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AMINO ACID ANALOG INCORPORATION INTO BACTERIAL PROTEINS

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SUMMARY

The amino acid analogs norleucine and para-fluorophenylalanine are shown to be incorporated into the proteins of *E. coli*. Analysis of proteins by an ion-exchange column showed that the proteins formed in the presence of the analogs are not radically different molecular species but are physicochemically similar to the proteins normally synthesized. The substitution of norleucine for methionine in the bacterial proteins was shown to occur in the same proportions in all of the "protein classes" resolved by the ion-exchange column.

INTRODUCTION

Considerable quantities of certain amino acid analogs may be incorporated into the proteins of *Escherichia coli*[1-6]. The analogs substitute for corresponding naturally occurring amino acids and cause various biological effects. In general, cellular growth becomes linear with time and specific enzymic activities may be lost, depressed, or remain unaffected. Such effects depend upon the degree and kind of substitution produced. Since the degree and kind of substitution can be controlled, the use of analogs provides a method for the quantitative examination of the relationship between altered molecular structure and enzymic activity. Evidence can also be adduced concerning susceptibility of bacterial protein types to analog substitution.

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PROCEDURES

Wild type *E. coli* ML 30 and a methionine-requiring mutant, ML 304d, were used in these experiments. The cells were cultured in vigorously aerated C medium*** with maltose (1.0 g/l) as the carbon source. Thiomethyl β-*d*-galactoside (TMG) $5 \cdot 10^{-4}$ *M* was used to induce the synthesis of β-galactosidase.

DL-[^{14}C]phenylalanine (10^{-4} *M*, $6 \cdot 10^{5}$ counts/min/μM), DL-[^{14}C]*p*-fluorophenylalanine ($5 \cdot 10^{-4}$ *M*, $4.3 \cdot 10^{4}$ counts/min/μM); L-[^{35}S]methionine (10^{-4} *M*, $6 \cdot 10^{5}$ counts/min/μM), DL-[1-^{14}C]norleucine ($2 \cdot 10^{-2}$ *M*) were the radioactive tracers used. The radioactive DL-[3-^{14}C]phenylalanine and DL-[3-^{14}C]*p*-fluorophenylalanine were synthesized by Dr. PICHAT of the Commissariat à l'Energie Atomique, France. L-[^{35}S]-methionine was obtained from the Abbott Laboratories, Chicago, Illinois; the DL-[^{14}C]-norleucine was obtained from the Volk Radiochemical Company, Chicago, Illinois.

Cell extracts

After growth in the presence of either the [^{14}C]phenylalanine or [^{14}C]*p*-fluorophenylalanine, the cells were harvested and washed twice with 0.02 *M* sodium phosphate buffer, pH 7.0, and resuspended in 10 ml of the same buffer at a bacterial concentration of 15 mg dry wt./ml. The cells grown in medium containing [^{35}S]-methionine or [^{14}C]norleucine were washed in TSM buffer** pH 7.6 and resuspended in 10 ml of the same buffer. In each case the cells were ruptured by extrusion through a small orifice under pressure (approximately 16,000 lb./sq. in.) in a modified*** French pressure cell[7]. The extruded material was centrifuged for 10 min at 13,000 rev./min in a Servall centrifuge to remove whole cells, cell walls, and other large fragments. An aliquot of the opalescent supernatant (approximately 8 ml) was placed on a N-diethylaminoethyl-cellulose (DEAE) ion-exchange column[8]. The remainder was used to determine the total radioactivity, total β-galactosidase, or other enzyme activities and for chemical fractionation[9]. Only a small fraction of the radioactivity and a negligible fraction of the enzyme activity were present in the large fragment pellets.

The amount of β-galactosidase was determined by the rate of hydrolysis of *M*/375 ortho-nitrophenyl β-*d*-galactoside in 0.05 *M* sodium phosphate buffer, pH 7, at room temperature by toluenized preparations of the whole cells prior to rupture, by the extract prior to fractionation through the column, and by aliquots of the individual tubes collected from the DEAE column. Phosphoglucomutase[10] and glucose-6-phosphate dehydrogenase[11] were also estimated in those aliquots.

DEAE-cellulose ion-exchange column

The DEAE-cellulose ion-exchange material was prepared as described by PETERSON AND SOBER[8]. The particular batch used throughout these experiments contained 1.0 mequiv. of nitrogen per gram dry weight. The exchanger was stored as a

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* C Medium: 2 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 3 g NaCl, 0.01 g Mg as $MgCl_2$, 0.026 g S as Na_2SO_4, 100 ml 10% maltose, and 900 ml distilled H_2O.

** TSM buffer: 0.01 *M* tris succinate, 0.005 *M* Mg as magnesium acetate.

*** The blind end of the pressure cell cylinder was bored out and a removable pressure seal inserted in order to facilitate loading, cleaning, and assembly. The operation of the cell was as described previously[7].

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moist slurry at room temperature in 0.1 *M* NaCl at pH 10. Immediately before use, a portion was washed in the centrifuge with 0.02 *M* phosphate buffer or TSM until the suspending fluid was neutral. Columns were prepared by settling several aliquots of the neutral slurry into a chromatographic tube (1 cm diameter) until the height of the column under 10 lb. pressure reached 20 cm. Such a column contained 2.5 g dry exchanger. The supernatant fluid of the pressure cell extract obtained from approximately 120 mg dry weight of bacteria was adsorbed to the column under pressure (10 lb./sq. in.). The column was eluted with a linear salt gradient (0.0 *M* to 0.8 *M* NaCl in 0.02 *M* phosphate buffer or TSM pH 7.6) supplied by the device of BOCK AND LING[12]. Eluates were collected in 20 drop (approx. 1 ml) fractions at a flow rate provided by an air pressure of 3–5 lb./sq. in. (about 0.15 ml/min). 100–200 tubes were collected at room temperature and stored at 4° until appropriate analysis could be made. Columns freshly prepared from the stock suspension were used for each experiment. Radioactivity, protein content[13], and enzyme activities were determined on aliquots from each of the fractions collected for each experiment.

RESULTS

The demonstration that amino acid analogs could be incorporated into bacterial proteins immediately raised many questions concerning the nature of the proteins produced. Investigations were carried out to determine whether the analogs are contained in radically different molecular species or in proteins similar to those normally synthesized. These investigations required (a) the use of an analog that would substitute for only one naturally occurring amino acid; and (b) a quantitative method for analyzing bacterial proteins.

The analog, *norleucine* substitutes for methionine in the proteins of *E. coli*. A reduction of about 38 % of the protein methionine is obtained when the methionine requiring mutant (ML 304d) is grown in C-medium containing DL-norleucine ($2 \cdot 10^{-2}$ *M*) and L-[^{35}S]methionine (10^{-4} *M*). This mutant was chosen in order to eliminate competitive reactions involving sulfur compounds other than methionine or the methionine analog. The separation of bacterial proteins into chromatographically resolvable "protein classes" was achieved through the use of the DEAE cellulose ion exchange column. Fig. 1 shows the elution pattern of an extract of *E. coli* grown for many generations in C-medium containing [^{35}S]methionine. Thiomethyl β-*d*-galactoside was added to induce the synthesis of β-galactosidase.

The bacterial extract was prepared from washed cells, ruptured by extrusion through a small orifice under pressure, and the extruded material centrifuged to remove whole cells and large cellular fragments. The opalescent supernatant was then used for the column analysis. Evident in this elution diagram are a number of well resolved regions showing a close correlation between the protein pattern (measured by the Folin procedure[13]) and the pattern of distribution of the radiomethionine. Two dimensional paper chromatograms of hydrolysates of an aliquot of the bacterial extract showed that the incorporated radioactivity was contained solely as methionine.

In Fig. 2 are shown the specific radioactivities of the individual fractions. The specific radioactivity is defined as the ratio of the quantity of radioactivity to the amount of protein synthesized after the addition of the radioactive amino acid.

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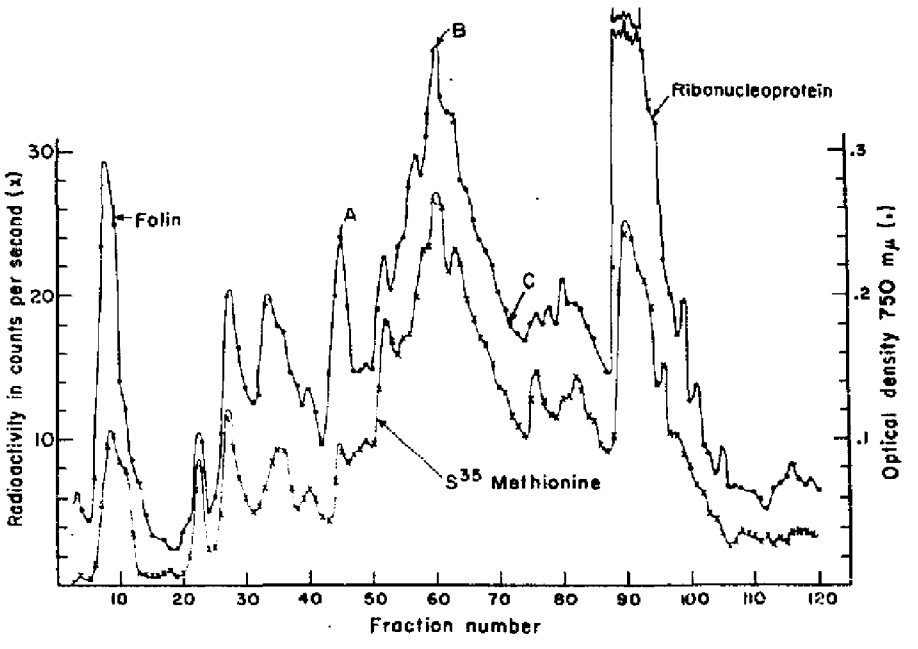

Fig. 1. Elution pattern of bacterial extract of *E. coli* obtained with ion-exchange column. Mutant cells (ML 304d) grown in C-medium containing L-[^{35}S]methionine (10^{-3} *M*); thiomethyl β-D-galactoside ($5 \cdot 10^{-4}$ *M*) and maltose.

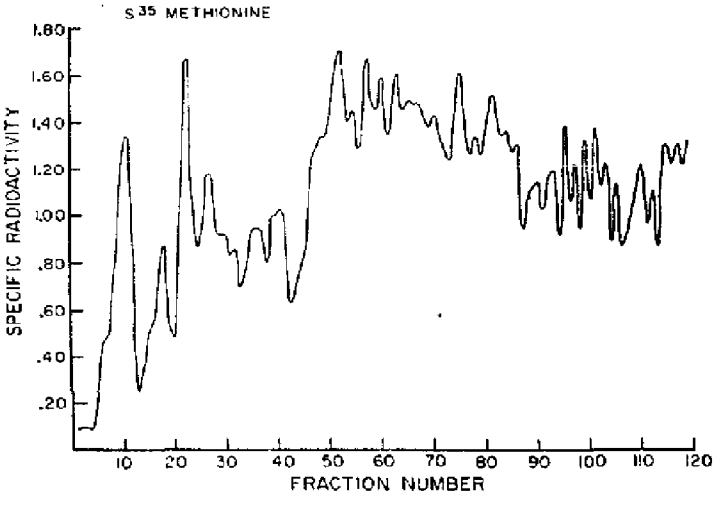

Fig. 2. Specific radioactivity of eluted column fractions (in arbitrary units). Data represent the ratio of radioactivity per fraction to the quantity of protein newly synthesized after the addition of the labeled methionine to the culture.

Fig. 3 shows the degree of resolution among the eluted proteins. Superimposed on the elution diagram are the locations of 3 enzyme activities; β-galactosidase (LAC), phosphoglucomutase (MUT), and glucose-6-phosphate dehydrogenase (ZW). Each enzyme activity can be correlated with an obvious protein peak. Other proteins

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having similar charge properties are, of course, contained in each region; nevertheless the partitioning of bacterial proteins into "protein classes" is apparent.

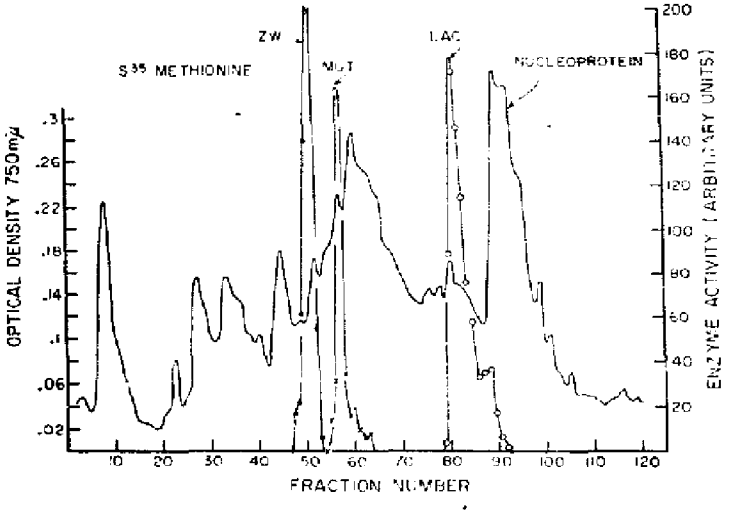

Fig. 3. Location of enzyme activities along elution diagram. Glucose-6-phosphate dehydrogenase (ZW); phosphoglucomutase (MUT); β-galactosidase (LAC).

Column analysis of bacterial extracts of cells grown in C medium containing DL-norleucine ($2 \cdot 10^{-2}$ *M*) and L-[^{35}S]methionine (10^{-4} *M*) gave elution patterns similar to Fig. 1. A significant difference was a *uniform reduction* in the specific radioactivities of these bacterial proteins compared to those of the control experiment (Fig. 2). The existence of certain markers (peaks, valleys, enzymes, etc.) along the elution diagram allows a quantitative comparison, marker for marker, among several column runs. Fig. 4 shows the specific radioactivities of seven well marked and separated regions obtained with the norleucine grown cells. These are compared to the same regions in the control experiment, where the specific radioactivity of each region was arbitrarily chosen to equal 100.

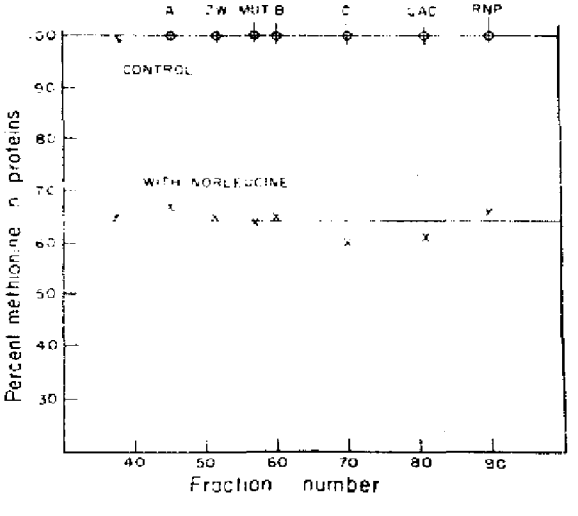

Fig. 4. Comparison of specific radioactivities of definite regions along elution diagrams obtained from *E. coli* ML 30d cells grown in C-medium containing L-[^{35}S]methionine (10^{-3} *M*) (control) and from cells grown in C-medium containing L-[^{35}S]methionine (10^{-4} *M*) plus DL-norleucine ($2 \cdot 10^{-2}$ *M*). Linear growth was obtained in the latter culture and the cells were harvested for analysis after more than a doubling of bacterial mass.

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The regions compared in Fig. 4 were: 2 well resolved and isolated protein peaks A and B; the ribonucleoprotein peak (Fig. 1); and the 3 peaks of enzyme activity (LAC, MUT, and ZW) (Fig. 3) easily measurable in both experiments, and region C (Fig. 1). Each point in Fig. 4 represents the arithmetical mean of the specific radioactivity of the maximum peak sample and the two samples immediately preceding and the two following this peak.

Fig. 5 shows an elution pattern obtained from cells grown in DL-[^{14}C]norleucine ($2 \cdot 10^{-2}$ *M*) and nonradioactive L-methionine (10^{-4} *M*). In this experiment there was a 43 % substitution of norleucine for methionine in the bacterial proteins. Radioautograms of two dimensional paper chromatograms of hydrolysates of the bacterial extract showed one radioactive, ninhydrin-positive spot having the same R_F as found with the labeled norleucine used in this experiment.

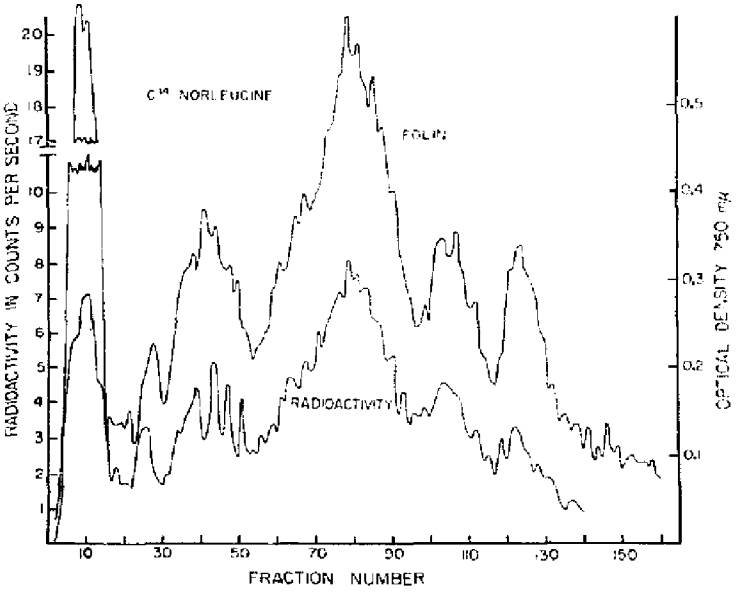

Fig. 5. Elution pattern of bacterial extract of DL-[1-^{14}C]norleucine grown cells. Mutant *E. coli* cells (ML 304d) grown for more than two doublings of bacterial mass in C-medium containing labeled norleucine ($2 \cdot 10^{-2}$ *M*) and L-[^{35}S]methionine (10^{-4} *M*).

There is a great deal of similarity in the elution diagrams obtained from the [^{35}S]methionine (Fig. 1) and the [^{14}C]norleucine labeled cells (Fig. 5). One significant difference, however, occurs in the first major peak of the elution diagrams. In these early fractions of eluted material are contained the non-protein amino acids (or analogs) concentrated by the cell from the environment. The quantity of "free amino acids" depends upon their external concentrations and in these experiments the ratio of DL-[^{14}C]norleucine to L-[^{35}S]methionine in the media was 100 to 1. Chemical fractionation of the eluted fractions showed that TCA-soluble material ("free amino acids") was mainly contained in the first 20 samples and dropped rapidly to a few per cent by the 35th sample, remaining low for the rest of the elution process. It has also been noted that the quantity of material contained in the ribonucleoprotein region varies from one column run to another and if the cells are ruptured in media

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containing phosphate buffer or in buffer containing an inadequate magnesium concentration this ribonucleoprotein is not seen at all.

DISCUSSION

The above results demonstrate that most of the proteins formed in the presence of the analog are not radically different molecular species, but are physicochemically similar to the proteins normally synthesized. The similarity of the elution diagrams obtained with the labeled methionine and norleucine (Figs. 1 and 3) also eliminates the suggestion that only certain proteins are susceptible to analog substitution. Indeed Fig. 4 demonstrates that the analog is incorporated into all of the proteins examined *in the same proportion*. Each methionine incorporation site thus seems to have an equal probability of analog substitution. The formation of a large quantity of uncompleted proteins caused by the joining of the analog by a peptide bond to one of its neighboring amino acids, but not to the other does not seem to be a probable event. Should such unfinished molecules be present, they would markedly alter the elution patterns obtained after the analog is incorporated.

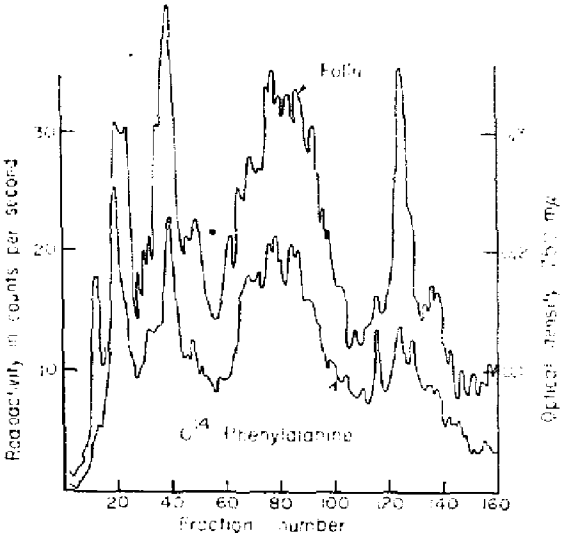

Fig. 6. Elution pattern of bacterial extract obtained with DEAE ion-exchange column. Wild type *E. coli*, ML 30 grown in C-medium containing DL-[3-^{14}C]phenylalanine (10^{-4} *M*) plus DL-parafluorophenylanine-($5 \cdot 10^{-3}$ *M*). Linear growth was obtained and the cells were harvested for analysis after more than a doubling of cellular mass.

These conclusions are strengthened by data obtained in expts. using other amino acid analogs. Fig. 6 shows the elution diagram obtained from wild type *E. coli* (ML 30) grown in C-medium containing DL-[3-^{14}C]phenylalanine (10^{-4} *M*) and DL-*p*-fluorophenylalanine ($5 \cdot 10^{-3}$ *M*). At these concentrations there is approximately a 50% substitution of the analog for protein phenylalanine, and linear growth occurs. The elution diagram obtained (Fig. 6) appears quite similar to that obtained from normal cells (Fig. 1). There is no evidence of different types of "protein classes" being formed as a result of analog substitution.

Fig. 7 demonstrates, within the limits of resolution of the column that *p*-fluorophenylalanine is incorporated into all the bacterial proteins. This elution diagram was obtained from wild type *E. coli* grown in C-medium containing DL-[3-^{14}C]*p*-fluorophenylalanine ($5 \cdot 10^{-3}$ *M*).

The use of another amino acid analog gave results which in every respect confirm

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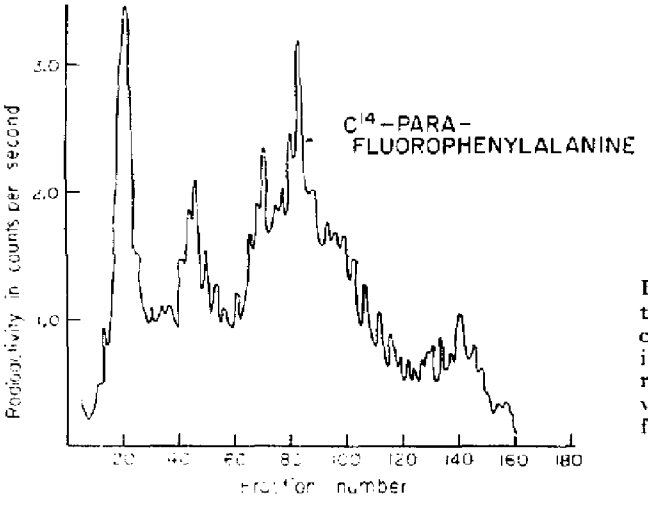

Fig. 7. Elution pattern of bacterial extract obtained with DEAE ion exchange column. Wild type *E. coli*, ML 30 grown in C-medium containing DL-[3-^{14}C] *p*-fluorophenyalanine ($5 \cdot 10^{-3}$ *M*). Linear growth was obtained and the cells were harvested for analysis after more than a doubling of cellular mass.

and augment the conclusions cited above. Selenomethionine *completely* substitutes for the methionine of the bacterial protein[4]. With this *uniform* replacement exponential growth was observed and the induction and synthesis of active β-galactosidase demonstrated. The constitutive enzymes, essential for exponential growth were obviously present in active forms. Under these conditions there can be little doubt that active altered proteins are synthesized, having biological as well as physicochemical properties similar to those of the normal cell.

The use of amino acid analogs other than selenomethionine has always resulted in linear growth of the cells whenever analog substitution in the protein was evident. Thus, it might be argued that at least one growth-rate-limiting enzyme was unusually susceptible to analog substitution, and the enzyme molecules are synthesized at a reduced rate, if they are synthesized at all. An alternative hypothesis would be that during analog incorporation the protein molecules continue to be synthesized but these would be proteins with reduced capacity for enzymic function. This elimination (or depression) of enzyme activity would depend upon the degree and kind of substitution involved and on the amino acid composition of the sites of enzyme action in the protein molecule. Some evidence supporting the latter hypothesis has accumulated and the investigation of this question is currently under way.

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