

## The mutagenicity of amino acid analogues in *Coprinus lagopus*

BY PHILIPPA J. TALMUD AND D. LEWIS

*Department of Botany and Microbiology, University College London*

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### SUMMARY

The amino acid analogues *p*-fluorophenylalanine (PFP) and ethionine (ETH) are strongly mutagenic in *Coprinus lagopus*. The most pronounced effect was found with suppressor mutations of the *met-1* locus. PFP, at a concentration of  $2.4 \times 10^{-4}$  M, increased the mutation frequency 500 fold and ETH, at a concentration of  $2.4 \times 10^{-3}$  M, 30 fold over the spontaneous mutation frequency. From the spectrum of suppressors of the *met-1* locus and the dominant revertants of the *ad-8*<sup>2</sup> locus, induced by analogue treatments, it was concluded that both analogues induce single base-change mutations. The dose response curves follow a sigmoid plot, revealing that within a certain range of analogue concentrations, mutagenesis is strongly dose dependent.

Using analogue resistant mutants, it has been shown that PFP mutagenesis is a function of its incorporation into protein. However, ETH mutagenesis is independent of protein incorporation but can be correlated with the degree of ethylation of nucleic acids. The synergistic effect PFP and ETH supports the evidence of the different mutagenic actions of the two analogues.

### 1. INTRODUCTION

It has been shown that many biochemical processes, related to nuclear function, can be mutagenic. They act by altering the accuracy of DNA replication as a result of malfunctioning biochemical and metabolic processes. There are now many examples of this; a mutant of gene 43, coding for the DNA polymerase in *T<sub>4</sub>*, has mutator gene activity (Speyer, 1965) and an increasing number of mutator strains have been identified in a variety of organisms (Drake, 1970), including several UV-sensitive mutants (Böhme, 1967; Jansen, 1972). Recently Springgate & Loeb (1973) have shown that the DNA polymerase of acute lymphatic leukaemia cells has greatly reduced fidelity. Furthermore, Zimmerman, Schwaier & Laer (1966), Auerbach & Ramsay (1967) and Kilbey (1969) have revealed that a number of factors (temperature, buffering, genetic background, etc.) indirectly modify mutation frequencies.

Amino acid analogues have a wide variety of effects on cellular biosynthesis, affecting feed-back inhibition and false regulation of protein, DNA and RNA synthesis (Fowden, Lewis & Tristram, 1967). The work of Janacek and co-workers demonstrated that the incorporation of a range of analogues into the  $\beta$ -galactosidase of *E. coli* could have a varying effect on its stability and activity (Janacek &

Rickenberg, 1964; Janacek, 1967; Janacek & Spizek, 1969; Spizek & Janacek, 1967, 1969).

Langridge (1968), in his study on the reversion of 56 amber mutants of  $\beta$ -galactosidase by suppressor mutants in *Escherichia coli*, concluded that it was the position in the polypeptide and not the amino acid itself that was the crucial factor in the restoration of active enzyme production.

It is plausible, therefore, that some analogue incorporation into DNA replication and repair enzymes could result in reduced fidelity and cause mistakes to be made.

Analogue induced mutagenesis has been reported in *Chlamydomonas* (McBride & Gowan, 1969) and in *Ustilago* (Lewis & Tarrant, 1971).

The aim of this report is threefold: first, to substantiate analogue mutagenesis in *Coprinus lagopus*, secondly, to define the mutations induced and finally to identify the mode of analogue mutagenesis by using a range of analogue resistant strains varying in the amount of analogue incorporated into protein and the degree of ethylation of nucleic acid fractions.

## 2. MATERIALS AND METHODS

### (i) *Strains*

Wild-type *Coprinus* is sensitive to only two out of a range of amino acid analogues tested – these are DL-*p*-fluorophenylalanine (B.D.H.) and DL-ethionine (Koch-Light). The mutagenic effect of these two analogues was tested on the reversion frequencies of two auxotrophic strains of different genetic backgrounds. Strain PR 2301 (*met-1*), spontaneous isolate which reverts by a series of five suppressor loci (Lewis, 1961) and strain NG 184 (*ad-8<sup>2</sup>*), induced by nitrosoguanidine by Dr L. Casselton. This strain reverts by back mutation. More recently a canavanine sensitive strain has been isolated by S. Senathirajah in our laboratory. The mutagenic effect of L-canavanine (Nutritional Biochemicals Corp.) was studied on strain RS 3 (*can<sup>s</sup> met-1*) and strain J 2397/54 (*can<sup>s</sup> met-1 arg-1*). Details of other strains used in this investigation are described in the text.

### (ii) *Culture media*

Complete, minimal and supplemented media for auxotrophs have been described by Lewis (1961) and Casselton (1965). Standard culturing techniques were used (Lewis, 1961) and unless otherwise stated all experiments were carried out on solid media.

### (iii) *Analogue treatment*

Comparison of equimolar concentrations of mutagens is not meaningful in cellular systems (Kølmark, 1956). Furthermore, strains of *Coprinus* vary in their sensitivity to these analogues. For this reason comparisons were made on the basis of the percentage growth inhibition obtained from radial growth measurements on analogue supplemented CM. Where possible, a range of analogue concentrations

Abbreviations: PFP, para-fluorophenylalanine; ETH, ethionine; CAN, canavanine; CM, complete medium; MM, minimal medium.

were used reaching a maximum of 60% growth inhibition. Details of the concentrations appear in the text. In all cases the analogues were added as filtered sterile solutions to CM.

Analogue treatment was divided into two parts. First, a short exposure to the analogue by inoculating the strain on analogue supplemented CM at a concentration determined by the growth tests. An analogue free control was set up. These plates were incubated at 37 °C for 48 h. Small inocula taken from the edge of these growing colonies were used to multi-inoculate a second set of analogue plates, always at the same concentration as the first set. (This guaranteed that oidia harvested from the second treatment plates would all have incorporated the analogue.) Treatment plates were incubated at 37 °C for 96 h in the dark. Oidia were harvested into sterile distilled water, filtered through a glass-wool filter and plated, at the appropriate density, onto MM reversion plates and viability plates of MM supplemented with the auxotrophic requirement. Viability plates were scored after 72 h. Mutation counts were made 4–7 days after incubation. All potential revertants were retested on MM and on MM supplemented with  $10^{-4}$  M PFP or  $6 \times 10^{-4}$  M ETH (depending on the mutagen) to test for resistance to the analogue.

#### (iv) Complementation test

This test was used for the characterization of suppressors of the *met-1* locus. In the case of recessive suppressors, non-complementation represents homozygosity of the suppressors and therefore growth on MM, while complementation restores the requirement and there is no growth on MM (Lewis, 1961). The five suppressors fall into three complementation groups. However, certain tester strains are rigorous in showing complementation between *su-3*, *su-4* and *su-5* which fall into complementation group 3.

#### (v) Incorporation of methyl- $^{14}$ C-methionine and ethyl- $^{14}$ C-ethionine into the various cell fractions

Oidia, harvested from 5 to 7-day-old cultures grown on CM, were used to inoculate 25 ml aliquots of liquid MM to give a final concentration of  $10^6$ – $10^7$  oidia/ml. Cultures were incubated for 36 h at 37 °C on a parallelogram action shaker, at the end of which time small discrete colonies could be seen.

8 ml samples were aerated in boiling tubes in a 30 °C waterbath and allowed to equilibrate for 30 min. At time zero the radioactive source was added to give a final concentration of  $0.625 \mu\text{C/ml}$  L-methyl- $^{14}$ C-methionine and L-ethyl- $^{14}$ C-ethionine (Radiochemical Centre, Amersham).

The sampling procedure was based on that of Tristram & Neale (1968). The level of radioactivity in the cold TCA (4°) insoluble and hot TCA (90°) insoluble fractions was assessed on a Packard scintillation counter.

Table 1. Results of the reversion frequencies of PR 2301 (met-1) after treatment with varying concentrations of PFP and ETH supplemented to CM

| Concentration         | PFP                    |                        |                        |                        |                        | ETH                    |                        |                        |                        |                        |
|-----------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
|                       | $5.0 \times 10^{-5}$ M | $7.5 \times 10^{-5}$ M | $1.0 \times 10^{-4}$ M | $2.5 \times 10^{-4}$ M | $5.0 \times 10^{-4}$ M | $1.2 \times 10^{-3}$ M | $1.8 \times 10^{-3}$ M | $2.4 \times 10^{-3}$ M | $3.0 \times 10^{-3}$ M | $3.6 \times 10^{-3}$ M |
| Growth inhibition (%) | Control                | 0                      | 26.0                   | 32.2                   | 53.0                   | 64.3                   | 24.0                   | 36.0                   | 64.0                   | 64.0                   |
| No. revertants        | 749                    | 650                    | 317                    | 134                    | 134                    | 125                    | 136                    | 112                    | 112                    | 112                    |
| Total viable no.      | $6.8 \times 10^7$      | $1.6 \times 10^8$      | $2.9 \times 10^8$      | $1.9 \times 10^8$      | $2.4 \times 10^8$      | $1.4 \times 10^8$      | $9.2 \times 10^8$      | $3.1 \times 10^8$      | $3.1 \times 10^8$      | $3.1 \times 10^8$      |
| Revertants/ $10^8$    | 1.1                    | 4.1                    | 18.5                   | 161                    | 555                    | 8.8                    | 14.7                   | 36.2                   | 36.2                   | 36.2                   |
| Increase over control |                        | $3.7 \times$           | $16.9 \times$          | $147 \times$           | $507 \times$           | $7.5 \times$           | $12.5 \times$          | $30.6 \times$          | $30.6 \times$          | $30.6 \times$          |

Table 2. The effect of analogue treatments on the viability of strain PR 2301

| Experimental repeats | Viability (%)          |                        |                        |                        |                        |                        |                        |                        |                        |                        |
|----------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
|                      | PFP                    |                        |                        |                        |                        | ETH                    |                        |                        |                        |                        |
|                      | $5.0 \times 10^{-5}$ M | $7.5 \times 10^{-5}$ M | $1.0 \times 10^{-4}$ M | $2.5 \times 10^{-4}$ M | $5.0 \times 10^{-4}$ M | $1.2 \times 10^{-3}$ M | $1.8 \times 10^{-3}$ M | $2.4 \times 10^{-3}$ M | $3.0 \times 10^{-3}$ M | $3.6 \times 10^{-3}$ M |
| Control              | 10.0                   | —                      | 9.0                    | 7.0                    | 5.0                    | 12.5                   | 11.7                   | 13.5                   | —                      | —                      |
| 1                    | 54.0                   | 41.0                   | 35.0                   | 50.0                   | —                      | —                      | —                      | —                      | —                      | —                      |
| 2                    | 13.6                   | —                      | —                      | —                      | —                      | 23.6                   | 14.6                   | 25.0                   | —                      | —                      |
| 3                    | 12.9                   | 10.0                   | 8.5                    | 10.0                   | 12.0                   | —                      | —                      | —                      | —                      | —                      |
| 4                    |                        |                        |                        |                        |                        |                        |                        |                        |                        |                        |

## 3. RESULTS

(i) *The mutagenic effects of PFP and ETH*

The reversion frequencies of strain PR 2301, after growth on varying concentrations of PFP and ETH are given in Table 1.

The dose response curve for PFP is sigmoid; a similar curve was obtained with ETH. At low analogue concentrations the reversion frequencies increase at rates that are almost quadratic, the increase becomes exponential and finally reaches a plateau.

Another striking feature of analogue mutagenesis is that even at analogue concentrations giving more than 60% growth inhibition, the viability of the oidia produced is not affected. The results in Table 2 show that although viabilities fluctuate between experimental repeats, they are constant within the same experiment covering a range of analogue concentrations. Even under the conditions of standardization maintained in these experiments it was impossible to control the factors which affect oidial sensitivity (Rahman and Cowan, personal communication).

Table 3. *The effect of PFP and ETH on the reversion frequency of NG 184 (ad-8<sup>2</sup>)*

|                            | Control                | 2.5 × 10 <sup>-5</sup> M PFP | 2.0 × 10 <sup>-4</sup> M ETH |
|----------------------------|------------------------|------------------------------|------------------------------|
| Growth inhibition %        | 0                      | 33.0                         | 36.5                         |
| Viability range            | 2.9%–18.7%             | 4.2%–17.7%                   | 1.8%–17.5%                   |
| No. revertants             | 330                    | 130                          | 70                           |
| Total viable no.           | 4.2 × 10 <sup>10</sup> | 5.7 × 10 <sup>8</sup>        | 4.3 × 10 <sup>8</sup>        |
| Revertants/10 <sup>9</sup> | 7.6                    | 225                          | 162                          |
| Increase over control      | —                      | 29.6 ×                       | 21.3 ×                       |

In the case of strain NG 184 (*ad-8<sup>2</sup>*) it was not possible to construct a dose response curve, the reasons being twofold. Firstly, the reversion frequency of the *ad-8<sup>2</sup>* locus is considerably lower than that of the suppressors of the *met-1* locus and secondly, at high analogue concentrations oidial production was greatly reduced so reversion frequencies could not be established. The combined results of the effect of PFP and ETH on the reversion frequency of the *ad-8<sup>2</sup>* locus, taken from four experiments, appear in Table 3.

A comparison of the mutagenic effects of PFP and ETH on the *met-1* and *ad-8<sup>2</sup>* loci will be discussed later.

(ii) *Problems arising from mutational studies*(a) *Grigg effect*

The increase of reversion frequencies and consequent lower concentration of oidia on reversion plates, after analogue treatment, posed the problem that the higher oidial concentrations on control reversion plates necessary to measure the lower reversion frequencies, might result in the 'Grigg effect', namely suppression of growth of revertants in the controls (Grigg, 1952). It was therefore necessary to set up a reconstruction experiment to test the 'Grigg effect' (Table 4).

Table 4. Reconstruction experiment to test the 'Grigg effect'

| No. of oidia plated                  | { PR 2301 |    | 0   | 2.8 × 10 <sup>6</sup> | 5.6 × 10 <sup>6</sup> | 2.8 × 10 <sup>6</sup> | 5.6 × 10 <sup>6</sup> | 2.8 × 10 <sup>7</sup> |
|--------------------------------------|-----------|----|-----|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
|                                      | { PRS 19  |    |     |                       |                       |                       |                       |                       |
| No. of colonies on MM                | 46        | 15 | 460 | 0                     | 46                    | 460                   | 460                   | 46                    |
| Expected no. of colonies on MM from  | —         | —  | —   | —                     | 2.8                   | 14                    | 28                    | 140                   |
| Total expected no. of colonies on MM | —         | —  | —   | —                     | 15                    | 141                   | 141                   | 15                    |
|                                      | —         | —  | —   | —                     | 17.8                  | 155                   | 169                   | 155                   |

Strain PRS 19 (*met-I su-4*) is a revertant of PR 2301 (*met-I*)

Table 5. Analysis of variance from fluctuation tests of PFP ( $7.5 \times 10^{-5}$  M) treatments of strain PR 2301

| Mean mutation frequency × 10 <sup>6</sup> | Control |      | 7.5 × 10 <sup>-5</sup> M PFP |       |
|---|---------|------|------------------------------|-------|
|   | 2.7     | 13.4 | 2.6                          | 5.3   |
| Variance between independent samples      | (MS)    | 2.7  | (MS)                         | 2.7   |
| Analysis of variance                      | DF      | 39   | DF                           | 49    |
| Between groups                            | F ratio | 27.2 | F ratio                      | 1.7   |
| Within groups                             | (MS)    | 0.1  | (MS)                         | 0.002 |

T-test between control and PFP: *t* = 10.8.

From these results we eliminated the likelihood of the 'Grigg effect' being operative.

(b) *Fluctuation test* (Luria & Delbrück, 1943)

Fluctuation tests were performed to investigate the high frequency of revertants induced by PFP in strain PR 2301. A routine test was always performed to see if revertants had been selected for by becoming analogue resistant. Over 1000 revertants were tested, not one was found to be resistant to either PFP or ETH. Furthermore, growth rate studies showed that revertants isolated from minimal medium reversion plates, after analogue treatment, had normal growth rates in the presence or absence of analogue, when compared to the mutant parent.

Revertants induced by PFP should show a higher variance value for independent cultures than for sample repeats from one culture. Experiments were performed using a PFP concentration of  $7.5 \times 10^{-5}$  M and a control was set up at the same time. Fifty samples after PFP treatment and forty of the control, from independent cultures, were tested for mutation frequencies and from each independent culture five samples were taken. The results were computerized and are shown in Table 5 as an analysis of variance.

The results show a significant difference between control and analogue treatments. However, an interesting point emerges from this study. While spontaneous reversions fit the Poisson distribution as expected, after PFP treatment there is a deviation from the Poisson distribution, indicating that most of the analogue induced mutations arise towards the end of the treatment period (Table 6).

Table 6. *Correlation of results from the fluctuation test with the Poisson distribution*

| Control                        |      |      |      |     |     |     |      |     |
|--------------------------------|------|------|------|-----|-----|-----|------|-----|
| No. of mutants                 | 0    | 1    | 2    | 3   | 4   | 5   | 6    | 7   |
| Expected frequency             | 2.3  | 6.9  | 9.6  | 8.9 | 6.1 | 3.3 | 1.5  | 0.6 |
| Actual frequency               | 1    | 7    | 13   | 8   | 5   | 3   | 2    | 1   |
| $\chi^2_{(6)} = 2.6, p = 0.8$  |      |      |      |     |     |     |      |     |
| $7.5 \times 10^{-5}$ M PFP     |      |      |      |     |     |     |      |     |
| No. of mutants                 | 0    | 1    | 2    | 3   | 4   | 5   | 6    |     |
| Expected frequency             | 13.3 | 17.6 | 11.6 | 5.1 | 1.6 | 0.4 | 0.09 |     |
| Actual frequency               | 5    | 28   | 13   | 4   | 0   | 0   | 0    |     |
| $\chi^2_{(5)} = 9.9, p = 0.07$ |      |      |      |     |     |     |      |     |

(iii) *The effect of CAN on the reversion frequency of the met-1 locus*

The mutagenic effect of CAN was tested in two strains of *Coprinus*, RS 3 (*met-1 can<sup>s</sup>*) and J 2397/54 (*arg-1 met-1 can<sup>s</sup>*), CAN, at a concentration of  $2.0 \times 10^{-4}$  M and giving 60% growth inhibition, had no effect on the reversion frequency of the *met-1* locus in either strain. Lewis & Tarrant (1971) reported that while CAN was not mutagenic in *arg<sup>+</sup>* strains of *Ustilago* it was highly mutagenic in an arginine auxotroph.



(iv) *Analysis of the types of revertants induced by PFP and ETH*

Spontaneous revertants of *ad-8*<sup>2</sup> are without exception dominant back mutations. Two UV induced revertants have been analysed as recessive suppressors mapping 2 map units from *ad-8*<sup>2</sup>. Twenty revertants from both PFP and ETH treatments were tested for dominance by crossing to a compatible strain MAE 218 (*ad-8*). All the revertants were dominant, suggesting a back mutation at the original site.

The *met-1* locus reverts by a series of five suppressors falling into three complementation groups (Lewis, 1961). While complementation groups 1 and 2, composed of suppressors 1 and 2 respectively, are thought to be by-pass suppressors (Shahriari & Casselton, 1973) and probably cover a wide range of mutations, complementation group 3 (*su-3*, *su-4* and *su-5*) has been suggested to be composed of missense suppressors specifying modified tRNA species (Todd & Casselton, 1972).

The revertants of PR 2301, induced by PFP, ETH and spontaneously, were classified into the three complementation groups on the basis of the complementation test. The results are given in Table 7.

Table 7. *The spectrum of induction of the 3 complementation groups of suppressors of the met-1 locus induced by a range of analogue concentrations*

| Treatment                    | No. of mutations/10 <sup>5</sup> viable oidia in |         |         |
|------------------------------|--|---------|---------|
|                              | Group 1  | Group 2 | Group 3 |
| Control                      | 0.7  | 0.2     | 0.06    |
| 5.0 × 10 <sup>-5</sup> M PFP | 3.4  | 0.1     | 0.4     |
| 7.5 × 10 <sup>-5</sup> M PFP | 8.2  | 0.1     | 8.2     |
| 1.0 × 10 <sup>-4</sup> M PFP | 150  | 8.1     | 320     |
| 2.5 × 10 <sup>-4</sup> M PFP | 148  | 93      | 167     |
| 1.2 × 10 <sup>-3</sup> M ETH | 5.3  | 0.2     | 2.8     |
| 1.8 × 10 <sup>-3</sup> M ETH | 8.1  | 0.7     | 5.2     |
| 2.4 × 10 <sup>-3</sup> M ETH | 7.7  | 3.8     | 18.3    |

Although not represented as independent observations, the suppressors from all treatments were taken from at least three independent experiments and fall roughly into the same spectrum, indicating that both PFP and ETH induce specific mutations.

PFP and to a lesser extent ETH causes a striking increase in group 3 suppressors of *met-1*.

(v) *Comparison of the increase of group 3 suppressors of met-1 and revertants of ad-8<sup>2</sup>*

A comparative analysis of the occurrence of group 3 suppressors with *ad-8*<sup>2</sup> revertants, based on previous results, is given in Table 8.

From Table 8 the mutagenic effect of the analogues for group 3 suppressors and *ad-8*<sup>2</sup> revertants is in the ratio of 4:1. If consideration is taken of the higher



Table 8. Comparison of the number of group 3 suppressors of met-1 locus and the number of revertants of ad-8<sup>2</sup> locus after PFP and ETH treatment

(Based on data from Tables 1, 3 and 7.)

|  | PR 2301 (met-1)        |                              |                              | NG 184 (ad-8 <sup>2</sup> ) |                              |                              |
|--|------------------------|------------------------------|------------------------------|-----------------------------|------------------------------|------------------------------|
|  | Control                | 7.5 × 10 <sup>-5</sup> M PFP | 1.8 × 10 <sup>-3</sup> M ETH | Control                     | 2.5 × 10 <sup>-5</sup> M PFP | 2.0 × 10 <sup>-4</sup> M ETH |
| Growth inhibition (%)                          | 0                      | 32.2                         | 36.0                         | 0                           | 33.0                         | 36.5                         |
| Group 3 suppressors or dominant revertants (%) | 5.8                    | 44.3                         | 30.4                         | 100                         | 100                          | 100                          |
| No. group 3 suppressors or dominant revertants | 6.2 × 10 <sup>-7</sup> | 8.2 × 10 <sup>-5</sup>       | 5.2 × 10 <sup>-5</sup>       | 7.9 × 10 <sup>-9</sup>      | 2.2 × 10 <sup>-7</sup>       | 1.6 × 10 <sup>-7</sup>       |
| Increase over control                          | ---                    | 132 ×                        | 83 ×                         | ---                         | 29.5 ×                       | 21.3 ×                       |

concentrations of analogues used for the suppressor experiments and the fact that there are three suppressor loci, the results show that the effects on the suppressors and *ad-8<sup>2</sup>* are the same.

(vi) *Analogue resistant mutants and the mechanism of analogue induced mutagenesis*

A number of analogue resistant loci, with alterations in the amount of analogue incorporated into protein, were studied to elucidate what effect these loci had on analogue induced mutagenesis.

*Pfp<sup>r</sup>-4* is the phenylalanyl-tRNA synthetase gene. On the basis of ATP-<sup>32</sup>P-PP exchange assay, Barker (1970) found that the ability of strain F 111 (*pfp<sup>r</sup>-4*) to activate PFP was reduced to a fifth of the wild-type control. From <sup>14</sup>C-PFP incorporation studies into strain F 111 she found that only 7% of the radioactivity incorporated by wild-type H2 was incorporated into the protein of F 111 (Barker, 1970).

We studied the effect of the *pfp<sup>r</sup>-4* locus on PFP induced mutagenesis in a recombinant strain PRS 54 (*pfp<sup>r</sup>-4 met-1*) derived from the cross F111 × PR2301. The results appear in Table 9.

Table 9. *The effect of PFP and ETH treatment on PRS 54 (met-1 pfp<sup>r</sup>-4)*

|                       | Control                | 1.0 × 10 <sup>-4</sup> M PFP | 2.1 × 10 <sup>-3</sup> M ETH |
|-----------------------|------------------------|------------------------------|------------------------------|
| Growth inhibition (%) | —                      | 4                            | 37.5                         |
| Mutation frequency    | 1.4 × 10 <sup>-6</sup> | 1.6 × 10 <sup>-5</sup>       | 1.4 × 10 <sup>-5</sup>       |
| Increase over control | —                      | 11.3 ×                       | 9.5 ×                        |

Comparing these results to the effect of both analogues on parental strain PR 2301, at comparable growth inhibition, ETH treatment gave normal ETH mutagenesis. However, PFP mutagenicity at 10<sup>-4</sup> M is reduced to 7.7% of the increase in mutation frequency found in PR 2301.

The correlation between the reduced mutagenicity (7.7%) of PFP in PRS 54 as compared to PR 2031 and the 7% incorporation of PFP from Barker's incorporation studies, led us to conclude that PFP activation and hence incorporation into protein (Arnstein & Richmond, 1964) was an essential aspect of PFP induced mutagenesis.

Two ETH resistant mutants were used in the investigation. Strain E2 (*eth<sup>r</sup>-1*) has an altered methionyl-tRNA synthetase, while strain MS 13 (*eth<sup>r</sup>-2 met-1 su-5*), although not fully characterized, incorporated large amounts of <sup>35</sup>S-ETH into its protein (Lewis, 1963). To study the effect of the *eth<sup>r</sup>* loci on ETH mutagenesis, two auxotrophic recombinants were extracted from crosses with NG 184 and the effect of ETH on the reversion frequency of *ad-8<sup>2</sup>* locus was investigated. The results appear in Table 10.

Two contrasting results emerge. ETH has no effect on the reversion of *ad-8<sup>2</sup>* in PK 4 (*eth<sup>r</sup>-2 ad-8<sup>2</sup>*) while it is strongly mutagenic in PK 49 (*eth<sup>r</sup>-1 ad-8<sup>2</sup>*). To clarify these results, studies were made of the distribution of methyl-<sup>14</sup>C-methionine and

Table 10. *The effect of ETH on the reversion frequencies of PK4 (eth<sup>r</sup>-2 ad-8<sup>2</sup>) and PK 49 (eth<sup>r</sup>-1 ad-8<sup>2</sup>)*

|                       | PK 4 (eth <sup>r</sup> -2 ad-8 <sup>2</sup> ) |                              | PK 49 (eth <sup>r</sup> -1 ad-8 <sup>2</sup> ) |                              |
|-----------------------|---|------------------------------|--|------------------------------|
|                       | Control                                       | 2.0 × 10 <sup>-3</sup> M ETH | Control  | 2.0 × 10 <sup>-3</sup> M ETH |
| Mutation frequency    | 3.5 × 10 <sup>-7</sup>                        | 1.4 × 10 <sup>-7</sup>       | 2.1 × 10 <sup>-7</sup>                         | 1.1 × 10 <sup>-5</sup>       |
| Increase over control | —   | 0.4 ×                        | —  | 51.5 ×                       |

Table 11. *Comparison of the incorporation of methyl-<sup>14</sup>C-methionine and ethyl-<sup>14</sup>C-ethionine into the cell fractions of strains H5, MS13 and E2 after 80 min*

(Expressed as cps/10<sup>-4</sup> g. dry weight.)

|                         | H5                   |                     | MS 13                |                     | E2                   |                     |
|-------------------------|----------------------|---------------------|----------------------|---------------------|----------------------|---------------------|
|                         | <sup>14</sup> C-METH | <sup>14</sup> C-ETH | <sup>14</sup> C-METH | <sup>14</sup> C-ETH | <sup>14</sup> C-METH | <sup>14</sup> C-ETH |
| Cold (4°) TCA insoluble | 341.0                | 14.0                | 254.3                | 10.0                | 108.5                | 8.6                 |
| Hot (90°) TCA insoluble | 294.2                | 7.5                 | 210.9                | 8.6                 | 62.5                 | 3.6                 |
| *Nucleic acid           | 46.8                 | 6.5                 | 43.4                 | 1.4                 | 46.0                 | 5.0                 |

\* By difference between the radioactivity in the cold TCA insoluble and hot TCA insoluble fractions.

Table 12. *Comparison of radioactivity on a percentage basis*

|  | H5  | MS 13 | E2   |
|--|-----|-------|------|
| Incorporation <sup>14</sup> C-ETH into protein fraction      | 100 | 111.4 | 48   |
| Incorporation <sup>14</sup> C-ETH into nucleic acid fraction | 100 | 23.8  | 77.5 |

ethyl-<sup>14</sup>C-ethionine into the cellular fractions of the two parental strains MS 13 and E2 and wild-type H5 (Table 11).

Despite the differential uptake of <sup>14</sup>C-methionine, the distribution of the label in cellular fractions is comparable in all three strains. <sup>14</sup>C-ethionine incorporation generally is greatly reduced and while most of the label can be traced to the protein fraction of MS13, the reverse is true for E2. By taking incorporation into wild-type H5 to be 100% the following results emerge (Table 12).

These results show that there is little correlation between the mutagenicity of ETH and its incorporation into protein, but led us to conclude that ETH mutagenicity is more likely a function of transethylation of nucleic acids.

(vii) *Mutational synergism*

The mutational synergism between chemical mutagens and UV irradiation has been reported in a number of organisms (Witkin, 1958; Shankel, 1962; Auerbach, 1969). In all cases the synergistic effect is explicable in terms of an interaction between the chemical mutagens and UV repair mechanisms. Since PFP and ETH seem to affect different cellular processes, it seemed likely that combined analogue treatments would reflect whether the mutational pathways of PFP and ETH are

different and interact. Synergism between the two analogues was tested by supplementing both analogue to CM and harvesting the oidia after the normal growth period. The combined treatment produced a reversion frequency of  $2.2 \times 10^{-3}$  as compared to the expected additive reversion frequency of  $6.3 \times 10^{-4}$ , that is a 3.5 fold increase.

From these results we concluded that the two analogues affect different but interacting processes.

#### 4. DISCUSSION

The conclusion that is drawn from the results is that both analogues behave as strong mutagens. The problem of mutation selection on both mutation and scoring media is resolved by the following evidence.

First, revertants from analogue treatments did not show an increased resistance to either analogue, nor did growth tests of revertants show increased growth rates in the presence or absence of analogue, by comparison with the mutant parent. Furthermore, scoring medium in both control and analogue treated experiments was identical, viz. minimal medium, allowing for equal selective advantages.

Secondly, results from the fluctuation test showed that after analogue treatment, the deviation from the Poisson distribution demonstrates that the mutations occur late in the treatment period; whereas if selection was operating the deviation would in fact be in the opposite direction.

Finally, if selection of spontaneous mutants was operating, sectors in the mycelium would be observed. None were found.

The results reveal the two major problems arising from the investigation.

1. Mutagenesis and lethality.
2. The mutagenic action of amino acid analogues.

##### (i) *Mutagenesis and lethality*

Both analogues ETH and PFP are strongly mutagenic in *Coprinus*. At  $2.4 \times 10^{-4}$  M PFP, the increase over the spontaneous reversion frequency of PR 2301 is comparable to a 6 minute dose of UV. An efficient mutagen is one which not only increases the mutation frequency, but has little effect on lethality. Analogue treatments differ from most mutagen treatments since they are incorporated into the growth medium and treatments are carried out over a period of 96 h. In *Coprinus* the mycelium which is treated, consists of uninucleate cells and tips with two to three nuclei in which nuclear division occurs. The mutations induced by analogues would occur in the tip cells during DNA replication. Any lethal mutations produced would therefore be selected against and would rarely, if ever, find their way into the oidia.

Furthermore, because the analogues are highly efficient in producing back mutations, they act by single base changes. Probably less than 1% of base changes are lethal. The low lethal production, the contraselection and the naturally low and variable germination rate of oidia are sufficient reasons for there to be no detectable effect of the analogues on lethality.

(ii) *The mutagenic action of amino acid analogues*(a) *Dose response curve*

We can infer something about the nature of the reaction of the mutagen and the genetic material from the dose response curves. Both analogues induce the same curves, which deviate from the 'one hit phenomenon'. Auerbach & Ramsay (1968), in their study of dose response curves in *Neurospora* K 3/17, reported that the complex curves induced by chemical mutagens reflect the secondary steps in the mutational process. With both ETH and PFP it is likely that a number of cellular events are involved in the mutagenic event which is best expressed in the dose-dependent exponential component of the curve, finally reaching a saturation point at the plateau.

(b) *Mechanism of analogue mutagenesis*

Despite the similarity in the dose response curves, it is evident that PFP and ETH differ in their mutagenic action. While PFP is mutagenic by protein incorporation, ETH mutagenesis is dependent upon transethylation of nucleic acids. The basis for this might be the fact that methionine is one of the rarer amino acids found in protein. This situation has probably been created by a demand for methionine in other cellular functions, so that the few methionine residues in protein are not at strategically placed catalytic sites. This might explain why, despite the large amount of ETH incorporation into *eth<sup>r</sup>-2* mutants, ETH has no effect on reversion frequencies.

Our findings are supported by the work of Faber (1963) who suggested that ETH carcinogenesis in rats might be due to the ethylation of nucleic acids. This has been substantiated by Rosen (1968) and Friedman, Shull & Faber (1969). Mendonça & Travassos (1972) speculated that ETH might be mutagenic by transethylating purines and so rendering them unstable. Ruddick & Runner (1972) have shown that ethyl-<sup>14</sup>C-ethionine specifically labels DNA in chick embryos.

In contrast, PFP mutagenicity is most likely to be due to the alteration of the fidelity of DNA replication and repair enzymes. This is supported by the behaviour of mutator genes and the mutator activity of *uvr* mutants (Böhme, 1967; Drake, 1970; Jansen, 1972).

The mutational synergism studies support this differential action of the two analogues and it is plausible that a number of enzymes involved in transmethylation and transethylation could have altered activity, due to PFP incorporation, favouring the latter process.

(c) *The molecular basis of analogue mutagenesis*

The results strongly suggest that despite their different mutagenic actions, both PFP and ETH induce single base change mutations. This is indicated by the dominant back mutations induced in *ad-8<sup>2</sup>* mutants, a mutation which was originally induced by nitrosoguanidine. This mutagen is known to induce single base changes (Smith *et al.* 1970; Baker & Tessman, 1968). Furthermore, both analogues increase the relative frequency of group 3 suppressors of the *met-1* locus

which are thought to be missense mutations (Todd & Casselton, 1972). The correlation between the induction of *ad-8<sup>2</sup>* revertants and group 3 suppressors is confirmed by the data in Table 8.

Since PFP mutagenesis in *Coprinus* appears to be a function of its incorporation into protein, it would follow that phenylalanine must occupy sites that are important for the catalytic activity of the DNA replication or repair enzymes or both. For this reason it is possible that PFP would not be mutagenic in organisms where the position of phenylalanine was not important for the activity of these enzymes.

This might explain the non-mutagenic action of CAN in *Coprinus*, while it is strongly mutagenic in *arg<sup>-</sup>* strains of *Ustilago* (Lewis & Tarrant, 1971); similarly ETH incorporation into the protein of *Coprinus*, even in large amounts, does not induce mutagenesis. However, ETH mutagenesis appears to be related to the extent of transethylation of nucleic acids. It is unlikely that ETH mutagenesis in bacteria would be due to transethylation, since ETH is not activated to S-adenosyl-ethionine (Peterkovsky, 1965); however, this does not exclude the possible mutagenicity of ETH by protein incorporation.

It is not possible, therefore, to generalize about the mutagenic action of amino acid analogues, since the factors controlling analogue mutagenicity might vary from organism to organism.

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