylalanine. The dotted lines, the solid lines and the enclosed areas represent areas of increasing intensities of ninhydrin reaction.

when FPA is added to sample F. A similar ninhydrin spot does not result in control I unless FPA is added to it. In Fig. 4, the chromatogram shows the following: (i) control sample G does not show a ninhydrin spot for FPA indicating that PTC treatment removed the free FPA added to the extent that it can be determined by qualitative paper-chromatographic methods, (ii) control sample H shows the presence of FPA, indicating that the acid hydrolysis and subsequent procedures do not cause an appreciable loss of FPA, (iii) sample F shows the presence of FPA and control sample I does not show FPA.

In order to assess the quantitative extent of the blocking of free FPA by PTC 2.21 mg of DL-FPA were treated with PTC and hydrolyzed according to the procedures outlined above. After removal of the acid, the residue was suspended in distilled water and taken to dryness. The residue was suspended in 0.3 ml of distilled water and brought to pH 5 with 0.4 ml of 0.05 *N* NaOH and assayed. After chromatographing the material with standard quantities of FPA, it was estimated that 10% of the FPA was not blocked by PTC treatment. A second experiment was set up like control sample G except that 30 mg of DL-FPA was added to a cytolyzate preparation from *L. arabinosus* grown in a complete medium without FPA. This was treated with PTC, hydrolyzed, and extracted with ether as before and the mixture diluted to 50 ml and used for assay. Charcoal absorption and elution was omitted. Chromatograms failed to resolve the amino acids sufficiently for a quantitative comparison of ninhydrin colors with FPA standards; accordingly, bioassay was used. This assay indicated that 16% of the free FPA was not blocked by PTC treatment.

IDENTIFICATION OF AMINO-BOUND FPA IN PEPTIDES FROM *L. arabinosus*

The experiments on the blocking of free FPA indicate that the blocking was 84–90%

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complete. To determine if the amount of FPA found in the cytolyzate of cells grown under stimulating conditions was more or less than could be attributed to incomplete blocking of free FPA, quantitative estimations of the total FPA and the amino-bound FPA were made on peptides obtained by the partial hydrolysis of the cytolyzate. The partial hydrolysis presumably also served to liberate free amino acids trapped in proteins, but not in peptide linkage. After incubation in 30 l of medium containing 39.6 mg DL-PA, and 98.9 mg DL-FPA for 48 h the *L. arabinosus* cells were harvested. Growth was followed in 10-ml cultures containing the same medium and the same medium without FPA. The cells were cytolyzed as described above and to the 250-ml volume, 250 ml of conc. HCl was added and the mixture was allowed to stand for 3 days at 37°. The acid was removed by distillation under reduced pressure. The residue was suspended in 100 ml of distilled water and then taken to dryness again. Resuspension of the residue in distilled water and concentration of the solution was followed by transferring the mixture to a test tube and adjusting to pH 6. After centrifuging, the supernatant and the four 5-ml washings of the precipitate were placed in 50-ml volumetric flasks and diluted to volume. This was a mixture of peptides from the partial hydrolysis of cytolyzate.

A 0.3-ml sample was completely hydrolyzed by adding 0.3 ml of conc. HCl and hydrolysis was carried out in an oven at 100° for 15 h. The excess acid was removed by evaporating to dryness in a vacuum desiccator over NaOH pellets. When dry, distilled water was added and the sample was taken to dryness again. Dilute NH_4OH was added to neutralize the remaining acid and the mixture was again dried. The

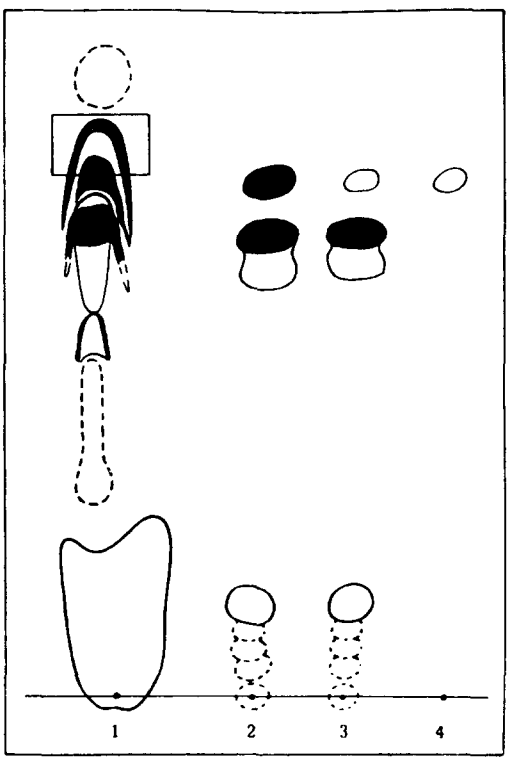

Fig. 5. Chromatographic separation of hydrolyzates of *L. arabinosus* cytolyzates. Left to right. Sample 1:20 µl sample of protein hydrolyzate of organisms grown in the presence of FPA partially hydrolyzed and treated with phenylisothiocyanate prior to acidic total hydrolysis. The rectangular area shows the area, from 3 companion chromatograms, cut for biological assay and rechromatographing, and the area in which most of the FPA is located. Sample 2: Mixed chromatogram of area eluted from sample 1 plus 10 µg of FPA. Sample 3: Chromatogram of area eluted from sample 1. Sample 4: 3 µg of FPA. The dotted outlines, the solid outlines and the filled- in areas represent increasing intensities of ninhydrin reaction.

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residue was suspended in 0.3 ml of distilled water and the FPA was determined by paper chromatography and biological assay. Table IV shows the results.

To another 0.3-ml sample, 0.3 ml of pyridine containing 43 mg of PTC was added. The reaction was carried out as described before and after removal of the excess pyridine and PTC with benzene, the remaining mixture was now totally hydrolyzed with an equal volume of conc. HCl in an oven at 100° for 15 h. Removal and neutralization of the acid was performed as above and the FPA was determined by paper chromatography and biological assay. Fig. 5 and Table IV give the results.

The results shown in Table IV and Fig. 5 agree qualitatively with the results in Fig. 1, *i.e.*, that FPA appears in the proteins of *L. arabinosus* under these growth conditions. In addition, the quantitative data in Table IV show that amino-bound FPA fraction (60–80 % of total FPA) is greater than would be expected (10–16 %) from the incomplete blocking of free FPA. Included in Table IV are the results of another experiment similar to the one just described.

DISCUSSION

The data presented indicate the incorporation of *p*-fluorophenylalanine into proteins of *Lactobacillus arabinosus*. Such a result is consistent with the fact that proteolytic enzymes which are not required to deal with such residues in nature, can nevertheless catalyze reactions involving them[30]. The spatial allowances of an organized polymolecular system such as a cell might be expected to be less, however, than is true for an isolated enzyme acting on a single substrate in a laboratory experiment. The experiments and analyses demonstrate that this much deviation from normal synthesis of protein is easily possible.

No alterations in protein concomitant with phenotypic variation were observed, inasmuch as no phenotypic variation was demonstrated. The conclusion that protein synthesis varies demonstrably was also reached from studies in the normal variation of protein and a designation of these as a Darwinian continuity of differing structures[31] *cf.* [32]. The process described here differs in being accomplished artificially with an unnatural amino acid. Although the adjustment to tolerance of *p*-fluorophenylalanine involves a genotypic change, the same cannot be said for the stimulatory response. In the present state of knowledge, it would seem most likely that development of FPA-tolerance involves a change in the genetic apparatus but that FPA-stimulation represents merely an alteration of protein synthesis within its normal limits without genic alteration. The evidence for the latter is principally the absence of a lag period in the enhancement of growth. Such interpretation is consistent with the belief that genic alteration requires a change in nature of the nucleic acid portion of an organism[33].

Early recognition that protein synthesis is subject to variation can be attributed to MACALLUM[1], who in 1926 said, " . . . proteins, composed as they are each of a variable number of amino acids and in variable proportions of these, cannot be predicated as having a uniform composition even absolutely in similar cells in the same organism, for thus mutation would never obtain in a species". MACALLUM's insight leaves open the possibility that variation in proteins is more of a cause than an effect, a point of view which would find little support today, but his reasoning as it applies to the possibility of variation in protein structure is now supported

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by experiments and analysis. In the light of the data presented, one can visualize that some latitude in protein synthesis is possible without mutation, whereas other variations require mutation.

SUMMARY

For the earlier suggested incorporation of *p*-fluorophenylalanine into the proteins of *Lactobacillus arabinosus* further evidence has been obtained. The cytolyzate of cellular products of culture in the presence of the unnatural amino acid have been partially hydrolyzed, treated with phenylisothiocyanate and totally hydrolyzed. The amino acid has been found and assessed in the hydrolytic product. The significance of these results has been discussed.

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