tions using polyamide sheets through step 35 during a degradation on 40 nmol of a 250 residue fragment from rabbit heavy chain 3381 (Table II) (M. Rosemblatt et al., 1975, manuscript in preparation). Selected residues may be detected in long runs depending on their background level. For example, a lysine at step 41 from degradation of 90 nmol of a citraconylated tryptic peptide from rabbit light chain, 150 residues in length, could easily be identified on polyamide sheets (M. N. Margolies, unpublished data).

Obtaining lengthy sequences on small amounts of material is an obvious advantage in the structural studies of proteins. These results reported here have permitted the regular application of the 0.1 *M* Quadrol program to sequence analyses employing 10–30% of the material previously required.

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Incorporation of Fluorotryptophans into Proteins of *Escherichia coli*†

E. A. Pratt and Chien Ho*

ABSTRACT: A tryptophan-requiring strain of *Escherichia coli* can go through two doublings of optical density after L-tryptophan is replaced in the medium by 4-fluorotryptophan, during which the fluoro analog displaces ~75% of the L-tryptophan in cell protein. One doubling occurs in the presence of 5- or 6-fluorotryptophan, with 50–60% replacement of L-tryptophan by analog. When β-galactosidase is induced at the time of addition of analog, it reaches 60% of the control specific activity in the presence of 4-fluorotryptophan, 10% of normal in the presence of 5- or 6-fluorotryptophan. Lactose permease activity is 35% of the control in the presence of 4- and 6-fluorotryptophan, less than 10% in the presence of 5-fluorotryptophan. D-Lactate dehydrogenase shows a specific activity twice that of the control in the presence of 4-fluorotryptophan, one-half with 5- or 6-fluorotryptophan. Thus fluorotryptophan can be incorporated into proteins and affect their activities, although the nature and magnitude of the effect cannot be predicted for any given enzyme. Such substituted proteins should be useful for the study of protein structure and function by ^{19}F nuclear magnetic resonance and other techniques.

In order to understand structure–function relationships in proteins, one needs to correlate the dynamics of protein–ligand interactions and subunit–subunit interactions at the atomic level with the structural information provided by X-ray diffraction studies. An ideal approach to investigate such problems is to introduce some kind of "probe" selectively into the system which will not significantly perturb the host and which can provide spectroscopic signals that are comparatively free from interference. A number of spectroscopic probes (such as the attachment of spin-labels and/or ^{13}C or ^{19}F nuclei and/or fluorescent dyes to selected amino acid residues on the protein and/or the ligands) have been used to gain insight into this important problem (for recent reviews, see Yguerabide, 1972; Dwek, 1973). Ideally, such studies should provide detailed and specific information about the conformational transitions and the molecular motion of the active sites, regulatory sites, etc., as well as about quaternary transitions in some cases. This type of study has contributed significantly to our understanding of the molecular mechanism of a number of enzyme systems. However, it requires the attachment of a spectroscopic

† From the Department of Biophysics and Microbiology and the Department of Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received February 13, 1975.* Supported by research grants from the National Institutes of Health (GM-18698) and the National Science Foundation (BMS 72-02534). This material was presented in part at the 1974 Biochemistry/Biophysics Meeting, June 2–7, 1974, Minneapolis, Minn.

probe to either the protein or the ligand molecule. Hence, it has serious limitations, especially when applied to those studies which require the covalent attachment of a probe to certain groups in a protein molecule. With the chemical modifying reagents presently available, only a few types of amino acid residues can be readily modified (Means and Feeney, 1971). In addition, not all of these groups are accessible to these reagents under nondenaturing conditions.

One alternative approach which retains the advantages of the spectroscopic probe technique and also overcomes the above mentioned limitations is to prepare a protein molecule specifically labeled with another nucleus. One may term this approach nucleus spin-labeling. This can be achieved by biosynthetically incorporating isotopically labeled amino acids into proteins. A number of isotopes (such as ^{2}H, ^{13}C, and ^{19}F) can be and have been used for nucleus spin-labeling studies in proteins (Katz et al., 1968; Markley et al., 1968; Putter et al., 1969; Browne et al., 1973; Sykes et al., 1974; Hull and Sykes, 1974). Sykes and coworkers have pointed out the advantages of ^{19}F as a label in studying fluorotyrosine alkaline phosphatase (EC 3.1.3.1) of *Escherichia coli*.

A number of attempts have been made to incorporate fluorinated amino acid analogs into bacterial proteins (Rennert and Anker, 1963; Gottlieb et al., 1965; Munier et al., 1967; Browne et al., 1970; Sykes et al., 1974). Although such analogs are generally highly toxic to the cell, limited growth and incorporation of analogs into proteins can take place in their presence. It is then possible to determine the effects of such incorporation on individual enzymes and to isolate them for nuclear magnetic resonance (NMR) studies. Induction of inducible enzymes at the time of addition of analog offers a particularly promising approach for incorporating large amounts of analog into a specific protein. Browne et al. (1970) found that they could induce β-galactosidase (EC 3.2.1.23) in *E. coli* in the presence of fluorotryptophans and Sykes et al. (1974) have prepared *m*-fluorotyrosine-labeled alkaline phosphatase from *E. coli*.

In this communication, we wish to report our work on induction of the lactose operon in the presence of 4-, 5-, and 6-fluorotryptophans, and also on the effect of analog incorporation on a membrane-bound enzyme, D-lactate dehydrogenase (EC 1.1.2.4) in *E. coli*.

Experimental Procedure

Purified *E. coli* β-galactosidase (grade IV), *o*-nitrophenol β-D-galactoside (ONPG),[1] isopropyl thio-β-D-galactoside (IPTG), thiomethyl β-D-galactoside (TMG), dichlorophenolindophenol, D-lactate (lithium salt), and 4-, 5-, and 6-fluorotryptophans were obtained from Sigma; [^{14}C]TMG was from New England Nuclear. Other chemicals were reagent grade from commercial sources.

Growth of Bacteria. *E. coli* W3110 *trp* A33 (Drapeau et al., 1968), obtained from Dr. C. Yanofsky, was used for all experiments. Cells were grown at 37° with aeration by shaking in M-9 medium (Anderson, 1946) with 0.4% succinate, supplemented with amino acids, purines and pyrimidines, and vitamins (Stanier et al., 1970). Turbidity was measured with a Fisher electrophotometer at 525 nm. A reading of 8 corresponds to 1×10^8 bacteria/ml. Protein concentration was measured by the method of Lowry et al. (1951).

Preparation of Sonicated Extracts. For sonication, the special microtip of the sonifier cell disrupter of the Heat Systems Company was used at its power limit; 2-ml samples of cell culture, chilled in an ice-water bath, were sonicated in 1-min bursts, with at least 1 min between each burst for cooling. Sonication was continued for 1–3 min depending on the density of the cell culture.

Induction and Assay of β-Galactosidase. The enzymes of the lactose operon were induced by addition of 1 m*M* IPTG. β-Galactosidase was measured by release of *o*-nitrophenol from ONPG by sonicated cell extracts at 25°. The assay buffer contained 0.1 *M* sodium phosphate buffer (pH 7), 1 m*M* $MgSO_4$, 0.2 m*M* $MnSO_4$, and 0.1 *M* β-mercaptoethanol (Revel et al., 1961). Enzyme was diluted as necessary to a final volume of 1 ml in this buffer; 0.2 ml of the buffer containing 0.4% ONPG was added to start the reaction. The reaction was stopped after development of an appropriate yellow color by the addition of 0.5 ml of 1 *M* Na_2CO_3 and the color measured with a Bausch and Lomb Spectronic 20 at 420 nm.

Assay of Lactose Permease. Cell samples were centrifuged and resuspended in 1 ml of supplemented M-9 medium lacking tryptophan and containing 0.02 mg of chloramphenicol. [^{14}C]TMG was added, and the mixture was incubated 15 min at 25°, filtered through a Millipore HA filter (25 mm in diameter, 0.45 μ in pore size), and washed three times with 3 ml of supplemented M-9 medium at 25°. The filter was dried, placed in a vial with Bray's solution, and counted in a Packard liquid scintillation counter.

Preparation of Anti-β-galactosidase Antiserum. Purified *E. coli* β-galactosidase (Sigma) was freed of $(NH_4)_2SO_4$ by passage through Sephadex G-25; 2 mg of β-galactosidase in Freund's adjuvant was injected subcutaneously into the back of a young white rabbit, followed 2 weeks later by a second injection. After another 2 weeks a blood sample was collected from an ear vein; the serum was obtained and stored frozen.

Precipitin Reaction between β-Galactosidase and Rabbit Anti-β-galactosidase. Sonicated extracts of β-galactosidase-containing bacteria were mixed with antibody in the presence of the assay buffer described above. The amount of β-galactosidase was kept constant, the amount of antiserum varied. The largest amount of antiserum added was sufficient to precipitate all enzyme activity. After 60-min incubation at 37°, an aliquot was taken for assay. The remainder was centrifuged for 30 min at 24,000*g* in a Sorvall RC2 refrigerated centrifuge and the supernatant assayed for β-galactosidase activity. The amount of antiserum needed to precipitate 50% of the β-galactosidase activity was determined from graphs of the results. From this value the units of normal or analog-containing β-galactosidase precipitated per ml of antiserum could be calculated.

Amino Acid Analysis. Samples of a bacterial culture were centrifuged; the cells were washed with 0.1 *M* sodium phosphate buffer (pH 7) and extracted twice with methanol–chloroform–water (2:1:0.8). The remaining pellet was resuspended in water, dialyzed overnight against water, and lyophilized. This material was subjected to alkaline hydrolysis and analyzed in a Beckman amino acid analyzer (Model 118), using the methods of Hugli and Moore (1972). Fluorotryptophans were readily separated from tryptophan under these conditions. Concentrations of tryptophan and analog in each sample were calculated from the

[1] Abbreviations used are: ONPG, *o*-nitrophenol β-D-galactoside; IPTG, isopropyl thio-β-D-galactoside; TMG, thiomethyl β-D-galactoside; 4F-Trp, 4-fluorotryptophan; 5F-Trp, 5-fluorotryptophan; 6F-Trp, 6-fluorotryptophan.

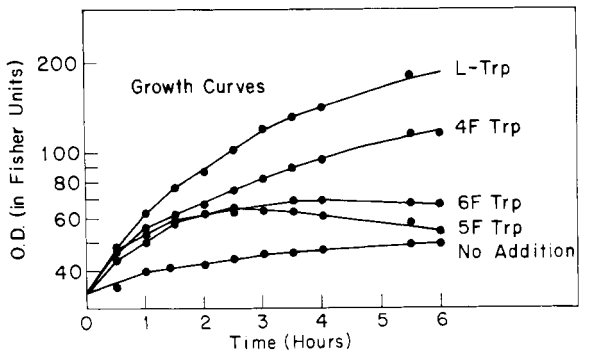

FIGURE 1: Growth of *E. coli* W3110 *trp* A33 after resuspension in medium lacking tryptophan, followed by additions as shown.

Table I: Percent of Tryptophan Replaced by Indicated Fluorine Analog.

	Hours after Addition			
Analog	2	3	4	6
4F-Trp	64	73	73	72
5F-Trp		51		
6F-Trp		59		

areas under the curves, after calibration with known amounts of each. The amount of analog could then be expressed as a percent of the total amount of tryptophan plus analog found in the sample.

Assay of D-*Lactate Dehydrogenase.* A 1-ml assay mixture contained 0.1 *M* potassium phosphate (pH 7.8), 10 m*M* D-lactate, and 20 μg/ml of 2,6-dichlorophenolindophenol (Futai, 1973; Kohn and Kaback, 1973). The reaction was started by addition of an appropriate dilution of sonicated cell extract. The decrease in absorbance at 600 nm was followed for 3 min in a Zeiss PMQ II spectrophotometer.

Results

Effect of Addition of Analog on Growth. For all experiments, exponentially growing cultures of *E. coli* W3110 *trp* A33 were centrifuged and resuspended, in medium lacking tryptophan, to a cell density of 2–3 × 10^8/ml. The culture was then divided, and the desired additions were made. In all experiments shown here, tryptophan and analogs were added to a concentration of 0.1 m*M*. Either more or less analog added results in less increase in optical density. Figure 1 shows the effect on growth as measured by increase in optical density (OD at 525 nm). If there is no addition of tryptophan, there is little growth of this tryptophan-requiring strain. If tryptophan is added, there is normal growth. When 4-fluorotryptophan is added, there are about two doublings of OD, with 5- or 6-fluorotryptophan about one doubling. Table I gives the results of amino acid analyses: 73% of the tryptophan in cell protein is replaced by 4-fluorotryptophan after 3 hr in the presence of the analog, and there appears to be no further incorporation thereafter. In the presence of 5- or 6-fluorotryptophan, 50–60% of the tryptophan is replaced by analog after 3 hr. Cells are not killed by 4-fluorotryptophan, but gradually lose viability in 5- and 6-fluorotryptophan.

Effect on Induction of the Lactose Operon. Figure 2 shows induction of β-galactosidase by 1 m*M* IPTG added at the same time as the fluorotryptophan analog. In the presence of 4-fluorotryptophan, β-galactosidase is induced to a specific activity 60% or more of that of the control containing tryptophan. When 5- or 6-fluorotryptophan is added, a specific activity 10% of the control is reached. If no tryptophan or analog is added, there is no induction of β-galactosidase.

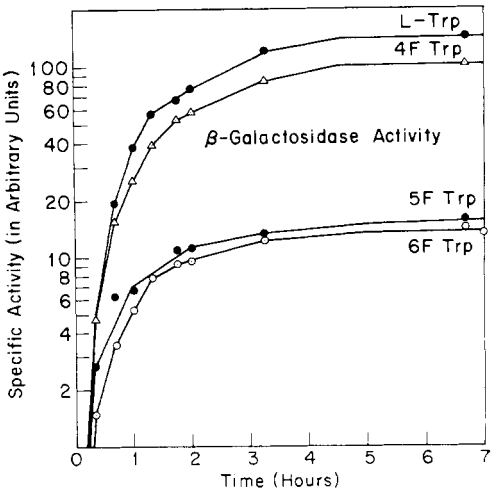

FIGURE 2: Induction of β-galactosidase by 1 m*M* IPTG after resuspension of *E. coli* W3110 *trp* A33 in medium lacking tryptophan, followed by additions as shown. Specific activity is expressed as *o*-nitrophenol released per minute (OD at 420 nm) divided by density of the culture at time of sampling (OD at 525 nm).

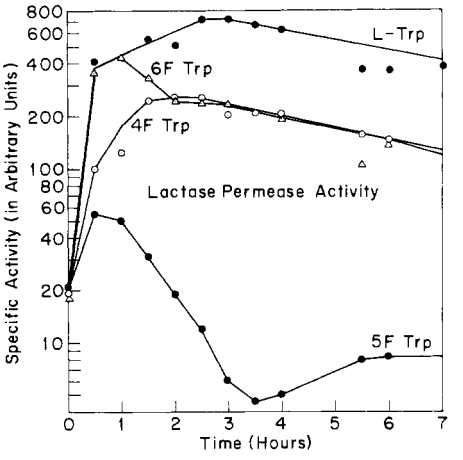

FIGURE 3: Induction of lactose permease activity by 1 m*M* IPTG after resuspension of *E. coli* W3110 *trp* A33 in medium lacking tryptophan, followed by additions as shown. Specific activity is expressed as cpm of [^{14}C]TMG taken up per sample divided by density of the culture at time of sampling (OD at 525 nm). [^{14}C]TMG, 0.05 mCi/0.7 mg of TMG, was mixed with unlabeled TMG. Addition of 10 μl to the reaction mixture gave a concentration of TMG of 2 × 10^{-4} *M* and 0.1 μCi.

Figure 3 shows induction of lactose permease, as measured by uptake of [^{14}C]TMG. In the presence of 4-fluorotryptophan there is induction to a level about 35% of the control. When 5-fluorotryptophan is added, there is only a slight induction of an activity which is rapidly lost. When 6-fluorotryptophan is added, induction is the same as in the tryptophan-containing control for the first hour, then the activity drops to the same level as in the presence of 4-fluorotryptophan. If no tryptophan or analog is added, there is no induction of lactose permease.

Kinetic measurements were made to detect further differences in the analog-containing proteins. Figure 4 shows

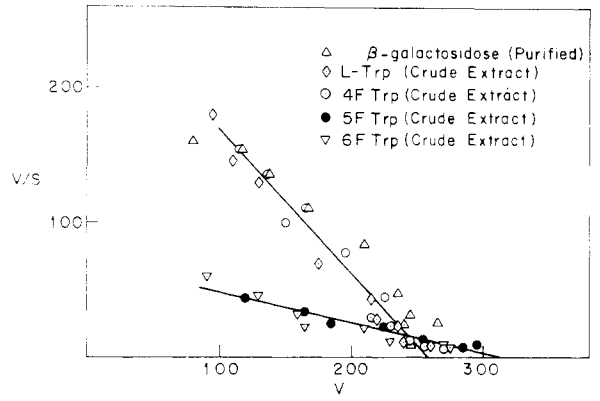

FIGURE 4: Eadie plot of β-galactosidase activity. Cells were harvested 3 hr after addition of IPTG and analogs. V is *o*-nitrophenol released per minute as measured by change in OD at 420 nm, and S is substrate concentration relative to the amount used in the standard assay.

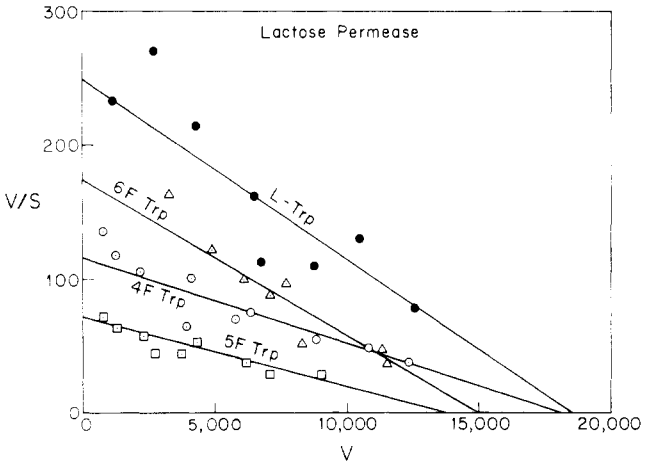

FIGURE 5: Eadie plot of lactose permease activity. Cells were harvested 1 hr after addition of IPTG and analogs. V is cpm of [^{14}C]TMG taken up in 15 min and S is μl of substrate added.

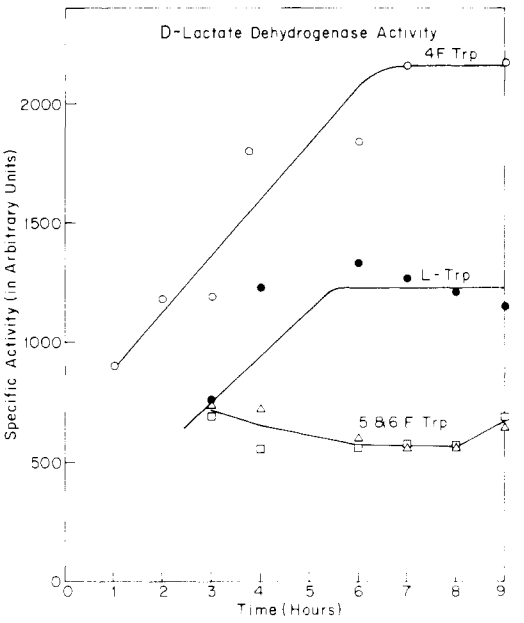

FIGURE 6: D-Lactate dehydrogenase activity after resuspension of *E. coli* W3110 *trp* A33 in medium lacking tryptophan followed by additions as shown. Specific activity is decrease in absorbance in 1 min at 600 nm divided by mg of protein/ml.

Table II: Ratio of Units of Fluorine Analog-Containing Enzyme to Tryptophan-Containing Enzyme Precipitated by a Given Volume of Antiserum.

L-Trp	4F-Trp	5F-Trp	6F-Trp
1.00	1.04	0.52	0.21

that the Eadie plots for commercially purified *E. coli* β-galactosidase, tryptophan- and 4-fluorotryptophan-containing crude extracts are essentially superimposable, while 5- and 6-fluorotryptophan-containing β-galactosidase appear to have an approximately fivefold increase in K_m. In the case of lactose permease activity (Figure 5), L-tryptophan- and 6-fluorotryptophan-containing cells appear to have the same K_m, while 4- and 5-fluorotryptophan-containing cells show an approximately twofold increase in K_m.

For β-galactosidase, an immunological test made possible an estimate of the actual amount of enzyme protein produced in the presence of analog. Using the precipitin test, the units of β-galactosidase precipitated per milliliter of antiserum can be calculated for normal and analog-containing enzyme. The ratios of these values obtained from averaging the results of several such experiments are given in Table II. With 5- or 6-fluorotryptophan-containing enzyme, fewer units are precipitated by a given volume of antiserum, suggesting that these samples have more protein in relation to the measured number of enzyme units than normal or 4-fluorotryptophan containing enzyme. This is not the result of a decreased ability to react with antibody, since supernatant taken from the apparent equivalence point between antibody and 5- or 6-fluorotryptophan-containing enzyme cannot precipitate additional normal β-galactosidase. The conclusion is that 4-fluorotryptophan-containing enzyme is equivalent to the normal enzyme, 5-fluorotryptophan-containing enzyme has two times as much protein in relation to units as normal enzyme, and 6-fluorotryptophan-containing enzyme five times as much protein. Thus in terms of actual amount of enzyme protein, in the presence of 4-fluorotryptophan 60% of normal is made, in the presence of 5-fluorotryptophan 20% and 6-fluorotryptophan 50%.

Effect on D-*Lactate Dehydrogenase Activity*. The specific activity of D-lactate dehydrogenase increases during growth of a normal, tryptophan-containing culture, leveling off toward the end of the logarithmic growth phase (Figure 6). When 4-fluorotryptophan is added, the specific activity becomes twice that of the normal enzyme, and continues to rise for 6 hr after addition of analog. In the presence of 5- and 6-fluorotryptophan, the specific activity decreases to half that of the normal enzyme. However, the K_m's of analog-containing enzymes could not be distinguished from that of the normal enzyme in crude extracts (results not shown).

Discussion

A number of amino acid analogs have been shown to be incorporated into protein. As has been pointed out (Richmond, 1962b; Fowden et al., 1967; Fowden, 1972), since such analogs must be acted upon by several enzyme systems, i.e., permeases and aminoacyl transferases, they must be close structural analogs of the natural amino acids they replace, similar in size, shape, and ionization. When an amino acid analog is added to a bacterial culture, slowing of growth within a generation, followed by complete inhibition

within 4–5 hr, is characteristic of analogs known to replace natural amino acids in protein (Richmond, 1962b).

A number of fluorinated amino acid analogs allow limited growth and are incorporated into protein (Munier and Cohen, 1959; Rennert and Anker, 1963; Gottlieb et al., 1965; Munier et al., 1967; Browne et al., 1970; Sykes et al., 1974; present work). What effect do they have on the structural and catalytic activities of these proteins? Richmond (1962b) suggests that since the analogs must be close structural analogs, it is unlikely that they will differ sufficiently to have much effect on the physical–chemical properties of proteins in which they are incorporated. The van der Waals radius of ^{19}F is only 0.15 Å larger than that of ^{1}H, but ^{19}F is much more electronegative than ^{1}H (Pauling, 1960). Thus, if the isotopic replacement of ^{1}H by ^{19}F alters the properties of a protein, this would suggest that the original group may be located in a hydrophobic region of the molecule or could play a role in the hydrophobic interactions. In a number of cases it has been shown that active enzymes are synthesized. In some cases they are reported produced to the same extent as in the normal cell. *E. coli* alkaline phosphatase in which the phenylalanines were 56% replaced by *p*-fluorophenylalanine showed no detectable effect on physical–chemical or enzymic properties. The addition of the analog had only a slight inhibitory effect on the rate of enzyme synthesis, and the specific activity of normal and analog-containing enzyme was the same (Richmond, 1962a).

In a number of cases a lowered specific activity is found. It is possible that an apparent low activity could result from synthesis of a less stable enzyme. Janecek and Rickenberg (1964) have shown that the β-galactosidase made in the presence of β-2-thienylalanine is more labile than the normal enzyme, although catalytic properties are unchanged when 50% of the phenylalanine is replaced. We also noted fluorotryptophan containing β-galactosidase to be less stable than normal unless protected by sulfhydryl reagents, such as β-mercaptoethanol. The galactoside permease formed in the presence of 5-fluorotryptophan appears to be destroyed in the cell. Degradation of abnormal proteins has been reported (Pine, 1967; Goldberg, 1971).

If the lowering of specific activity is not the result of instability, the question then arises as to whether the analog-containing enzyme has less intrinsic activity than the normal form or is synthesized in smaller amount. One must be able to compare enzyme activity and amount of enzyme protein. Sykes et al. (1974) found that when alkaline phosphatase was induced in the presence of *m*-fluorotyrosine, although the kinetic properties of 70% substituted enzyme were essentially the same as the normal, they isolated only 10% of the normal amount. With *Bacillus cereus* exopenicillinase, Richmond (1960) found that when 75% of the phenylalanine was replaced with *p*-fluorophenylalanine, the specific activity was 50% of normal and the material was changed immunologically. Yoshida (1960) studied *Bacillus subtilis* α-amylase and found that when two phenylalanines out of 18 were replaced with *p*-fluorophenylalanine, activity was 85% of normal, and when four were replaced, 70% of normal, although physical properties were the same.

Browne et al. (1970) working under conditions of catabolite repression found 28% of the normal amount of β-galactosidase produced in the presence of 4-fluorotryptophan, 5.6% in the presence of 5-fluorotryptophan, and 0.7% with the 6-fluoro analog. Our figures, using succinate rather than glucose as carbon source, are 60, 10, and 10%, respectively. Incorporation of 4-fluorotryptophan results in an enzyme unchanged enzymatically or immunologically. When 5- or 6-fluorotryptophan is incorporated, there is kinetically altered activity; with the 5-fluoro analog one-fifth as much protein is made, with the 6-fluoro analog one-half as much protein as in a normal culture. Our results with 4-fluorotryptophan incorporation into D-lactate dehydrogenase are the first where an enzyme is reported to have a higher activity in the presence of an analog. We have not yet determined amount of protein synthesized.

In general, it cannot be predicted what the effect of a given analog on a given enzyme will be. Browne et al. (1970) suggest that on the average, enzymes containing 4-fluorotryptophan are more like the normal than those containing 5- or 6-fluorotryptophan. This is true of β-galactosidase; but not of lactose permease, although the permease in general seems to be more labile to analog incorporation. Thus, Cohen and Munier (1959) and Bowman et al. (1964) find that fluorophenylalanine allows formation of β-galactosidase while inhibiting permease action, and Janecek and Rickenberg (1964) find the same for 2-thienylalanine. With D-lactate dehydrogenase, 4-fluorotryptophan seems to increase activity over the normal, 5- and 6-fluorotryptophan to decrease it. Fluorinated amino acid analogs appear in many cases to have marked effects on the activities of enzymes, and are thus promising probes for study of structure and function using NMR as well as other techniques.

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