THE EFFECT OF AMINO ACID ANALOGUES ON GROWTH AND PROTEIN SYNTHESIS IN MICROORGANISMS

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I. Introduction

Since the work of Woods (92) on the mechanism of action of sulfonamides, it has frequently been observed that inhibitors of biological interest are structural analogues of some essential metabolite. Such inhibitors often compete with the natural metabolite at the active center of an enzyme, and this holds true for the useful structural analogues of the 20 natural amino acids found to occur universally in proteins. In this case, however, there is an additional dimension to the problem because certain analogues are so similar to the natural compounds that they become incorporated into proteins in the place of the natural amino acids. The proteins formed in this way are sometimes altered in their specific enzyme activity, and since some of these proteins will themselves be the enzymes which handle the analogue as a substrate, the uptake of an effective analogue into a cell may have far-reaching and complex effects, leading finally to complete growth inhibition.

In practice, many amino acid analogues have been found to inhibit the growth of microorganisms. First, therefore, the effect of different analogues on growth will be examined to see if any common patterns of inhibition emerge. Then, the effect of these analogues on the mechanism of protein synthesis and the properties of proteins synthesized in the presence of the analogues will be considered. This evidence allows some tentative conclusions to be drawn concerning: (i) how some analogues exert their growth inhibitory effect, (ii) the factors determining amino acid incorporation into proteins, and (iii) the effects of replacement of a natural amino acid by an analogue on the enzymic and immunological properties of certain proteins. Evidence obtained with mammalian systems has only been considered in the review where it bears directly on the processes in microorganisms, and no attempt has been made to discuss in detail effects of amino acid analogues on the formation of virus particles. This subject has not yet been reviewed, but a number of papers on this topic have recently appeared (1, 2, 80, 89, 96).

A number of structural analogues of amino acids not found in proteins have been synthesized and found to be antimetabolites. They include, for example, structural analogues of the β-alanine portion of pantothenic acid, but they have not been considered further (93).

A preliminary section is included to consider the analogues that have inhibitory properties, to decide what chemical changes can lead to the formation of potent analogues.

It should, perhaps, be pointed out that two compounds of similar shape are correctly called "isosteres," but in this review the term "analogue" has been employed throughout since it is common usage.

II. Chemical Structure of Analogues

Two criteria have to be fulfilled for an "unnatural" amino acid to act as an effective antimetabolite.

TABLE 1. *Amino acid analogues of biochemical interest grouped according to the change in structure that leads to antimetabolite properties*

Alteration	Analogue	Natural amino acid	Key references
—F for —H	*p*-Fluorophenylalanine	Phenylalanine	(17, 64, 65)
	o-Fluorophenylalanine	Phenylalanine	(63)
	m-Fluorophenylalanine	Phenylalanine	(58)
	3-Fluorotyrosine	Tyrosine	(12)
	5-Fluorotryptophan	Tryptophan	(8, 9)
	6-Fluorotryptophan	Tryptophan	(69)
—O— for —CH_2—	Canavanine	Arginine	(75)
	O-Methylthreonine	Isoleucine	(71)
—S— for CH_2—	*S*-(2-Aminoethyl)cysteine	Lysine	(72)
—CH_2— for —S—	Norleucine	Methionine	(23)
—O— for —S—	Methoxinine (*O*-methylhomoserine)	Methionine	(79)
—Se— for —S—	Selenomethionine	Methionine	(82, 86)
—CH= for —CH_2—	Methallylglycine	Leucine	(28)
—CH(OH)— for —CH_2—	*β*-Phenylserine	Phenylalanine	(47, 57)
Increase in chain length by —CH_2—	Ethionine	Methionine	(95)
	Homoarginine	Arginine	(88)
	ω-Methyllysine	Lysine	(54)
—NH_2 for —OH	*p*-Aminophenylalanine	Tyrosine	(10)
—Cl for —CH_3	*α*-Amino-*β*-chlorobutyric acid	Valine	(11)
—N= for —CH= in ring systems	Azatryptophan	Tryptophan	(69)
	Tryptazan	Tryptophan	(13)
—CH_3 for —H in a phenyl ring	4-Methyltryptophan	Tryptophan	(85)
	5-Methyltryptophan	Tryptophan	(59)
	6-Methyltryptophan	Tryptophan	(52)
Change in the nature of rings	a. Thienyl for phenyl		
	β-2-Thienylalanine	Phenylalanine	(65)
	β-3-Thienylalanine	Phenylalanine	(28)
	b. Benzothienyl for indolyl		
	β-3-Benzothienylalanine	Tryptophan	(37)
	c. Furyl for phenyl		
	β-2-Furylalanine	Phenylalanine	(28)
	β-3-Furylalanine	Phenylalanine	(28)
	d. Thiazole for imidazole		
	2-Thiazolealanine	Histidine	(60)
Miscellaneous	3-(Aminoethyl)cyclohexaneglycine	Lysine	(26)
	Cyclohexaneglycine	Isoleucine	(33–36, 43, 51, 68)
	β-(Cyclopentane)alanine	Leucine	
	β-(1-Cyclopentene)alanine	Phenylalanine	

1) The molecule must have a similar shape and size to the naturally occurring molecule.

2) If the substituent group is one that can ionize, it must either produce the same type of ion (e.g., —C—N—C(=NH)—NH_3^+ and —O—N—C(=NH)NH_3^+) or be un-ionized at physiological pH values.

In practice, all the useful amino acid analogues have been close structural isomers of naturally occuring amino acids. Apart from a report (66) that D-norleucine inhibits the growth of *Escherichia coli*, all amino acid analogues have the L configuration and compete with the natural L-amino acids. Table 1 shows the compounds considered in this review grouped according to the change in structure that has produced an active antimetabolite. Most frequently, four types of change seem to have been effective: (i) replacement of hydrogen in a ring system by fluorine, (ii) replacement of phenyl by some other resonant ring structure, (iii) replacement of one type of heterocyclic ring by another, and (iv) replacement of a residue from the backbone of an amino acid by another with similar size and shape.

Replacement of hydrogen in a ring system by

TABLE 2. *Comparison of size and shape of analogous groups in amino acid analogues and their natural competitors*

Group	Compound	Interatomic distance (Angstrom)	Bond angle degrees	Radius of group involved (Angstrom)
Phenyl C—F	*o*, *m*, and *p*-Fluorophenylalanine; fluorotryptophan; 3-fluorotyrosine	1.3	Planar	0.64*
Phenyl C—H	Phenylalanine, tryptophan, tyrosine	1.08	Planar	0.37*
C—C—C	Norleucine	1.54	108	1.84
C—S—C	Methionine, *S*-(aminoethyl)cysteine	1.81	105	1.02
C—Se—C	Selenomethionine	1.98	98	1.13
C—O—C	Methoxinine, *O*-methylthreonine	1.43	108	0.6
C—CH=CH—	Methallylglycine	1.33	108	1.84
Phenyl C—OH	Tyrosine	1.36	Planar	
Phenyl C—NH_2	*p*-Aminophenylalanine	1.47	Planar	
C—C—CH_3	Valine	1.54	108	1.86
C—C—Cl	α-Amino-β-chlorobutyric acid	1.76	110	1.05

* Van der Waals radii: given by Bergmann (8). Other data from "Interatomic Distances" (46).

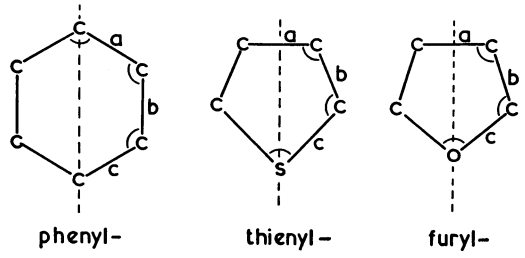

FIG. 1. *Phenyl* (C_6H_5—), *thienyl* (C_4H_3S—), *and furyl* (C_4H_3O—) *drawn to scale to show relative bond lengths and angles. All radicals are planar and symmetrical about the dashed lines (see reference (46)).*

Bond lengths (A)

Phenyl: $a = b = c = 1.40$.
Thienyl: $a = 1.44$, $b = 1.35$, $c = 1.74$.
Furyl: $a = 1.46$, $b = 1.35$, $c = 1.46$.

Angles

Phenyl: $\angle ab = \angle bc = \angle cc = 120°$.
Thienyl: $\angle ab = 113°$, $\angle bc = 112°$, $\angle cc = 91°$.
Furyl: $\angle ab = 106°$, $\angle bc = 111°$, $\angle cc = 107°$.

Dimensions (A):

$a = 1.33$	$a = 1.33$
$b = 1.33$	$b = 1.33$
$c = 1.47$	$c = 1.38$
$d = 1.54$	$d = 1.43$
$e = 2.47$	$e = 2.32$
$\angle cd = 110°$	$\angle cd = 111 \pm 3°$

fluorine. Although the fluorine atom is appreciably larger than hydrogen, the aromatic C—F and C—H bonds are very similar in length (Table 2). In addition fluorine in such a combination is very unreactive (44), has similar electronic properties to hydrogen, and, like hydrogen, produces minimal disturbance of the electronic resonances of the phenyl radical.

So far this transposition has produced *o*-, *m*- and *p*-fluorophenylalanine (phenylalanine analogues), 3-fluorotyrosine (tyrosine analogue), and 5- and 6-fluorotryptophan (tryptophan analogues). Bergmann has recently discussed the synthesis of other fluorine-containing antimetabolites (8). Substitution of hydrogen by halogens other than fluorine rarely leads to potent analogues. However, replacement of —CH_3 by —Cl or —Br is sometimes effective (11).

Replacement of phenyl by another resonant ring system. The replacement of the phenyl residue of phenylalanine by thienyl- or furyl- has led to the formation of active antimetabolites. However, the thiophen and furan rings differ from phenyl since the C—C bond length varies depending on the distance of the bond from the O or S atom in the molecule (Fig. 1). The slightly different potency of β-2- and β-3-thienyl or furyl alanines (27) probably reflects the fact that substitution in the 2 position gives a slightly closer similarity to a substituted phenyl residue than substitution at the 3 position. The thiophen

analogue of tryptophan (β-(benzothienyl)alanine) is an analogue of doubtful potency (5, 37).

Replacement of one type of heterocyclic ring by another. Moyed (60) has recently shown that replacement of imidazole by thiazole produces an effective histidine analogue. This compound is a potent inhibitor of the histidine biosynthetic pathway, and its effect is reversed by L-histidine. Unfortunately the relative size and shape of the imidazole and thiazole radicals are not accurately known.

Replacement of carbon in a ring system by nitrogen often produces active antimetabolites, and has led to the design of the tryptophan analogues α-amino-β-(3-indazole)propionic acid (tryptazan) and 7-azatryptophan. No crystallographic data for these compounds exist, but in pyridine the analogous system =C—N=C— has interatomic distances of 1.37 A and 120° which is close to that found for =C—C=C— in benzene derivatives, and clearly the replacement of carbon by nitrogen in 7-azatryptophan will have little effect on shape of the molecule when compared with tryptophan. The same is almost certainly true for the introduction of the second nitrogen into the five-membered ring of tryptazan. As the replacement of nitrogen for carbon in the six-membered ring of tryptophan is so effective, it is surprising that β-2(pyridyl) alanine is such a poor analogue of phenylalanine (51).

Replacement of a residue from an amino acid backbone. Changes of this type involve the replacement of —S— by —CH_2— (or vice versa), —CH_2— by —O—, —S— by —O— and —S— by —Se—. Table 2 shows the interatomic distances, bond angles, and atomic radii associated with changes of this kind. It will be seen that certain of these changes introduce a considerable modification in the structure of the molecules when compared with the changes considered above; thus the ω-methyl of norleucine will lie 2.49 A from the δ-carbon of the backbone, whereas the distance is 2.87 A in methionine, a shortening of 13.2%. Further, when one considers that selenomethionine can presumably replace at the same sites as norleucine, the difference in that measurement becomes 2.49 A to 2.99 A, or ±10%.

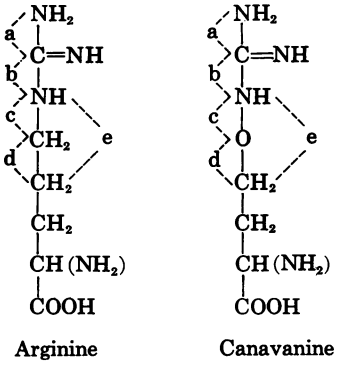

FIG. 2. *Structures of arginine and canavanine*

An interesting compound that falls into this group is canavanine (Fig. 2). Here the replacement of —C— by —O— leads to a shortening from 2.47 A in arginine to 2.32 A in canavanine, although the bond angle is scarcely affected. A further effect of the substitution is to lower the dissociation constant of the guanidino group from pH 12.5 in arginine to about pH 8.0 in canavanine. It is surprising, therefore, that canavanine is capable of taking part in the majority of metabolic interconversions undergone by arginine (48). Despite the fall in dissociation constant, however, the majority of canavanine molecules will have an ionized guanidino group at physiological pH values, and from this point of view be indistinguishable from arginine.

Another example in which two compounds with very different substituent groups can act as analogues when un-ionized is shown by *p*-aminophenylalanine and the reversal of growth inhibition caused by this compound with tyrosine. The shape and size of the —NH_2 and —OH groups are similar, and both groups can be ionized (Table 2). The hydroxyl of tyrosine forms RO^- and H^+ with an acid dissociation constant of about 10^{-9}, whereas the —NH_2 of *p*-aminophenylalanine has a constant of about 4×10^{-4} (*unpublished observations*), and forms RNH_3^+. This means that, at physiological pH values, both amino acids are present in the un-ionized form. The relative shape and size of substituents in other useful analogues is summarized in Table 2.

Size limits for analogues of individual amino acids. Although many amino acid analogues have now been examined, it is not possible to generalize about changes that will produce active analogues of any specific amino acid. The systems responsible for handling the natural amino acids seem to vary greatly in the divergence from the

normal structure that they will tolerate in their substrates. Under certain circumstances enzymes normally specific for methionine ($R \cdot CH_2 \cdot S \cdot Me$) will act on methoxinine ($R \cdot CH_2 \cdot O \cdot Me$), selenomethionine ($R \cdot CH_2 \cdot Se \cdot Me$), norleucine ($R \cdot CH_2 \cdot CH_2 \cdot Me$) or ethionine ($R \cdot CH_2 \cdot S \cdot CH_2 \cdot Me$). On the other hand, organisms do not seem to mistake glycine for alanine or leucine for isoleucine or valine to any great extent—all of which changes are of the same order of size as the changes possible for methionine. In certain cases the structure required in an amino acid antimetabolite seems to be extremely ex-

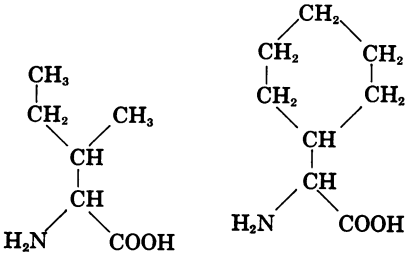

FIG. 3. *Comparison of structures of isoleucine and cyclohexaneglycine.*

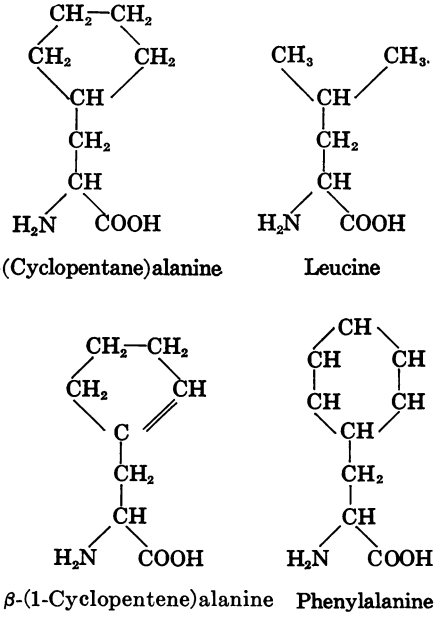

FIG. 4. *Comparison of β-(cyclopentane)alanine with leucine and β-(1-cyclopentene)alanine with phenylalanine.*

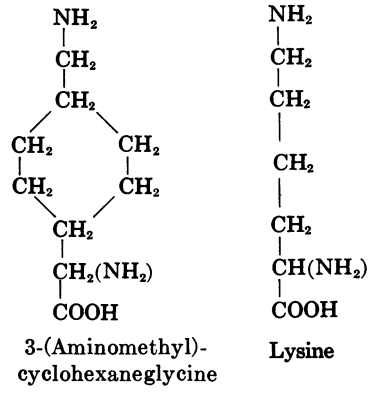

FIG. 5. *Comparison of structures of 3-(aminomethyl)cyclohexaneglycine and lysine.*

acting as far as the relative positions of certain atoms are concerned, whereas the over-all size of the molecule is relatively unimportant. Harding and Shive (43) have synthesized a range of antimetabolites which illustrate this point well. These workers first synthesized cyclopentaneglycine, which was found to inhibit growth of *E. coli*. This compound has a puckered ring system, and in the actual molecules the carbon atoms 1–5 and 8 in cyclohexaneglycine have a very close spatial similarity to the carbon atoms 1–6 of isoleucine (Fig. 3). Growth tests showed that isoleucine reversed the growth inhibition due to the antimetabolite. In a similar way β-(cyclopentane)alanine (which has a puckered ring system) was found to be a leucine analogue, whereas β-(1-cyclopentene)alanine has a planar ring and resembled phenylalanine (Fig. 4) (68). Synthesis of further cyclohexane and cyclohexene derivatives of alanine and glycine led to antimetabolites specific for leucine, isoleucine, and valine. In the above examples the relative positions of the carbon atoms in the antimetabolites are very critical, whereas the over-all size and shape of the molecules are quite dissimilar (33–36). These principles of design have led to 3-(aminomethyl)cyclohexaneglycine (a lysine analogue), in which the relative positions of the α-carboxyl, α-amino, and ω-amino groups are close to those found in lysine. The shape of the molecule is, however, quite different (Fig. 5) (25, 26).

Table 1 shows that effective antimetabolites have been made only for the larger amino acids. To date no effective analogues of glycine, serine,

cysteine, threonine, alanine, aspartic acid, glutamic acid, or proline have been reported, and it is tempting to suggest that the biological sites that deal with these amino acids are more exacting to molecular shape and size than those handling the larger amino acids. Probably this is not so, for changes in the structure of the small amino acids (unless the replacing group is identical in shape and size with the natural compound) will produce a relatively larger change in over-all shape and size than in a large amino acid. Furthermore, certain of the changes used to produce antimetabolites from the larger amino acids cannot be used with the smaller, since the resulting compounds are too unstable, for example, fluoroglycine or the introduction of sulfur into the backbone chain of threonine or glutamic acid in the place of $—CH_2—$. The absence of effective proline analogues is puzzling, but azetidine-2-carboxylic acid might prove to be interesting in this respect (39).[1]

III. Effect of Amino Acid Analogues on Growth of Bacteria

Two points will be considered in this section. First, what are the characteristic effects of amino acid analogues on the growth of microorganisms and secondly, is it possible to deduce anything about the function of an analogue from the type of inhibition observed?

The majority of amino acid analogues, whether designed as antimicrobial, antiviral, or antitumor agents, are screened routinely for possible antimetabolite activity against bacteria, and a mass of reports exists, therefore, on the effect of analogues on bacterial growth. Usually *E. coli* or some organism with a well characterized amino acid requirement such as *Leuconostoc mesenteroides* P60 is inoculated into a simple synthetic medium containing suitable concentrations of the compound under test, and the total yield of bacteria obtained after overnight incubation is measured. It is not possible to deduce much about the detailed effect of an amino acid on growth from experiments of this kind since no idea of the time sequence of inhibition can be obtained. Also, growth in the test could be due to the selection of resistant forms (3, 59, 60). These criticisms apply even more strongly to tests in which the suspected analogue is mixed with varying quantities of the natural amino acid in an attempt to show competition.

The first careful work on the growth kinetics following addition of amino acid analogues to bacterial cultures was carried out by Cohen and Munier (17, 64, 65). Addition of 4- or 5-methyltryptophan, *p*-fluorophenylalanine, β-2-thienylalanine, norleucine, or β-phenylserine to cultures of *E. coli* growing exponentially in a simple synthetic medium resulted in an immediate change in growth rate. The growth curve obtained was described as "linear," that is, the mass of the culture measured turbidimetrically increased at a linear rate from the point of addition of the analogue, whereas the control culture continued to increase exponentially. Linear growth continued for periods up to 5 hr and was associated with an increase of optical density of 3- to 4-fold. A similar type of inhibition was found on adding ethionine to cultures of *E. coli* (40).

Cultures of *Bacillus cereus*, on the other hand, showed little change in growth rate (measured turbidimetrically) for about 20 min after addition of *p*-fluorophenylalanine. Thereafter growth slowed, and eventually ceased about 4 hr after addition of the analogue (76). Addition of 7-azatryptophan to *E. coli* B caused an immediate fall in growth rate, but the growth curve remained exponential. In this case the growth curve was measured both turbidimetrically and by colony counts of viable organisms (69).

The implications of linear growth are rather interesting. Linear increase in *mass* of individual cells between divisions followed by cell division to two parts at intervals so that the mean cell size remains constant results in an exponential increase in cell mass in the culture, i.e., an exponential growth rate. A continued linear growth rate could be achieved in a number of ways. For example, it would occur if one of the daughter cells formed on division ceased to grow, whereas the other continued to grow at full rate. Experiments on clones of organisms growing in the presence of *p*-fluorophenylalanine have shown that cell division continues after addition of the analogue, and that both daughter cells continue to reproduce for at least 90 min. If both the daughter cells are equally potent, a linear growth rate must imply that on division both daughter cells continue to grow, but only at half the rate of the parent. Cohen and Munier (17) suggest

[1] Tests have now shown that azetidine-2-carboxylic acid replaces proline in protein from *E. coli* and certain higher plants (39*a*).

that such a type of growth could arise if addition of an analogue caused an immediate halt in formation of an enzyme whose function immediately became growth limiting. Such a situation would indeed lead to linear growth, but it is difficult to see how this could continue for very long without some imbalance in cell metabolism leading to a fall in growth rate. In practice a linear growth rate of precisely the type suggested by Munier and Cohen does not occur, since the slope of the curves obtained after addition of *p*-fluorophenylalanine or thienylalanine (16, 17, 83) is less than would be expected from the exponential rate of the control culture. If the growth rate and the mass of an exponentially growing culture are known at the point of addition of the analogue, the expected slope of the subsequent linear growth curve can be calculated accurately. The rates published for *p*-fluorophenylalanine- and thienylalanine-grown cells are between 10 and 30% less than would be expected from the exponential rate at the point of addition of the analogues.

In view of these considerations it seemed important to determine whether addition of *p*-fluorophenylalanine did indeed lead to true linear growth or only to an approximately linear rate. The analogue was therefore added to cultures of *E. coli* growing exponentially in a simple synthetic medium. The growth curve was followed by (i) turbidimetry, (ii) colony counts of viable organisms, and (iii) cell number. In addition samples were removed at intervals and cell length was determined by direct measurement of fixed and stained preparations (20). Preliminary results suggest that addition of the analogue causes an immediate onset of a growth rate between exponential and truly arithmetic when measured turbidimetrically, but that this rate becomes progressively slower as incubation continues. Colony counts, dry weight, and total cell number increased in parallel with turbidity, and although cell division continued, the mean size of the cells increased. These results are very similar to those reported for yeast (16), but rather different from those with *E. coli* (65).

Quite apart from determining whether addition of an analogue leads to perfect "linear" growth or not, the growth curve obtained after addition of an antimetabolite to a culture growing exponentially in a single synthetic medium can, in practice, suggest how the analogue may act. If growth is immediately inhibited completely, yet the inhibition is reversible by the natural amino acid, it is likely that the antimetabolite inhibits some essential step in the biosynthetic pathway of the natural amino acid. In this case it is most unlikely to be incorporated into cell protein. On the other hand, onset of "linear" growth (either immediately or within a generation under current cultural conditions), followed 4 to 5 hr later by complete inhibition, is characteristic of all the analogues known to replace natural amino acids in proteins. Thus, from the data published by Moyed (60), it is likely that 2-thiazolealanine is incorporated into the proteins of *E. coli* and that it partially inhibits the biosynthesis of histidine. Similarly, cyclohexenealanine has a growth-inhibitory effect on *E. coli* similar to that of *p*-fluorophenylalanine, and may be a further example of a structural analogue of phenylalanine that can replace the natural amino acid in protein (33). The inhibitory action of all useful analogues is immediately and completely reversed by the natural structural analogue, but not by any other amino acid (except where free metabolic interconversion to the natural competitor is possible). Thus, L-*p*-fluorophenylalanine inhibition is immediately reversed by L-phenylalanine and phenylpyruvic acid, but not by tyrosine. Nonspecific reversals such as those obtained by Bergmann, Sicher, and Volcani (10) for a number of phenylalanine derivatives suggest that the inhibitions observed in these cases were not due to any of the mechanisms considered below. Of course, the effect of an analogue on growth rate only gives an indication of the mechanism of action; so far, however, all analogues useful in studies on bacterial metabolism have shown some effect on growth within a generation, provided the natural competitor was absent. This last point brings up another limitation of the method, since only relatively few bacterial species will grow in defined media and allow effective growth tests.

IV. Effect of Amino Acid Analogues on Mechanism of Protein Synthesis and Properties of Proteins

In practice the majority of amino acid analogues inhibit growth by interfering with either protein synthesis or the function of the proteins synthesized in the presence of the analogues. In the first case they prevent or reduce the rate of

formation of normal proteins; in the second, they replace natural amino acids in proteins, and this may, secondarily, affect protein synthesis by altering the properties of the enzymes involved. These two processes will be dealt with separately, although certain compounds will be considered in both sections.

Effect of analogues on protein synthesis: relation to growth. An early paper by Halvorson and Spiegelman (42) was interpreted, and has been widely reported, as showing that a number of analogues (e.g., ethionine and *o*-, *m*-, and *p*-fluorophenylalanine) completely inhibited protein synthesis in yeast. The experiments were carried out in media containing all the requirements for growth except a nitrogen source. Under these conditions yeast is able to synthesize a limited amount of protein from the free amino acid pool contained in the organisms, and it was suggested that protein synthesis could be measured by following the disappearance of amino acids from the pool. Addition of the analogues led to an immediate complete inhibition of the disappearance of pool amino acids, and this was interpreted as a complete inhibition of protein synthesis.

Subsequent work by Cohen and Munier (17) has shown that addition of *p*-fluorophenylalanine to exponentially growing cultures of *E. coli* slows but does not stop protein synthesis. When the quantity of protein synthesized was plotted as a differential plot (increase in amount of protein vs. increase in bacterial dry weight) it was found that the analogue had no *preferential* inhibitory effect on protein synthesis; that is, growth (as measured turbidimetrically) and protein synthesis were inhibited to the same extent. In view of these results, Cohen *et al.* (16) have reinvestigated the effect of *p*-fluorophenylalanine on protein synthesis in yeast. Addition of the analogue to exponentially growing cultures led immediately to "linear" growth and had no effect on the differential rate of protein synthesis. The 1952 experiments were therefore reinterpreted: the nondisappearance of amino acids from the pool of yeast was due to an increased flow of amino acids to the pool rather than to a specific inhibition of protein synthesis. This reinterpretation was supported by experiments showing an increased rate of protein turnover in the presence of *p*-fluorophenylalanine (16). On the basis of these and other experiments it is now well established that certain analogues (e.g., *o*-, *m*-, and *p*-fluorophenylalanine, ethionine, thienylalanine, norleucine, canavanine, *p*-aminophenylalanine, selenomethionine, and tryptazan) cause partial growth inhibition but do not have a preferential inhibitory effect on protein synthesis. This is not true of 5-methyltryptophan. This compound causes an immediate complete inhibition of protein synthesis, and has therefore a similar effect to that suggested by Halvorson and Spiegelman for all analogues.

In the following sections an attempt will be made to locate the step at which the analogues cause a partial or complete inhibition in the rate of protein synthesis and of growth.

A. Effect on Assimilation and Supply of Amino Acids

Protein synthesis involves two main processes. First, the provision of the amino acids necessary to make the proteins, and, secondly, the process of ordering the amino acids to form specific proteins. In most bacteria some amino acids are taken up from the surrounding environment and some are synthesized within the cell from precursors, which in turn have been assimilated from outside the cell. Both processes are susceptible at various stages to inhibition by amino acid analogues.

It is now fairly well established that the amino acids in the "pool" in bacteria are in rapid equilibrium with the protein-synthesizing sites in the cell (14). Any inhibition by analogues of the assimilation of essential amino acids into the pool will therefore interfere with protein synthesis. Cohen and Rickenberg (15) have studied this problem by incubating *E. coli* in the presence of an amino acid-C^{14} until the pool is maximally labeled, and then transferring the organisms to media containing a variety of single nonradioactive amino acids. When the second amino acid is the nonradioactive isotope of the first and at equimolar concentration, the level of radioactivity in the pool falls rapidly to a value of about 30% of the maximal level characteristic of that amino acid. In practice it was found that L-isoleucine, L-norleucine, and L-leucine were capable of exchanging with valine-C^{14} in the pool but methionine, proline, and phenylalanine were not. Similarly, L-phenylalanine-C^{14} could be displaced by *p*-fluorophenylalanine (if present at 20 times the concentration of phenylalanine) but not by

phenylserine or isoleucine, and L-methionine-C^{14} could be displaced by L-norleucine but not by D-norleucine, D-methionine, proline, or phenylalanine. These experiments were interpreted as showing that there are structural requirements involved in holding an amino acid in the pool and for an exogenous amino acid to displace this amino acid from the pool. Furthermore, certain analogues could pass freely into the cell, probably using the same catalytic sites as the naturally occurring structural isomers. It is interesting that L-norleucine will displace both valine and methionine from the pool, yet methionine will not displace valine, nor valine displace methionine. This means, presumably, that norleucine is sufficiently similar to both valine and methionine to react on the same catalytic sites as those amino acids, whereas valine and methionine are too dissimilar to have an overlapping specificity.

The use of the membrane filter technique has allowed Cowie (21) to study the assimilation of *p*-fluorophenylalanine-C^{14} into the pool of *E. coli* and the effect of phenylalanine on this process. Radioactive *p*-fluorophenylalanine flowed rapidly into the pool, and thence into protein, when no phenylalanine was present in the growth medium. When equimolar concentrations of phenylalanine and *p*-fluorophenylalanine were present, the phenylalanine passed rapidly to the pool while the *p*-fluoro analogue remained in the medium. Only when the *p*-fluorophenylalanine concentration in the medium was four times that of the phenylalanine, did any analogue pass into the pool. In a similar way the phenylalanine passed from the pool to the cell protein preferentially. It follows almost certainly that phenylalanine and the *p*-fluoro analogue use the same route into the pool. Further, these results explain why relatively low exogenous levels of phenylalanine will completely protect cultures from the inhibitory effects of *p*-fluorophenylalanine. Similar experiments carried out with *B. cereus* and *Salmonella typhimurium* have shown that phenylalanine was used in preference to *p*-fluorophenylalanine in both cases. Quantitatively *S. typhimurium* was similar to *E. coli* but with *B. cereus* *p*-fluorophenylalanine was used significantly only when the concentration of analogue in the medium rose to about ten times the phenylalanine concentration ((76, 77) and *unpublished observations*). Unfortunately, similar experiments have not been carried out with other analogues, but the kinetics of inhibition found with β-2-thienylalanine and the reversal by phenylalanine suggest that this analogue behaves in the same way as *p*-fluorophenylalanine (49).

Dunn (29, 30) and co-workers (31, 32) have studied the effect of a range of di- and tripeptides containing β-2-thienylalanine on *E. coli*. These compounds are as effective growth inhibitors as thienylalanine itself, and the inhibition is reversed by phenylalanine. However, the kinetics of reversal suggest that thienylalanine is taken up into the cell by a different route from that used by the peptides. This deduction is only tentative since there is no conclusive evidence that the peptides are not hydrolyzed to the free amino acids before exerting their inhibitory effect.

In summary, therefore, it seems likely that analogues are taken into the cell by the same route as their natural competitors, and that the transport system discriminates against the unnatural compound. No analogues have shown inhibitory properties in broken cell preparations made from bacteria and yet been inactive against intact organisms because of their inability to pass the permeability barriers of the cell, a situation which is of course theoretically possible.

Analogues as inhibitors of endogenous synthesis. Recent work on the regulation of the endogenous supply of amino acids has shown that the rate of synthesis of the amino acids may be controlled by two processes known as feedback inhibition and repression. Feedback inhibition implies that the compound formed by the biosynthetic pathway will inhibit the functioning of the enzymes of the pathway if it is allowed to accumulate either in the cells or the growth medium. There is no change in the *quantity* of the biosynthetic enzymes that are synthesized; it is their function that is inhibited. Repression, on the other hand, is a process whereby accumulation of the product of a biosynthetic pathway leads to a block in the *formation* of the biosynthetic enzymes. In practice both inhibition and repression by analogues have been reported. It is difficult to see, however, how repression by an analogue can be proved unless the enzymes in question are purified so that the actual quantity of enzyme protein that is synthesized can be measured, because the incorporation of analogues into enzyme proteins is known in some cases to affect specific enzyme activity.

Some of the earliest work in which amino acid analogues were used concerned the inhibition of

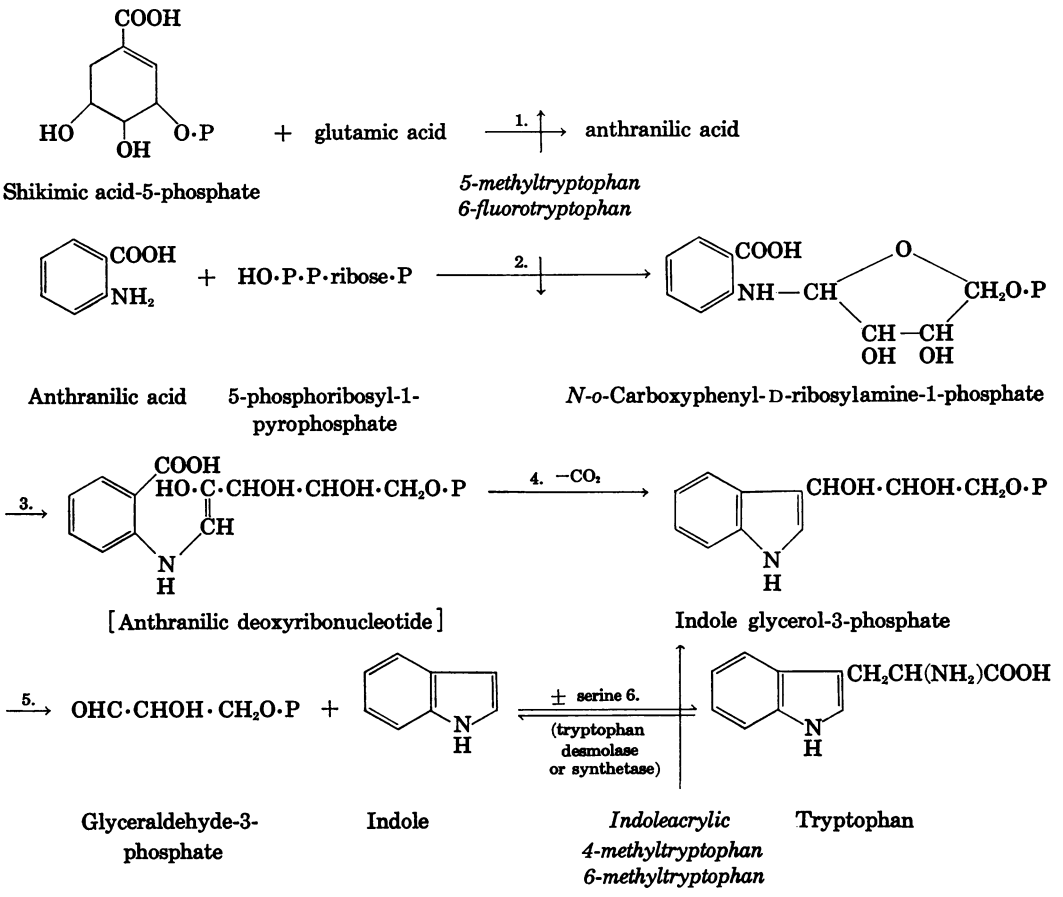

FIG. 6. *Tryptophan biosynthetic pathway*

tryptophan synthetase, an enzyme involved in the biosynthesis of tryptophan. This enzyme catalyzes the condensation of indole and serine to form tryptophan (Fig. 6), and Fildes (38) showed that in intact organisms it is competitively inhibited by indoleacrylic acid. Subsequently the isomeric 2-, 4-, 5-, 6-, and 7-methyltryptophans were tested on the same enzyme: 2-methlytryptophan was inactive, whereas the others were inhibitory in the order 4 > 5 > 6 > 7. 6-Methyltryptophan has subsequently been shown to be a genuine repressor of a number of enzymes of the tryptophan biosynthetic pathway (52), whereas the 4-methyl derivative was confirmed as a strong inhibitor of purified tryptophan synthetase (85). Pardee and Prestidge (70) have reported that *E. coli* mutant 19-2 (a tryptophan requirer) releases anthranilic acid into the medium and that this process is blocked by 7-azatryptophan. It seems likely, therefore, that 7-azatryptophan blocks an enzymic step prior to the formation of anthranilic acid.

Other tryptophan analogues inhibit at other stages of tryptophan biosynthesis (Fig. 6). Moyed and Friedman (62) report that 6-fluorotryptophan inhibits the condensation of anthranilic acid with phosphoribosylpyrophosphate, whereas 5-methyltryptophan inhibits the conversion of shikimic acid-5-phosphate + glutamic acid to anthranilic acid (59). Bergmann (7) and coworkers (9) have found that 5-fluorotryptophan blocks the conversion of anthranilic acid to indole. Probably this analogue acts on the same enzyme as 6-fluorotryptophan (62), but it is not possible to be certain since the experiments were carried out on whole organisms, unlike the experiments with the 6-fluoro analogue, where a partly purified enzyme system was used.

Munier and Cohen (65) have tried to measure the effect of *p*-fluorophenylalanine and β-2-

thienylalanine on the biosynthesis of phenylalanine, and of norleucine on methionine synthesis. When *p*-fluorophenylalanine or β-2-thienylalanine are present in the culture medium there seems to be a decrease in the total amount of phenylalanine synthesized by the organism. As the authors point out, these experiments are not conclusive since only a single sample (taken at an unspecified time after addition of the analogues) was analyzed, and decomposition of amino acid in the growth medium was not measured. With these provisos the results are consistent with either inhibition or repression of the biosynthetic pathway. Inhibition seems the more likely, since repression of enzymes of the phenylalanine pathway must be restricted to the last stages of synthesis. The synthetic route to this amino acid has many steps in common with tyrosine, and neither *p*-fluorophenylalanine nor thienylalanine have any inhibitory effect on tyrosine synthesis (65). Under similar conditions norleucine has no effect on the biosynthesis of methionine although it does have a profound effect on the incorporation of methionine into protein. In this case the analogue increased enormously the amount of methionine released into the medium (65).

Recently the histidine biosynthetic pathway has been shown to be inhibited by 2-thiazolealanine (60). This compound inhibited the enzymic formation of "compound III" (61) from ribose-5-phosphate and adenosine triphosphate (ATP).

In summary, therefore, it is clear that certain analogues, on gaining entry to the cell, inhibit the endogenous supply of the natural competitor. As will be seen later, this greatly facilitates the incorporation of the analogue into proteins by increasing the relative concentration of analogue to competitor that is supplied to the protein-synthesizing enzymes.

B. Effect on Process of Protein Synthesis

As has been mentioned previously, analogues either interfere with the normal process of ordering the amino acids in the peptide chains of the protein, or the proteins that contain the analogue in place of a natural amino acid are less effective, whether in a structural or catalytic role. The formation and function of abnormal proteins will be dealt with later; at this stage, discussion will be confined to the effect of analogues on the individual steps of the synthetic process, and for this purpose an outline of the steps involved may be helpful (Fig. 7).

The reports of many workers with mammalian cells suggest that one site at which the amino acids are arranged into peptide chains is the microsome. In bacteria the situation is less clear-cut, since to date no synthesis of a specific enzyme protein has been shown unequivocally to occur on any of the many different sized ribo-

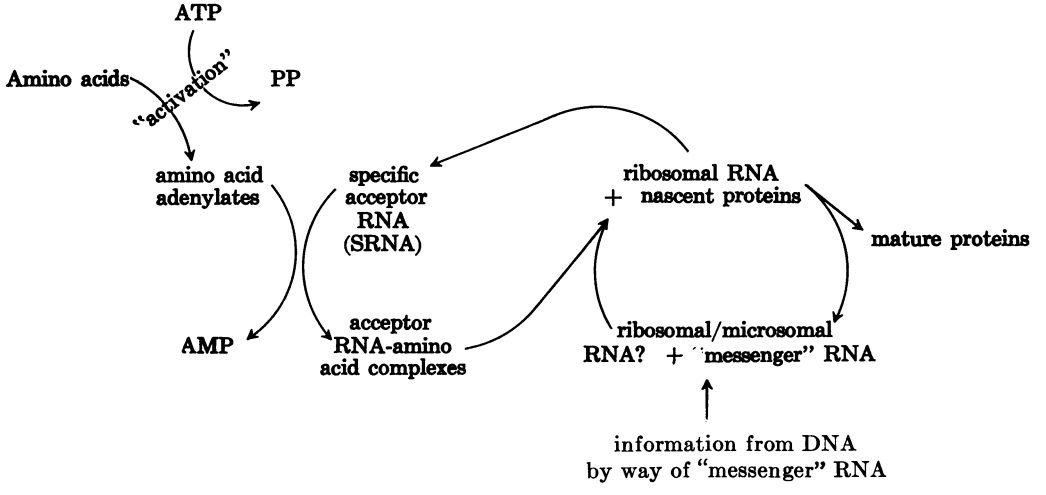

FIG. 7. *Postulated mechanism for the insertion of free amino acids into peptide chains of proteins. ATP, adenosine triphosphate; PP, pyrophosphate; AMP, adenosine monophosphate; RNA, ribonucleic acid; SRNA, soluble RNA; DNA, deoxyribonucleic acid.*

nucleoprotein particles found in bacteria (45). However, the experiments reported by several groups of workers (55, 78) make it likely that the 70S ribosome is the structure in bacteria analogous to the microsome of mammalian tissues. Two problems exist, therefore. First, how is the genetic information contained in the deoxyribonucleic acid (DNA) of the organism transferred to the particles in which the synthesis of the peptide chains occurs; and secondly, what changes do the amino acids undergo before they are incorporated into the peptide chains of the individual proteins? The early steps in the transfer of genetic information to the ribosome (or microsome) do not concern us directly here, since the reactions involved are, as yet, only tentative, and the effect of amino acid analogues on them is undetermined. The changes undergone by the amino acids, on the other hand, are relevant.

The first step in the reaction sequence is the "activation" of the amino acids to form amino acid adenylates (45, 84). The process is catalyzed by "activating enzymes" present in the "pH 5 enzyme fraction" of bacterial cells. Since the reaction proceeds according to the following equation:

$$\text{ATP} + \text{amino acid} \underset{\rightleftharpoons}{\overset{Mg^{++}}{\rightleftharpoons}} \text{amino acid adenylate} + \text{pyrophosphate},$$

the reaction may be followed experimentally either by measuring the formation of the adenylate or by "pyrophosphate exchange" (24). The second method is more commonly used, but may give rise to misleading results since pyrophosphate exchange per se is not necessarily evidence for the formation of an amino acid adenylate.

Next, the amino acid residue of the adenylate is transferred to a low molecular weight ribonucleic acid (RNA) specific for the amino acid residue being incorporated. These low molecular weight RNA molecules are usually called the acceptor or transfer RNA, and are part of the "soluble" RNA (SRNA). From the transfer RNA, the amino acid residue is probably passed to its specific place on the RNA of the ribosome (or microsome) with the liberation of the transfer RNA, which can now accept another activated amino acid residue. It is on the RNA of the ribosome, it is thought, that the formation of the peptide bonds occurs, with the liberation of the intact peptide chain. It seems probable that the amino acids carried on the specific transfer RNA are inserted into their correct position in the peptide chain by a sequence of determinants contained in the base sequence of the "messenger" RNA on the ribosome.

TABLE 3. *Activation of structural analogues of natural amino acids by a preparation from Escherichia coli**

Amino acid	Pyrophosphate exchange (mμmole PP^{32} incorporated into ATP: hr: mg protein $\times 10^{-3}$
L-Valine	8.0
D-Valine	0
DL-Norvaline	6.7
α-Amino-β-chlorobutyric	7.5
L-Methionine	4.6
D-Methionine	0
DL-Selenomethionine	4.8
DL-Ethionine	1.5
DL-Norleucine	0.8
L-Phenylalanine	1.1
DL-*p*-Fluorophenylalanine	0.1
L-Tryptophan	7.0
DL-5-Methyltryptophan	0

* Results recalculated from Nisman and Hirsch (67). Activation measured by "pyrophosphate exchange."

Effect on Amino Acid Activation. Nisman and Hirsch (67) have shown that norvaline, α-amino-β-chlorobutyric acid, *p*-fluorophenylalanine, selenomethionine, ethionine, and norleucine are activated by extracts from *E. coli* (Table 3). Both norvaline and α-amino-β-chlorobutyric acid are activated at approximately the same rate as L-valine, and selenomethionine as rapidly as methionine. Ethionine and norleucine stimulate to 30 and 15% of the methionine rate, and *p*-fluorophenylalanine only reaches 10% that of phenylalanine. 5-Methyltryptophan is not activated. Since *p*-fluorophenylalanine will replace the majority of phenylalanine in the proteins of *E. coli* under conditions where the rate of protein synthesis is about half that found in the absence of the analogue (65), the rate of activation of an analogue has little relation to its rate of incorporation into protein. For example, extracts of a phenylalanine-requiring mutant of *E. coli* (strain

C_2) activated thienylalanine at the same rate as phenylalanine, yet thienylalanine could not support RNA and protein synthesis in the absence of phenylalanine (91). Equimolar quantities of phenylalanine and thienylalanine were activated at the same rate as either amino acid alone (measured by the hydroxamate method), yet no information was given about the relative amounts of the two amino acids converted to the adenylate. Experiments with the purified tryptophan-activating enzyme from pancreas (81) have shown that azatryptophan, tryptazan, and 6- and 5-fluorotryptophan can be activated by the tryptophan-activating enzyme, but that 5-methyltryptophan, 6-methyltryptophan, and 6-methyltryptazan are not. The formation of adenylates was measured both by the hydroxamate tests and by pyrophosphate exchange. Furthermore, azatryptophan hydroxamate was actually isolated from the reaction products. Though it is true that all the amino acids incorporated into proteins (including analogues) have now been shown to undergo an activation step, it is not possible to argue that the activation of amino acid means that an amino acid is incorporated into protein (81).

Sharon and Lipmann's work with the pancreatic enzyme does show, however, that certain analogues can inhibit competitively the action of the activating enzyme. Thus, 5- and 6-methyltryptophan at 200 times the concentration of tryptophan inhibit activation of the natural amino acid by about 70%. The physiological significance of this is rather doubtful since a relative concentration of this order within the cell could only be achieved if the analogue, but none of the natural amino acid, was present in the growth medium.

Recently Berg (6) and Bergmann et al. (11) have shown that certain amino-acid-activating enzymes are also capable of transferring the amino acids to either the 2′- or 3′-hydroxyl group (which group is uncertain) of a terminal adenylic acid in the specific acceptor RNA. They have called the enzymes RNA synthetases. The valyl RNA synthetase was found to transfer one of the structural isomers of α-amino-β-chlorobutyric acid at the same rate as L-valine, although the K_m of the enzyme for the analogue was 3.3 times as great. The other isomer, the allo-α-amino-β-chlorobutyric acid, was much less active. Presumably this must mean that the active sites in the enzymes responsible for handling valine can distinguish between the two methyl groups in the 4,4 positions of valine. The kinetics were determined by "pyrophosphate exchange," and there is, therfore, no evidence that the α-amino-β-chlorobutyric acid actually forms a stable complex with the acceptor RNA. The effect of this analogue has also been investigated on hemoglobin synthesis in reticulocytes (73). Addition of valine-C^{14} to a partially purified hemoglobin-synthesizing system led to a flow of valine-C^{14} to the microsomal particles, to soluble proteins, and to hemoglobin. If the system was incubated with α-amino-β-chlorobutyric acid, hemoglobin synthesis was blocked. However, if small quantities of valine-C^{14} were added after prior incubation with the analogue, valine-C^{14} was found in the microsomal particles, but no valine-C^{14} could be detected in hemoglobin, even if precautions were taken (e.g., addition of "carrier" hemoglobin) to trap all the hemoglobin synthesized. Addition of larger quantities of valine led to restoration of hemoglobin synthesis. Complete reversal of inhibition was obtained by an analogue to valine molar ratio of 2:1. Similar results with lysine-C^{14} make it unlikely that the analogue is blocking only the incorporation of valine into protein. Taken in conjunction with the results obtained with *E. coli* (11), these data make it seem likely that α-amino-β-chlorobutyric acid is activated, passed to the acceptor RNA, and thence to the microsome (or ribosome). Whether it is true that no hemoglobin is formed, or whether some abnormal protein is synthesized which cannot be detected in the normal hemoglobin detection system, cannot yet be decided.

It follows from the experiments discussed in this section that the majority of analogues can be activated to a greater or lesser extent by the systems active on the natural amino acids. Most experiments have been carried out in vitro with broken cell preparations and in the absence of exogenous natural competitors. Clearly this is an artificial situation which is unlikely to decide whether competitive inhibition of amino acid activation by an analogue can cause growth inhibition. In the one case in which an activating enzyme is inhibited by an analogue (5-methyl- and 6-methyltryptophan) under physiological conditions, relatively enormous quantities of the analogue are required. There is not enough evidence to show whether analogues can inhibit

growth by blocking the transfer of activated amino acids to the acceptor RNA or by ordering the amino acids on the microsome (or ribosome). The results obtained with α-amino-β-chlorobutyric acid on hemoglobin synthesis suggest that this compound reaches the acceptor RNA but is not incorporated into protein.

C. Incorporation of Amino Acid Analogues into Cell Proteins

The majority of useful amino acid analogues inhibit protein synthesis in bacteria to some extent, but a 3- to 4-fold increase in cell protein from the point of addition of the inhibitor is frequently found. Analysis of the amino acid content of the protein formed in this way often shows that the analogue amino acid has been incorporated in place of the naturally occurring competitor. To obtain formal proof of the incorporation of an analogue into protein it is necessary to isolate from the cell protein peptides containing the analogue. Usually it is sufficient to show that the analogue is present in a protein fraction so that neither the —COOH nor the $—NH_2$ group of the analogue are accessible to reagents without hydrolysis. The presence of free $—NH_2$ groups can be shown by reaction with fluorodinitrobenzene followed by isolation of the *N*-dinitrophenyl derivative of the analogue (75). The presence of free —COOH groups can be detected by treating the protein before hydrolysis with ninhydrin. If the analogue is involved in a peptide bond, it will not be accessible to ninhydrin, and should be recoverable after hydrolysis.

The incorporation of an unnatural amino acid into the total mixed proteins of a tissue was first shown by Levine and Tarver (53) using ethyl-C^{14}-labeled ethionine. Although the ethionine was not reisolated from the protein in these experiments, subsequent work with *Tetrahymena pyriformis* confirmed that ethionine-C^{14} was incorporated and ethionine-C^{14} peptides could be isolated (41). It was not possible, however, to decide whether the ethionine incorporation occurred at the expense of the methionine normally present in the protein.

Subsequently it has been shown that norleucine (65); tryptazan (13); 7-azatryptophan (69); *o*-, *m*-, and *p*-fluorophenylalanine (63, 65); canavanine (50, 75); β-2-thienylalanine (65); selenomethionine (86); and *p*-aminophenylalanine (*unpublished observations*) are incorporated into the protein of bacteria, and there is strong circumstantial evidence that β-phenylserine is also incorporated (47). It is certain that incorporation of norleucine occurs at the expense of methionine, and not leucine or isoleucine, and *p*-fluorophenylalanine and thienylalanine at the expense of phenylalanine, but not tyrosine. Incorporation of *p*-fluorophenylalanine or thienylalanine has no effect on the incorporation of any amino acid in the protein other than phenylalanine, and the composition of the protein synthesized in the presence of the analogue is therefore unchanged except for the phenylalanine-*p*-fluorophenylalanine or phenylalanine-thienylalanine transposition (65, 77). Although a number of analogues are incorporated, the level that they reach in the cell protein varies markedly. Munier and Cohen (65) report that *p*-fluorophenylalanine or thienylalanine replaces phenylalanine by about 75% in the total mixed cell protein of *E. coli*, and a similar degree of replacement was found in *B. cereus* (77). In addition, norleucine and ethionine replace 50% of the methionine of *E. coli*, whereas 7-azatryptophan only reaches about 30% of the tryptophan normally found in *E. coli* proteins. The tyrosine analogue, *p*-aminophenylalanine, only reaches a maximal level of about 2 to 5% of the tyrosine content of protein. Canavanine replaces arginine (at least partially) in the Walker carcinoma system, since addition of canavanine leads to a fall in the arginine content of protein (50), but whether the uptake of canavanine exactly compensates for the fall in arginine in this system or bacteria is not known (75).

It is possible that selenomethionine can replace methionine completely (22). The analogue will allow exponential growth of a methionine-requiring mutant of *E. coli* in the absence of methionine, although the doubling time of the culture is 75 min rather than 55 which is achieved normally. Since the mutant can use neither inorganic sulfate nor cysteine as a source of methionine, it follows that the analogue must replace all the methionine residues in the protein. Furthermore growth can continue in the presence of selenomethionine alone for up to 100 generations under conditions in which there is little chance of carry-over of methionine being responsible for the growth. An unexplained finding is that organisms growing exponentially

in selenomethionine will not grow when transferred to solid media containing selenomethionine to satisfy their methionine requirement.

Addition of *p*-fluorophenylalanine to *B. cereus* results in replacement of 75% of the phenylalanine of the protein, and this occurs at the same rate in the total cell protein, the total exocellular protein, and the purified exopenicillinase synthesized by the organism (77). It seems virtually certain, therefore, that *p*-fluorophenylalanine replaces phenylalanine to about the same extent throughout all the proteins of the cell. In *E. coli*, Cowie and his collaborators (23) have shown that either norleucine or *p*-fluorophenylalanine show an approximately equal degree of replacement in a number of crude protein fractions. The organisms were grown in radioactive analogue, and the soluble protein was chromatographed on diethylaminoethyl (DEAE)-cellulose. The specific analogue concentration (micromoles of analogue incorporated per milligram of protein) was determined in certain fractions from the column, and no significant variation was found for either compound. These results also suggest an equal degree of replacement in all bacterial proteins. However, this is not absolutely certain since the specific analogue concentration was determined only at a relatively few points in the elution diagram, and the experimental errors involved do not allow calculations to be made with an accuracy of better than $\pm 20\%$. Furthermore, the soluble protein may not be representative of the total cell protein.

Yoshida and Yamasaki (95) have studied the problem of replacement of amino acids by analogues in purified α-amylase from *Bacillus subtilis*. This molecule normally contains four methionine residues. The synthesis of α-amylase was carried out in a medium containing ethionine-S^{35} and the purified enzyme was digested with trypsin. In this way Yoshida was able to isolate three of the four expected methionine and ethionine-containing peptides and measure the methionine to ethionine ratio. The ratios were: peptide 1, 0.64:0.36; peptide 2, 0.61:0.39; peptide 3, 0.60:0.4 against a ratio in the total mixed protein from the cell of 0.59:0.41. These results suggest random replacement. However, Yoshida was unable to find the fourth peptide, and this inevitably leaves open the possibility that the replacement is not completely random.

Incorporation of analogues into enzyme proteins. It is not possible to deduce much about the effect of analogues on specific enzyme activity unless one is in a position to compare enzyme activity and quantity of enzyme protein directly. Various analogues have been reported to affect the activity of enzymes in microbial cultures. However, only in a few cases has care been taken to see whether the inhibitory effect is specific for the enzyme and not a reflexion of the general inhibitory effect that most analogues have on protein synthesis. To establish a *prima-facie* case that an analogue has a specific effect on an enzyme it is necessary to show that the differential rate (increase in enzyme activity vs. increase in culture density) of appearance of enzyme activity is lowered. Even an effect of this kind must be interpreted cautiously, for an artificial limitation of the growth rate of *E. coli* can lead to differential effects on the rate of enzyme synthesis (56). In this case inhibition was caused by increased "glucose repression" following the "sparing" of glucose that occurs when growth is limited artificially. Thus the addition of an analogue may lead to an effect on the differential rate of enzyme synthesis because of increased glucose repression rather than any effect on the specific enzyme activity of the protein synthesized in the presence of the analogue. Azatryptophan almost certainly causes inhibition by a "sparing" action on glucose, since the analogue completely inhibited β-galactosidase synthesis in *E. coli*, whereas aspartyl-carbamyl transferase was inhibited 50% and D-serine deaminase was unaffected (70). For this reason, too, the experiments reported by Munier and Cohen (65) with a range of analogues are subject to some ambiguity. In these experiments the rate of oxygen uptake was to measure the quantity of respiratory enzymes present in the culture, and addition of analogues such as β-thienylalanine, 5-methyltryptophan, norleucine, and *p*-fluorophenylalanine led to impaired oxygen consumption. This could indicate that the analogue inhibited the further synthesis of the respiratory enzymes to a greater or lesser extent. The suggestion that this was caused by the incorporation of analogues into the enzymes resulting in inactive proteins is, however, less likely than that "glucose repression" of the enzymes becomes more marked after addition of the analogues. Similarly, the effects of analogues on β-galactosidase (17) and β-glucuronidase (83) synthesis are difficult to interpret, paricularly since H. V. Rickenberg (*personal communication*) has recently suggested

that the β-galactosidase synthesized in the presence of β-thienylalanine is less stable under conditions of assay than the normal enzyme.

Some direct method of measuring the amount of enzyme protein present is therefore essential before deductions can be made about the effect of analogues on specific enzyme activity. To date the effect of analogues has been measured on two bacterial enzymes, the exopenicillinase synthesized by *B. cereus* (76, 77) and the exocellular α-amylase of *B. subtilis* strain H (94).

Since α-amylase from this strain is normally prepared from organisms in the stationary phase of growth, it is not possible to obtain a differential plot of appearance of enzyme activity in the presence of *p*-fluorophenylalanine that is very meaningful. However, Yoshida (94) purified α-amylase from cultures grown in 72 and 150 μg of DL-*p*-fluorophenylalanine per ml, and determined the degree of replacement of phenylalanine by the analogue, together with the effect of this replacement on specific enzyme activity. The enzyme synthesized in the lower concentrations of the analogue contained about 2 *p*-fluorophenylalanine molecules per molecule of enzyme; that synthesized in the higher concentration, about 4. The specific enzyme activities of the preparations were 85% and 70% of normal, respectively. The incorporation of analogue occurred at the expense

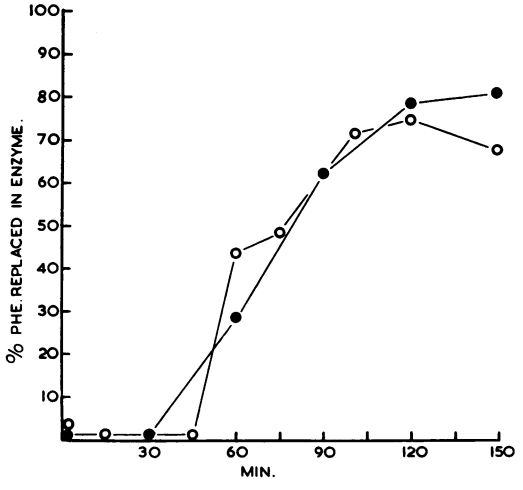

FIG. 8. *Percentage of total phenylalanine (PHE.) in exopenicillinase replaced by p-fluorophenylalanine in the course of incubation in the presence of the analogue. Normal exopenicillinase contains 7 phenylalanine residues. Symbols ● and ○ denote separate experiments.*

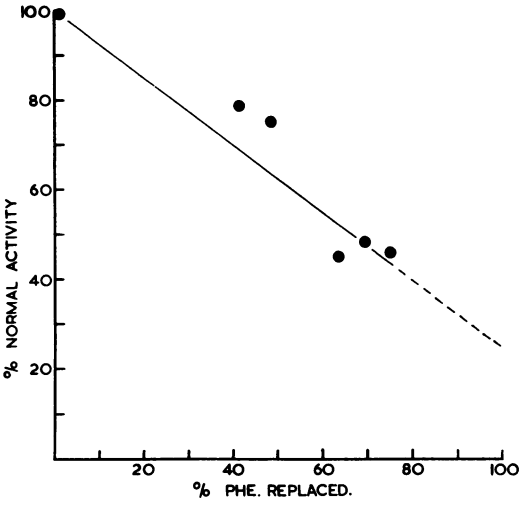

FIG. 9. *Effect of replacement of phenylalanine (PHE.) by the p-fluoro derivative on the specific enzyme activity of exopenicillinase.*

of phenylalanine only, but it was impossible to decide whether replacement was random or confined to certain residues. It was not possible to separate any enzyme with impaired specific activity from the complete mixture, although clearly some such protein must be present. Interpretation of the results is made more difficult by the possibility that the active enzyme molecule arises by the addition of a peptide to a previously synthesized amylase precursor.

The exopenicillinase, isolated from *B. cereus* grown in an amino acid medium + *p*-fluorophenylalanine, was found to contain a maximum of about 75% of the normal phenylalanine residues replaced by the *p*-fluoro derivative. The kinetics of replacement following the addition of the analogue are shown in Fig. 8. The normal exopenicillinase of *B. cereus* contains 7 phenylalanine residues, so in this experiment an average of 5 to 6 out of the 7 residues seem to be replaced. In fact, replacement may be complete in the protein synthesized at the latter stages of incubation, since (unlike the incorporation of *p*-fluorophenylalanine into the total protein of *E. coli* (65)), the degree of replacement in *B. cereus* is not constant with time, but increases. In these experiments the specific enzyme activity of the exopenicillinase fell to about 50% of normal under conditions where about 75% of the phenylalanine had been replaced. The results presented in Fig. 9 show that there is fair agreement between the degree of replacement and the net level of specific

enzyme activity of the mixture of enzyme molecules. As with the α-amylase system, there is no evidence as to whether replacement is random or not, but at least one type of enzyme molecule must be synthesized under these conditions with an altered specific enzyme activity. Attempts to separate this molecular type from the mixture of enzyme proteins obtained in this way have, so far, been unsuccessful (77). The immunological properties of exopenicillinase containing *p*-fluorophenylalanine showed that the reaction of the enzyme with normal antiserum was altered (77).

In both the bacterial enzymes studied so far, incorporation of *p*-fluorophenylalanine led to impaired specific enzyme activity. Vaughan and Steinberg (87) have followed the incorporation of the same analogue into the lysozyme and ovalbumin synthesized by a hen oviduct preparation. The lysozyme showed no fall in specific enzyme activity. Recently Westhead and Boyer (90) prepared aldolase and glyceraldehyde-3-phosphate dehydrogenase with normal specific enzyme activities from rabbits which have been fed on diets rich in *p*-fluorophenylalanine. The two enzymes contained 25% and 16% of their phenylalanine replaced by the *p*-fluoro analogue, respectively. Incorporation of the analogue does not, therefore, always lead to lowered specific activity. The impaired specific enzyme activity with α-amylase and exopenicillinase could be because phenylalanine is involved either directly in the active center or in controlling the configuration of the protein near the active center. The effect with α-amylase is particularly dramatic, since an average of only 4 out of the 18 residues is replaced. Unfortunately, no studies on the enzyme kinetics of normal and analogue-containing enzymes have yet been reported. Such studies might throw some light on changes at the active center.

V. Effect of Amino Acid Analogues on Synthesis of Bacteriophage

Various amino acid analogues have been shown to block the maturation of viruses within cells, but in this review only the effect of analogues on the development of bacteriophage will be considered. 5-Methyltryptophan completely blocks the appearance of mature T_2 or T_4 phage in *E. coli* (18, 19). It was suggested (4) that the analogue blocked tryptophan synthesis by the bacteria, which were then unable to supply an essential constituent of the phage. Subsequent work has shown that this interpretation is probably correct (see earlier).

Pardee and Prestidge (70) have shown that azatryptophan inhibits the multiplication of phage T_2r in *E. coli*. At 2 to 5 μg of analogue per ml, less than 20% of the infective centers were maintained, but it was not possible to block phage infection completely, even at high analogue concentrations. The inhibition of phage production was not caused by nonrelease of otherwise mature phage. If azatryptophan was added to a culture within 10 min of infection and the analogue then reversed by tryptophan at various intervals, no mature phage was found on further incubation, although this treatment reversed the growth inhibition of the receptor cells. During the first 10 min after infection, azatryptophan had little effect on protein synthesis (65% of normal), but DNA synthesis was inhibited by 75%. Since the bacteriophage DNA contains 5-methylcytosine, it is possible to show that after 16 min in the presence of the analogue, phage DNA is less than 30% of the level in the uninhibited system. The proteins whose synthesis is necessary for the formation and release of mature phage can be classed in three main groups: (i) those necessary for the development of the phage, (ii) those which are to become part of the structure of phage, and (iii) those involved in lysis of the cell to liberate the phage. Pardee and Prestidge suggest that azatryptophan interferes with the synthesis of proteins of the first class, and that it may be the synthesis of 5-methylcytosine which is blocked. Since the total bacterial + phage protein at this point contains about 0.5% azatryptophan, they suggest that the inhibition is caused by the incorporation of the analogue into the proteins concerned. Investigation of the phage structural proteins showed that few viable phages were synthesized in the presence of the analogue, and no intact T_2 phage could be seen on electron micrographs. The majority of the phage DNA is liberated from the cells without its protein envelope, whose synthesis is presumably disrupted by the analogue. The purified phage proteins obtained in the presence of the analogue will not kill or adsorb to bacteria. Titration of the abnormal protein with anti-T_2 serum showed that only 20% of the

protein was precipitable, whereas with normal T_2 protein the reaction went virtually to completion.

It seems likely from these experiments, therefore, that azatryptophan blocks phage formation by interfering primarily with the synthesis and function of proteins and thereby with the synthesis of phage DNA.

VI. Conclusions

The foregoing sections have dealt primarily with the experimental facts about the effects of analogues on growth and protein synthesis in bacteria. From these results it is possible to draw some tentative general conclusions about the ways in which bacteria respond to a structural analogue of a natural amino acid. The response depends largely on three factors:

1) The extent to which the analogue satisfies the substrate specificity of the enzymes which normally handle the natural amino acid.

2) The availability of the natural amino acid.

3) The extent to which the incorporation of the analogue into proteins in the place of the natural amino acid modifies the structure of the proteins and the enzymic activity of enzymes.

Clearly the vast majority of compounds which are formally amino acids (i.e., having the general formula $R \cdot CH(NH_2) \cdot COOH$) have no effects on bacteria, certainly not those of the type characteristic of the useful structural analogues. This is, presumably, because the bacteria are impermeable to such compounds.

If an amino acid analogue does have a sufficiently similar shape, size, and ionic configuration to the natural compound to use a common transport system, then in most cases the analogue will satisfy the structural requirements of the other enzymes involved in using the amino acids for protein synthesis to some extent. In the case of analogues that are incorporated into protein, there is nothing to suggest that the active centers of the enzymes involved in protein synthesis become more exacting toward their substrates the later they lie in the reaction sequence leading to peptide bond formation. Certain amino-acid-activating enzymes, however, activate structural analogues at rates of the same order as those achieved with the "natural" substrates, yet the analogues are incorporated into proteins only at 1% or less of the rate that this implies. Clearly, in this case some reaction with a higher specificity must lie between activation and the formation of the intact protein. Work with α-amino-β-chlorobutyric acid suggests that the limiting step is the one involving actual peptide formation (73). The methyl-substituted tryptophans are also exceptions. These compounds do pass into the cell, but act as repressors or inhibitors of tryptophan biosynthesis. The compounds are not activated or incorporated into protein, and it is doubtful even whether they pass into the cell by the same route as tryptophan. There is every reason to believe, however, that norleucine; selenomethionine; *o*-, *m*-, and *p*-fluorophenylalanine; thienylalanine; azatryptophan; tryptazan; and ethionine are taken up into the cell by the route used by the natural amino acid and are then incorporated into proteins by the enzymes normally used for that purpose by the natural compound. In all the cases that have been tested so far, amino acid analogues are found to enter proteins by replacement of naturally occurring structural analogue, but no other amino acid. This suggests that analogues are selected for insertion into specific positions in the cell proteins by the determinants used for the natural amino acids.

In practice this means that when an amino acid analogue which satisfies the structural requirements for active transport into the cell is added to a bacterial culture (which lacks exogenous competitor), the compound will be incorporated randomly in the place of the natural amino acid in all residues in all proteins synthesized after addition of the analogue. Furthermore, unless there is any of the natural amino acid present free in the organism, replacement by the analogue will be complete. (See, for example, the effect of canavanine on a strain of *Staphylococcus aureus* exacting as to arginine (74), and of selenomethionine on a methionine-requiring mutant of *E. coli* (23).)

If the natural amino acid is present in the culture, either in the medium or the free amino acid pool, competition will occur with the analogue for the active centers of the enzymes involved, and the quantity of the analogue flowing to protein will be reduced. Often it is found that the natural amino acid is handled preferentially by the transport system when a mixture of structural analogues is present in the growth medium.

If the natural amino acid is not present in the growth medium, the analogue flows unhindered

into the cell. This means that the enzymes responsible for synthesis of protein will be presented with a mixture of analogue and natural amino acid at a ratio determined by the rate of inflow of analogue through the transport system, the rate of production of the natural amino acid by the biosynthetic system, and the rate of loss of the biosynthetic compound from the free amino acid pool to the medium. If the analogue has no effect on the supply of endogenously synthesized competitor, the enzymes of the cell will continue to be fed with a mixture of structural analogues, and the degree of replacement in the protein will be determined by the relative K_m values of the enzymes for the amino acids. Maximal replacement by an analogue should be expected, therefore, when the external analogue concentration is sufficient to saturate the free amino acid pool. Ethionine is an analogue that probably acts in this way since it does not seem to inhibit or repress methionine biosynthesis.

A number of analogues, however, (e.g., *p*-fluorophenylalanine, thienylalanine, 6-fluorotryptophan) do inhibit the endogenous synthesis of the natural amino acid either by feedback inhibition or repression. In this case, as soon as the analogue enters the free amino acid pool of the cell it will compete with the natural amino acid for the active centers of the enzymes involved in protein synthesis, but also will start to block the flow of the endogenously synthesized natural amino acid. As time goes on (and providing that the level of analogue in the free amino acid pool is maintained by an adequate external concentration), the ratio of analogue to natural amino acid presented to the enzymes will move in favor of the analogue, and this will result in a larger replacement in protein.

The kinetics characteristic of this process depend upon whether the block in endogenous supply is caused by feedback inhibition of an enzyme in the biosynthetic pathway or repression in the formation of all the enzymes of biosynthetic pathway. If inhibition occurs and is complete, the endogenous supply of amino acid will be halted virtually instantaneously, and competition with the analogue will last only as long as the level of the natural compound in the free amino acid pool lasts. This will be shown by an almost immediate and maximal flow of analogue to the proteins and an immediate effect on growth. On the other hand, if repression occurs (without any inhibition), the rate of endogenous synthesis may be as much as 50% of normal after one generation, and the ratio of analogue to natural amino acid fed to the enzymes will change relatively slowly. This will be reflected in an increasing rate of incorporation of analogue into protein and a delayed effect on growth. It is possible that the differences between the effects of *p*-fluorophenylalanine on growth and protein synthesis in *E. coli* and *B. cereus* (see earlier) could be explained on the basis of an inhibition of endogenous supply in *E. coli* but repression in *B. cereus*.

Replacement of a natural amino acid by an analogue in an enzyme can affect specific enzyme activity, and this may have a number of repercussions on the response of organisms to amino acid analogues. First (since analogues are thought to be incorporated at random into all sites) replacement will occur in the proteins responsible for the biosynthesis of all amino acids. If such enzymes have a lowered specific activity, this process could further reduce the supply of endogenously synthesized competitor. On the other hand, this process may equally cause a lowering of the specific activity of the enzymes responsible for protein synthesis, and this would affect a natural amino acid and an analogue equally. The over-all effect would probably be a net lowering in the rate of total protein synthesis. Only if a low degree of incorporation into an enzyme carrying out a normally rate-limiting reaction causes complete loss of enzyme activity will the rate of growth and protein synthesis immediately become linear with time. Cohen and Munier (17, 65) have suggested that this does occur when *E. coli* is treated with β-thienylalanine.

Effect of incorporation of analogues on physicochemical properties and activity of enzymes. Since an analogue must satisfy the specificity of the active sites of several enzymes to be incorporated successfully into enzyme proteins, it must be a close structural analogue of an amino acid normally found in proteins. It is unlikely, therefore, that an effective analogue will differ in structure sufficiently from the normal compound to have much effect on the physicochemical properties of proteins into which it is incorporated. This is particularly so since changes of this sort in the protein would only occur with changes in the ionic, stereochemical, or hydrogen-bonding properties of an analogue, and these are just the

sort of changes that would be likely to make the analogue unacceptable to the cell.

It is less possible to be dogmatic about the effect of analogues on specific enzyme activity since the way in which an active center operates is so little understood. So far exopenicillinase and α-amylase have been shown to be affected by incorporation of analogues, whereas alkaline phosphatase of *E. coli* (77*a*), lysozyme from hen oviduct preparations (87), and aldolase and glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle were unaffected (90). In general it is possible to suggest tentatively that the effect of an analogue on the activity of an enzyme may depend upon the following factors: (i) the nature of the analogue and the degree to which it differs from the natural amino acid, (ii) whether a residue implicated in the active center is replaced (There is no evidence yet to suggest that such a residue would be less accessible to replacement than any other in the protein.), and (iii) whether any residues are replaced which are involved in maintaining the tertiary structure of the enzyme. Too little is yet known about the mechanism of enzyme action to make application of these factors more than tentative.

Future prospects for use of analogues. In the past the use of amino acid analogues has not conferred any particular advantage over the use of the natural amino acids in elucidating the steps in biochemical reactions, particularly protein synthesis. Usually the processes have been worked out with natural amino acids and then the tolerances allowable in the various steps have been tested by analogues.

The discovery that analogues can be incorporated into individual enzyme proteins has opened up the possibility of using these compounds to study the mechanism of action of the active center. It would be particularly interesting in this connection to develop an analogue which could be incorporated into a protein in vivo and then allowed to react in various ways after isolation and purification of the proteins. Such compounds as *p*-aminophenylalanine could be ideal for such purposes. A similar approach could also yield valuable information about the mechanism of enzyme-antibody interactions, since the reaction with the analogues in situ could be used to complement the studies made by treating the amino acids occurring naturally in proteins with various reagents and studying the effect on enzyme neutralization.

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