THE INHIBITION OF ENZYME FORMATION BY AMINO ACID ANALOGUES[1]

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It has been established in a variety of instances (Doudoroff *et al.*, 1943; Spiegelman *et al.*, 1947; Monod and Torriani, 1948; Klein and Doudoroff, 1950; Sleeper *et al.*, 1950; Pollock, 1950) that microbial enzymatic adaptation involves enzyme formation. This has been accomplished by exhibiting active homologous enzyme in extracts prepared from cells incubated with substrate. Similar preparations from cells not exposed to substrate are found to contain little or no detectable enzyme. As pointed out by Stanier (1951), in no case where an adequate search has been conducted has a failure to find proof of enzyme formation been reported.

The existence of this phenomenon raises the question of the identity of the precursor converted into active enzyme. Many possibilities can be entertained as to the origin and nature of the precursor material, as well as for the number and extent of the reactions required to transform it into functional enzyme. A plausible approach is to pose the problem in the form of the following question: Is it possible for precursor to become active enzyme without the participation of the free amino acid pool? Putting the question this way accepts no preconceived assumptions about the complexity of the precursor being transformed into enzyme. In principle it could range all the way from a fully formed protein down to the individual amino acids. This formulation of the problem quite pointedly directs attention to the kind of experimental analysis which could provide pertinent information. The use of analogues of amino acids as agents to prevent the incorporation of free amino acids into proteins recommends itself immediately as one potentially fruitful method of analyzing the problem. It is the purpose of the present paper to summarize experiments performed along these lines.

An experimental analysis of the question formulated would meet with extraordinary difficulty in adaptive responses which require as a *sine qua non* the presence of an exogenous nitrogen source. The nonparticipation of the free amino acid pool in enzymatic adaptations conducted under such circumstances would be difficult to imagine. Consequently, a prerequisite for informative experiments on free amino acid involvement would be enzyme formation under conditions in which the nitrogen used for new protein synthesis is derived solely from existent cellular material. It has fortunately been possible to demonstrate the synthesis of enzymes in the complete absence of a nitrogenous source with cells suspended in buffer solutions of substrate. The experiments reported here employ a system of this nature, adaptation to maltose utilization by yeast cells.

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METHODS AND MATERIALS

Organism used and conditions of growth. The medium used for growing cells was prepared by adding the following to one liter of water: 5 g peptone (Difco); 2.5 g yeast extract; 6.0 ml of 60 per cent sodium lactate; 0.25 g $CaCl_2$; 0.25 g $MgSO_4 \cdot 7H_2O$; 2 g KH_2PO_4; 6 g $(NH_4)_2SO_4$; and 60 g of glucose. A diploid representative of *Saccharomyces cerevisiae*, strain K, was employed. Cultures in the log phase were prepared by inoculating 500 ml of the glucose medium with 0.2 ml of a 24-hour culture. After 12 hours of standing incubation at 30 C the cultures were harvested by centrifugation and used immediately. The cells were washed twice in chilled water and suspended in cold buffer to a density of 2.6 mg dry weight per ml with the aid of a calibrated Klett-Summerson photoelectric colorimeter. Unless otherwise specified the buffer was M/10 phosphate—M/10 succinate adjusted to pH 4.5.

Methods of measuring adaptation and growth. Two manometric methods were employed to follow the course of the adaptation. One was the usual two cup method (Umbreit, Burris, and Stauffer, 1950) which permits the simultaneous measurement of Q_{O_2} and $Q_{CO_2}^{O_2}$ and allows for a continuous observation of the adaptive process. The other method involves taking samples at intervals and measuring the $Q_{CO_2}^{N_2}$ of the cells on the adaptive substrate. Manometric measurements were conducted with conventional Warburg equipment at 30 C. Anaerobic conditions were established by flushing with nitrogen. The maltose used was Merck's maltose, further purified by recrystallization from 50 per cent alcohol.

Effects of the analogues on growth were studied in Burkholder's (1943) synthetic medium modified as follows: the asparagine was omitted and the ammonium sulfate increased to 4.0 g per liter; 5.9 g per liter of succinic acid were added to increase the buffering capacity. The resulting medium was brought to pH 4.5 by the addition of KOH. Amino acids and their analogues were adjusted to the same pH and sterilized separately.

Ten ml of the modified Burkholder's medium were placed in a 50 ml Erlenmeyer flask constructed with an enlarged neck permitting the insertion of a two holed rubber stopper containing an inverted Klett tube and an aeration tube. The flasks were incubated at 30 C while shaking at a rate of 252 oscillations per minute with a Burrell wrist action shaker. Growth was followed by inverting the flask contents into the Klett tube and taking turbidity readings in a Klett-Summerson photoelectric colorimeter employing a blue filter. In certain instances direct counts were made to follow the onset of growth. The flasks were inoculated with 0.1 ml containing approximately 4×10^5 cells from a 24-hour culture grown on the synthetic medium.

Methods of measuring enzyme content. To compare the enzymatic activity of the intact cells with their enzyme content, "fast dried" preparations were made in the following manner. Washed cells were spread over unglazed porcelain plates to remove excess moisture. Then they were spread on the inside walls of beakers, placed in a vacuum desiccator over anhydrous $CaCl_2$, and evacuated for a period of at least four hours. The maltose splitting capacity of such preparations is quite stable to storage in the cold. About 85 per cent of the enzymatic activity can be easily extracted in soluble form from the dried cells. As a routine practice,

however, the activity of the entire ground dried powder suspended in M/15 phosphate buffer at pH 6.8 was measured.

Enzymatic activity was measured manometrically by the rate of glucose released using a maltose negative yeast strain, *Torula monosa*. The method is analogous to that of Keilin and Hartree (1948) who employed a preparation of glucose oxidase. The latter method was found to be inapplicable to our enzyme system due to a severe inhibition by the accumulating gluconate.

For measurement purposes *Torula monosa* was grown in the complete glucose medium at 30 C in standing cultures for 24 hours. Cellular suspensions then were flushed with nitrogen for about four hours to increase their fermentative capacity and stored at 3 C for future use. Under such conditions cells are usable over a period of several weeks. When desired for assay the cells were centrifuged and washed twice with cold water and resuspended in buffer to 25 per cent by volume of wet cells. A 0.5 ml sample of this suspension was added to each Warburg vessel and, under anaerobic conditions, can release 2,400 mm^3 of CO_2 per hour from glucose. Endogenous controls were run in the course of all assays which were corrected for endogenous fermentation. Preparations were assayed in duplicate at dilutions yielding activities which increased by a factor of 2 when the amount of enzyme was doubled. The validity of this procedure was checked and confirmed by a direct analysis for increase in reducing power as determined by the method of Somogyi (1945).

Method of collecting and analyzing free amino acid pools. Free amino acid pools were obtained by the method of Gale (1947). A thick suspension, containing 100 mg dry weight of washed cells per ml, was prepared by the addition of either M/5 acetate or water to a calibrated centrifuge tube. Acetate was used when it was desired to analyze for glutamic acid by the decarboxylase method and water when the amino acids were to be analyzed by paper strip chromatography. The suspension was placed in a boiling water bath for 20 minutes, the coagulated proteins removed by centrifugation, and the supernate retained for analysis.

The free amino acids were determined by the method of McFarren (1951). Descending chromatograms were run using Whatman paper no. 1 suspended in 24 by 12 inch cylinders equilibrated at 30 C. Only eight of the 21 amino acids could be located with ease using the three solvents systems *o*-cresol at pH 6.2, *m*-cresol at pH 8.4, and phenol at pH 12.0. The different ninhydrin reacting components were compared and identified by the use of internal and external standards of crystalline preparations.

The glutamic acid content was quantitatively estimated in M/5 acetate extracts at pH 4.25 by the decarboxylase method of Gale (1945). A preparation specific for glutamic acid was obtained by aging a dried 24-hour culture of *Escherichia coli* (Crooke's strain).

EXPERIMENTAL RESULTS

Survey of the effect of amino acid analogues on growth and adaptation. Figure 1 exhibits typical experimental findings with one of the effective analogues, *o*-fluoro-phenylalanine, and illustrates the methodology of these experiments.

It is evident that *o*-fluoro-phenylalanine exerts a strong inhibitory effect on both growth and adaptation. Thus, the rates of growth and adaptation in the control curves, as determined from the slopes of the linear portions of the corresponding semilogarithmic plots, are 0.141 and 0.208, respectively. These compare with 0.023 for the growth rate and 0.080 for the rate of enzyme formation in the presence of the analogue. The length of the lag periods can be obtained by the extrapolations indicated in the lower curves of figure 1. In instances

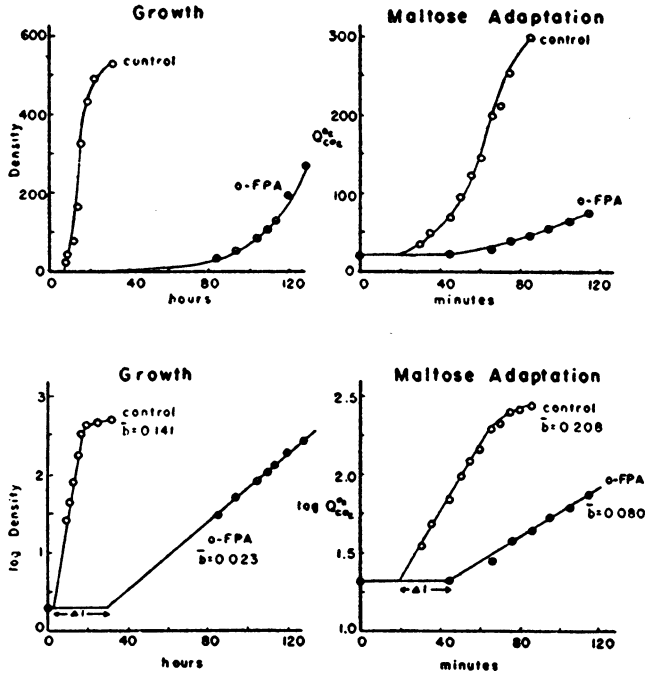

Figure 1. The effect of *o*-fluoro-phenylalanine (*o*-FPA) on growth and adaptation. The lower curves are semilog plots of the corresponding upper ones. The b values noted in the lower curves represent the slopes of the linear portions of the corresponding curves. Length of lag periods is determined from the extrapolations to the zero time ordinates indicated in the lower curves.

of extremely severe inhibitions (>99 per cent) adequate quantitative estimations of effects on the length of the lag periods become virtually impossible.

Several features of the results obtained with *o*-fluoro-phenylalanine (as well as with the other effective analogues) may well be detailed here because of their implications for the mechanism of the inhibition obtained with these agents. It is evident from the lower curves of figure 1 that the presence of analogue influences the kinetics in two experimentally distinguishable ways. The rate of protein formation, as measured by either growth or adaptation in the exponential phase, is decreased. The other effect is seen in the increase in the lag period which precedes active synthesis in the two processes studied. None

of the analogues depressed the eventual total yield of cells formed in a growth experiment.

Table 1 summarizes the data obtained in the survey of 23 analogues of amino acids. The rate of growth and adaptation in the presence of the analogues are

TABLE 1

Effects of analogues of amino acids on growth and adaptation to maltose by yeast cells

All of the analogues were tested at a level of 0.02 M. Both growth and adaptation were carried out at 30 C, the former in synthetic medium and the latter in phosphate succinate buffer at pH 4.5. Growth was determined photometrically and checked by direct counts at low levels. Adaptation was followed manometrically. Rates of both processes were obtained from the slopes of the corresponding semilogarithmic plots and lag periods by extrapolation of the latter to zero time levels of density and activity. The usual control lag periods encountered were 2.0 hr for growth and 0.3 hr for adaptation.

AMINO ACID ANALOGUES	GROWTH		ADAPTATION	
	Per cent of control rate	Lag of experimental / Lag of control	Per cent of control rate	Lag of experimental / Lag of control
DL-1 Amino cyclohexane carboxylic acid	100	1.0	100	1.0
α-Amino-β-ethoxy isobutyric acid	100	1.0	100	1.0
DL-Allothreonine	100	1.0	100	1.0
DL-Alloisoleucine	100	1.0	100	1.0
L-Cystathionine	100	1.0	100	1.0
N-Methyl alanine	100	1.0	100	1.0
p-Bromo-phenylalanine	100	1.0	100	1.0
p-Chloro-phenylalanine	100	1.0	100	1.0
α-Amino butyric acid	100	1.2	100	1.0
DL-Norvaline	100	1.4	100	1.5
S-Methyl-L-cysteine	100	3.0	100	1.8
N-Ethyl glycine	100	1.0	100	1.0
DL-Norleucine	99	1.9	84	1.8
γ-Ethyl amide of glutamic acid	98	1.0	98	1.0
α-Amino enanthic acid	97	1.0	100	1.0
Methyl amino methane sulfonic acid	95	1.9	61	1.9
o-Chloro-phenylalanine	92	1.0	98	1.0
Amino methane sulfonic acid	83	1.4	76	1.4
Ethionine	70	1.2	83	1.0
m-Chloro-phenylalanine	54	1.2	59	1.3
m-Fluoro-phenylalanine	22	5.0	50	1.3
o-Fluoro-phenylalanine	16	5.5	38	2.2
p-Fluoro-phenylalanine	<1	—	27	2.5

recorded as percentages of the control rates. The effects on the lag periods are expressed as the ratio of the lag of the experimentals to that of the controls.

It is evident from the results summarized in table 1 that a parallelism exists between the inhibitory effectiveness of the analogues on growth and adaptation. In general, those without influence on the rate of adaptation were also incapable of inhibiting growth. On the other hand, those found to interfere with growth to a detectable degree also were able to suppress adaptation. No analogue was

discovered with a severe effect on growth which was not coupled with the corresponding capacity to inhibit adaptation.

The rather detailed nature of the correspondence between the effects on growth and adaptation is particularly well illustrated by S-methyl-L-cysteine. This latter compound had no detectable effect on the rates of either growth or adaptation but quite markedly lengthened the lag periods of both processes. The good correlation between the effects on growth and adaptation suggests interference with reactions common to both processes.

Of the compounds examined, it is evident that the halogen substituted phenylalanines are the most effective. Much of the subsequent work to be discussed was done with these agents with particular attention focused on *p*-fluorophenylalanine.

TABLE 2

Direct demonstration of inhibition of enzyme synthesis by analogue

Washed log-phase cells suspended in pH 4.5 buffer to a density of 2.6 mg dry weight per ml were used. Ninety-two ml aliquots were adapted aerobically for 100 min at 30 C in the presence and absence of 0.01 M *p*-fluoro-phenylalanine. The adapting mixture contained 20 mg of maltose and 0.25 mg of glucose per ml. At the end of the incubation the cells were centrifuged and washed in the cold, and enzymatic activity of intact cells was determined anaerobically. Enzyme contents were estimated with *Torula* using "fast dried" preparations made from aliquots of the same cells by the methods described. Activities are expressed as mm^3 CO_2 released anaerobically at 30 C from maltose (3 per cent) per hr per mg dry weight of sample. Average deviations from the mean of duplicate assays are given.

		$Q^N_{CO_2}$ (MALTOSE)
Controls:		
No *p*-fluoro-phenylalanine	Intact cells	250 ± 5
	Dried cells	240 ± 6
Experimentals:		
0.01 M *p*-fluoro-phenylalanine	Intact cells	22 ± 1
	Dried cells	20 ± 1

Demonstration that amino acid analogues inhibit enzyme formation. The possibility of direct inhibition of adaptive substrate metabolism by the analogues was eliminated. None of the 23 analogues tested exhibited any detectable capacity to depress the rate of maltose fermentation by adapted cells. Further, no measurable effects on the rate or characteristics of glucose utilization at either high or low levels of this substrate were observed.

A direct demonstration that the analogues which prevent the appearance of enzymatic activity actually inhibit the synthesis of enzymes was achieved by enzyme assay of dried cell preparations. The methods employed and results obtained are summarized in table 2. A small amount of glucose (0.25 mg per ml) was added to the adapting mixture. It serves as an initiating energy supply (Spiegelman, Reiner, and Cohnberg, 1947) and was included in all experiments where rapid onset of adaptation was desired. This amount of glucose is almost completely consumed in the first ten minutes after tipping and does not interfere with the subsequent observations on the course of the adaptation.

The data of table 2 clearly indicate that the ability of *p*-fluoro-phenylalanine to prevent the appearance of adaptive activity in the intact cell derives from its capacity to prevent the formation of enzyme. A number of similar experiments were conducted with this as well as other effective analogues of amino acids with essentially identical results.

It will be noted that excellent quantitative correspondence was obtained between the enzymatic activity of the intact cells and their enzyme content as determined with dried cell preparations derived from them. Good agreement between such parallel measurements is invariably obtained during the exponential portion of the adaptation curve. During this period the capacity of the cells to ferment the adaptive substrate maltose is well below the rate of glucose fermentation and hence the adaptive system is rate limiting. Consequently, within the region delimited, measurements of intact cell activity provide an adequate quantitative estimate of enzyme content.

TABLE 3

Reversal of o-fluoro-phenylalanine inhibition by phenylalanine

Growth took place in synthetic medium at 30 C and was measured tubidimetrically. Adaptation was measured manometrically as $Q^{O_2}_{CO_2}$ at 30 C in phosphate succinate buffer. Rates represent the slopes of the linear portions of the curves obtained in a semilogarithmic plot against time.

SYSTEM	GROWTH RATE	ADAPTATION RATE
Cells	0.182	0.198
Cells + phenylalanine (0.02 M)	0.182	0.194
Cells + *o*-fluoro-phenylalanine (0.02 M)	0.025	0.058
Cells + *o*-fluoro-phenylalanine (0.02 M) + phenylalanine (0.02 M)	0.183	0.196

Reversal of analogue inhibition by the homologous amino acids. If the analogues of amino acids prevent the formation of enzymes by acting as specific antagonists of the amino acids, it would be expected that the addition of the corresponding homologues would overcome the inhibition. Experiments to examine this question were performed with all of the analogues found to be effective inhibitors. The influence on both growth and adaptation was studied by the methods already described. The extent of the reversals achieved may be illustrated by a typical set of results obtained with *o*-fluoro-phenylalanine and summarized in table 3. Similar studies, carried out with the other effective analogues which could be completely reversed, are summarized in table 4. Two of the effective antagonists, methylaminomethane sulfonic acid and aminomethane sulfonic acid, thus far have not been reversed by the addition of any one of a number of compounds tried.

Where easy and complete reversal of the antagonists was achieved by the addition of the natural amino acid, relatively rigid specificity requirements were observed. Thus for example, in the case of the most effective analogue,

p-fluoro-phenylalanine, all of the 21 natural amino acids in addition to glutamine, asparagine, and ammonia were tried. The only compound which was detectably effective was phenylalanine and this gave complete reversal.

The effect of analogues on the utilization of the free amino acid pool. The relative specificity and effectiveness of the homologous amino acids in reversing the inhibition of both growth and adaptation by the analogues strongly imply that these latter agents suppress protein synthesis via interference with reactions involving free amino acids. These results suggested the desirability of a more direct experimental analysis of the influence of these analogues on the utilization of the free amino acids in the internal environment of the cell. The experiments of Gale (1947) and his coworkers have provided several more or less equivalent methods for the separation of the free amino acid pool.

Qualitative and semiquantitative analysis of the individual amino acids in the free amino acid pool was accomplished chromatographically. The following eight free amino acid pool components could be followed with comparative ease:

TABLE 4

Reversal of amino acid analogue inhibition of growth and adaptation by the natural amino acids

Reversibility of the inhibition on both growth and adaptation was tested and found to parallel each other. Analogues and amino acids were at 0.02 M.

ANALOGUES OF AMINO ACIDS	REVERSED BY
Norleucine	Leucine and isoleucine
Ethionine	Methionine
meta-Chloro-phenylalanine	Phenylalanine
meta-Fluoro-phenylalanine	Phenylalanine
para-Fluoro-phenylalanine	Phenylalanine
ortho-Fluoro-phenylalanine	Phenylalanine

glutamine, glutamic acid, aspartic acid, valine, lysine, arginine, phenylalanine, and histidine. All of these were identified with the aid of known purified preparations.

From a comparison of the size and intensity of the ninhydrin spots, the major components in the free amino acid pools derived from cells in the log phase appear to be glutamic, glutamine, aspartic, and lysine. These results are in agreement with those of Roine (1947) and Freeland and Gale (1947).

The investigations of Virtanen and his collaborator (1948, 1949), and of Roine (1947) in particular, suggested a method for the experimental analysis of the effect of amino acid analogues on the flow from the free amino acid pool to protein. Roine, working with torulae, showed a 75 per cent decline in the amount of "free" amino nitrogen in the course of a nitrogen starvation procedure carried out by allowing cells to metabolize glucose in the absence of an external nitrogen source. As pointed out by Roine, and confirmed in the course of the present investigation, no detectable amounts of nitrogenous material pass out of the cells into the medium in the course of a treatment of this sort. Thus, active metabolism in the absence of exogenous nitrogen leads to the

incorporation of the free amino acids into components which are no longer extractable in a soluble form by methods which yield the free amino acid pool.

To examine the effect of analogues of amino acids on this process of amino acid incorporation during nitrogen starvation, the following type of experiment was performed. Freshly harvested and washed log-phase glucose grown cells were resuspended in 270 ml of M/15 KH_2PO_4-M/20 succinate at pH 4.5 to a density of 2.84 mg dry weight of cells per ml. One-third of this suspension was taken as a zero time sample, centrifuged in the cold, and the cells resuspended

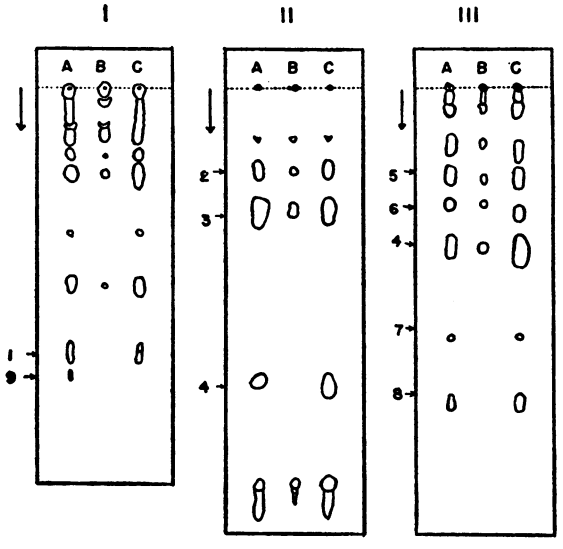

Figure 2. The effect of *p*-fluoro-phenylalanine (*p*-FPA) on flow from the free amino acid pool. The three strips represent scale reproductions of chromatograms of the free amino acid pools derived from cells variously treated. The A columns correspond to cells incubated for 65 min in 4 per cent glucose in the presence of 0.02 M *p*-FPA, the B columns to control cells incubated without the analogue, and the C columns to zero time samples. The three strips correspond to the three solvent systems employed. Strip I was developed for 24 hr with *o*-cresol, strip II with phenol for 24 hr, and strip III with *m*-cresol for 40 hr. The numbers indicate the following identified components: (1) phenylalanine, (2) aspartic acid, (3) glutamic acid, (4) glutamine, (5) lysine, (6) arginine, (7) histidine, (8) valine, and (9) *p*-fluoro-phenylalanine.

in 5.0 ml of H_2O, placed in a 100 C water bath for 20 minutes, and the supernate chromatogrammed. The remainder of the suspension was divided into two equal aliquots. To each was added sufficient glucose to yield a final concentration of 4 per cent. One received in addition, *p*-fluoro-phenylalanine (0.02 M) and the other served as a control. Both aliquots then were incubated aerobically at 30 C while shaking in large Warburg flasks. At the end of 65 min they were removed and the cells treated in the manner described for the zero time control.

Figure 2 reproduces to scale the results of the chromatographic analysis of the free amino acid pools obtained from the three groups of cells. A comparison

of the zero time samples (columns C) and the 65 min control cells (columns B) reveals clearly the sharp drop in free amino acid content which attends active metabolism of glucose in the absence of an exogenous nitrogen source. The difference is even more striking than can be seen from figure 1 which permits a comparison based only on the size of the spots but fails to exhibit the marked differences in intensity. The concentration of three of the components, i.e., phenylalanine, glutamine, and valine, has been depressed in the nitrogen starved cells to the point where they are no longer detectable.

It is evident from a comparison of the A columns in the three strips with columns B and C that the presence of *p*-fluoro-phenylalanine effectively inhibits the net incorporation of the free amino acids into insoluble nitrogenous com-

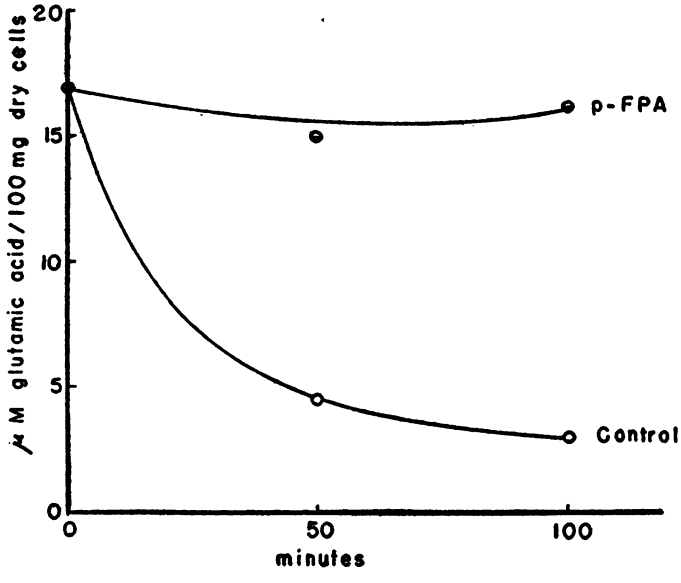

Figure 3. The effect of 0.02 M *p*-fluoro-phenylalanine on utilization of glutamic acid in the free amino acid pool.

ponents. The extra spot (no. 9) observable immediately below phenylalanine in strip I, and not seen in either one of the control columns of this strip, corresponds to *p*-fluoro-phenylalanine, the analogue employed in this experiment.

A significant finding of these experiments is that the presence of any one analogue of amino acid can prevent the incorporation not only of its homologue but of all of the other amino acids as well. Since all of the free amino acid appear to behave in a parallel fashion, it is possible to check the results obtained with paper chromatography by quantitative analysis of any one of the identified amino acids. Glutamic acid, a major component, was followed by the use of a specific decarboxylase as described by Gale (1945).

The type of experiment performed was in principle identical to those previously described which followed the free amino acid pool chromatographically.

Suspensions of freshly harvested and washed log-phase cells were allowed to metabolize glucose in nitrogen free phosphate succinate buffer at pH 4.5 in the presence and absence of 0.02 M *p*-fluoro-phenylalanine. Samples of cells were removed at intervals, the free amino acid pool separated, and the glutamic acid analyzed enzymatically. The results obtained in a representative experiment are described by the curves of figure 3.

The data and conclusions are indistinguishable from those derived by the chromatographic method. A rapid incorporation of glutamic acid from the free amino acid pool occurs during glucose metabolism in the absence of an external supply of nitrogen. This incorporation is severely inhibited by *p*-fluoro-phenylalanine.

DISCUSSION

Mode of action of the amino acid analogues. The available data (see Dittmer, 1950, for review) lead to the conclusion that the analogues of amino acid function by interfering with reactions involving free amino acids. This interpretation is supported by the reversal and free amino acid pool experiments of the present investigation. The possibility that the effective inhibitors can influence reactions other than those controlling amino acid incorporation into protein (e.g., synthesis, interconversion, etc.) cannot be eliminated by the data obtained. However, it may be noted that the experiments which examined the behavior of the free amino acid pool in the presence of analogue failed to provide evidence for their existence.

It is evident that, for example, *p*-fluoro-phenylalanine, which prevents the net incorporation of phenylalanine and the other seven components identified, cannot be functioning solely by preventing phenylalanine synthesis either primarily or by interconversion. Phenylalanine should then have been incorporated to the extent that it was available. However, the amount of phenylalanine found at the end of the incubation period in the presence of analogue was not detectably different from that which obtained at zero time. This, despite the fact that in the same period the control cells lowered the phenylalanine content to undetectable levels. Furthermore, as may be seen from a comparison of columns A and C in the three strips of figure 1, no peculiar unbalances in favor of one or more of the components attended the incubation with the analogue. Such might well have been expected had the interference of the analogue been restricted specifically to any extent to interconversion or other metabolic reactions amongst the amino acids involving its homologue.

It seems difficult to explain the observations recorded without invoking a mechanism involving the active inhibition of the incorporation of the free amino acids into insoluble, heat precipitable, components of the cell.

Implications for the precursor problem of enzyme formation. The experiments described examined the process of enzyme formation in the absence of exogenous nitrogen. They can in principle, therefore, provide information on the nature of the *cellular* nitrogenous components which are converted into active enzyme. Further discussion of possible interpretations and mechanisms may

best be developed in terms of the following set of mutually compatible reaction sequences;

(1) Precursor → → → Enzyme
(2) Precursor + Free Amino Acids → → → Enzyme
(3) Free Amino Acids → → → Enzyme

Reaction (1) assumes the preexistence in the unadapted cell of a precursor which can be converted into active enzyme without the involvement of free amino acids. This latter property distinguishes it from reactions (2) and (3) and permits an experimental decision. Reactions (2) and (3) both assume that amino acids are necessary participants in the process of enzyme synthesis. Reaction (3), however, entertains the possibility of enzyme molecule fabrication directly from free amino acids with no stable complex precursor intervening.

The particular variation of reaction (1) which would suggest that enzymatic adaptation is akin to the transformation of trypsinogen into trypsin has been made unlikely by earlier investigations. Severe competitive interactions amongst enzyme forming systems have been shown to exist by Monod (1942) in the bacteria and by Spiegelman and Dunn (1947) in the yeasts. Cells incubated in the presence of two metabolically unrelated substrates do not normally adapt simultaneously to both despite the fact that either substrate functioning alone can induce its homologous enzyme. Such observations make difficult the acceptance of any concept of substrate induced enzyme synthesis which depends on the transformation of an inactive precursor already fully and uniquely specified as to its enzymatic potentiality. These results, however, did not exclude other variations of reaction (1) involving a complex precursor utilizable as a protein source for two or more enzymes.

Direct transformation of a preexistent precursor presumes a pathway of enzyme formation independent of free amino acid incorporation. Its existence would have one or more of the following consequences for analogue inhibited adaptations induced in the absence of external nitrogen: (a) absence of parallelism in the effects on growth and adaptation; (b) inhibition on rate of enzyme formation with no effect on lag; (c) a break in the velocity of enzyme formation prior to the attainment of full activity. None of these predicted consequences was observed. If direct transformation does occur, it constitutes a quantitatively minor portion of the enzyme synthesizing process.

The data obtained indicate that those analogues which interfere effectively with utilization of internal free amino acids suppress growth and enzyme formation in nongrowing cells. They imply that free amino acid metabolism constitutes a major component of the enzyme synthesizing mechanism.

It is important to note that complete suppression of either growth or adaptation was not obtained at the concentrations of the analogues attainable. An interesting feature of the inhibition exerted by these agents on protein synthesis is the constancy of the rates established in their presence. Once out of the lag period the rate of growth or adaptation is maintained at a level characteristic for the amino acid analogue employed and the physiological condition of the cells. One might suppose that the gradual accumulation of the corre-

sponding homologue would ultimately lead to an escape from the inhibitory effect of the analogue. The fact, however, that no such recovery process is observed strongly suggests a rigid coupling between utilization and formation of the amino acids.

Other than pointedly emphasizing the importance of the free amino acids, the experiments reported here tell us little about the nature or complexity of any other nitrogenous substance which may, or may not, be components of the enzyme forming mechanism. Experimental support does indeed exist suggesting that fully formed and active enzyme molecules can be involved in enzymatic adaptations. Spiegelman and Dunn (1947) have shown that adaptations of yeast cells carried out in the absence of an external supply of nitrogen are accompanied by the loss of one or more of the enzymes preexisting in the cells. Such losses may include even members of the socalled "constitutive" group of enzymes and can be alleviated by the addition of an external nitrogen source during the adaptation. Striking observations along analogous lines have been recently reported by Cohn and Torriani (1951) who have uncovered a relationship between lactase in *E. coli* and an enzymatically inactive but antigenically related protein which decreases during adaptation.

Such observations do not establish the participation of complex protein molecules as nitrogen precursors in enzyme synthesis. Much more detailed information will have to be acquired before such instances of interactions between enzyme forming systems and existent protein molecules will receive adequate interpretation.

The mechanism whereby the free amino acids can serve as precursors for enzyme formation is as yet obscure. One of the simplest and most direct pictures one can entertain is a stepwise formation of peptides of increasing complexity and chain length. This would suggest peptides of various sizes as intermediates in the chain of reactions leading from the individual amino acids to protein. The work of Anfinsen and Steinberg (1951) on ovalbumin synthesis supplies supporting evidence for this view. The interesting feature to emerge from the present study is, however, the absence of any indication for a process of this nature. A careful comparative search was made amongst both the identified and unidentified separable components of the free amino acid pool using five solvents. No components corresponding to peptides, or other elements, could be detected which were unique to samples derived from cells incubated in the presence of analogue. Though such negative results cannot as yet be accepted as conclusive, they are highly suggestive, and particularly so when taken in conjunction with the finding that the presence of any analogue suppresses the incorporation of all the other amino acids studied along with that of its homologue. It is difficult to see why the analogue of a particular amino acid should prevent the formation of di- and tri-peptides involving other unrelated amino acids, if such simple peptides do represent normal stages in protein synthesis. On the other hand, these findings can be understood if the first intermediate formed in protein synthesis is already of such complexity as to involve the utilization of a large proportion of the various amino acids. Under these cir-

cumstances, if one were missing or its use specifically prevented, none of the other amino acids would be utilized. This view of protein synthesis is in complete agreement with the conclusions recently deduced by Geiger (1950) from data obtained in nutritional studies with animals.

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SUMMARY

A study has been carried out on the effect of a series of analogues of amino acids on substrate induced enzyme formation in the absence of an exogenous source of nitrogen.

A parallelism was found between the capacity of an analogue to inhibit growth and its ability to supress enzyme synthesis in the nongrowing cells of *Saccharomyces cerevisiae*. Complete and specific reversal was achieved by the addition of the corresponding homologous amino acids.

It was demonstrated that analogues of amino acids which suppress enzyme formation inhibit net incorporation from the free amino acid pool. Chromatographic and decarboxylase analyses of free amino acid pool components indicate that the presence of any one analogue prevents the incorporation of its homologue and of all the other amino acids as well.

No evidence for a direct, amino acid independent, transformation of complex precursor into active enzyme was obtained. The data lead to the conclusion that the primary pathway of substrate induced enzyme formation in nongrowing cells involves the utilization of the internal free amino acids. They suggest that the first intermediate formed is of such complexity as to demand the participation of a large proportion of the various amino acids present.

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