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The Effect of an Amino Acid Analogue, *p*-Fluorophenylalanine, on Longevity of Mice

R. Holliday and A. Stevens[1]
National Institute for Medical Research, London

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Abstract. Male and female inbred CBA mice were treated with different concentrations of *p*-fluorophenylalanine (pFPA) for 4 weeks and their subsequent longevity was recorded. Young males treated with 4×10^{-4} *M* pFPA had their average longevity reduced by 123 days in comparison to controls, but a five-fold higher concentration had less effect. 1-year-old females treated with 4×10^{-4} *M* pFPA had their subsequent average longevity reduced by 77 days, and again a higher concentration had no significant effect. The longevity of 3- to 4-week-old females and 1-year-old males was also unaffected by the analogue. The control and treated mice were marked by ear punch and mixed in cages, so environmental effects could be largely discounted. The results indicate that the incorporation of an amino acid analogue into protein can reduce longevity, as predicted by the protein error theory of ageing. It is also clear that there is a complex interrelationship between the analogue dose, the age and sex of treated animals, which will require further study.

A prediction of the protein error theory of ageing is that the partial replacement of a natural amino acid with an analogue will tend to mimic the effect of spontaneous errors in translation and therefore accelerate the normal process of ageing (*Orgel*, 1963). An essential feature of the theory is that once errors have begun to accumulate, there will be a feedback of defects into the complex machinery for transcription and translation, leading eventually to a lethal level of errors in protein synthesis. A short analogue treatment may therefore prematurely launch an irreversible build up of errors, the effect of

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which is seen only long after the treatment has been terminated. Some years ago an experiment of this type was carried out with *Drosophila melanogaster,* in which it was shown that feeding larvae with a mixture of amino acid analogues significantly shortened the lifespan of adult flies (*Harrison and Holliday,* 1967). It is now demonstrated that low concentrations of *p*-fluorophenylalanine (pFPA, an analogue of phenylalanine) added to the diet of inbred CBA mice for a 4-week period can significantly reduce their subsequent lifespan. Surprisingly, higher concentrations of the analogue had less or no effect. There were also differences in the response of male and female mice.

Methods

3- to 4-week-old inbred CBA male mice were placed in small cages and provided with drinking water containing 2×10^{-3}, 4×10^{-4} or 8×10^{-5} *M* pFPA for 4 weeks, together with a normal laboratory solid diet (Dicksons PRM). The mice were weighed before and after treatment and the volume of liquid taken was measured. After treatment the four groups of mice were marked by ear punch (left, right, both or neither ear) and randomly distributed among seven large cages, each containing approximately 20 animals. Subsequently the same experiments were set up with CBA females. The number of mice used, weight increase and volume of liquid taken in these two experiments are given in table I. In addition, experiments with a similar number of male and female mice were set up in which the 4-week treatment with the same three concentrations of pFPA, was not given until the mice were 1 year old. The details of this experiment are also given in table I.

The mice were examined every other day and any dead animals were recorded and removed. No systematic attempt was made to determine the cause of death by post-mortem examination. CBA is a very long-lived inbred strain with no specific ageing pathology and a very low incidence of tumours (*Russell,* 1966). There was no evidence of any deaths from infections.

Results

The average lifespans of the mice treated with 2×10^{-3}, 4×10^{-4} and 8×10^{-5} *M* pFPA at 3–4 weeks are given in table II. Males treated with the intermediate level had a mean lifespan 123 days less than the controls. Student's t test (table III) shows that this reduction in lifespan is statistically significant ($p < 0.01$). The other treated groups also had reduced lifespans, but the difference from the control is suggestive rather than significant (table III). Survival curves for the untreated and 4×10^{-4} *M* pFPA-treated mice are shown in figure 1. This indicates that the treatment tends to cause a number of early deaths and that the remaining mice live as long as controls. This is substantiated by statistical tests. For instance, if mice are divided arbitrarily into those living less than 600 days and those living longer, a χ^2 test, with Yate's correction, on a 2×2 contingency table (control and 4×10^{-4} *M*) gave $\chi^2_1 = 6.1$, $p = 0.01–0.02$. Thus, most of the

Table I. Setting up the longevity experiments

Animals age and sex	pFPA treatment *M*	Number of mice	Increase in weight, %	Volume of liquid taken, ml/g body weight
3–4 weeks male	none	38*	60.2	46.1
	8×10^{-5}	36	68.2	43.6
	4×10^{-4}	36	71.3	43.4
	2×10^{-3}	35*	70.6	41.0
3–4 weeks female	none	35*	81.3	40.5
	8×10^{-5}	35*	75.0	42.1
	4×10^{-4}	35*	76.0	38.8
	2×10^{-3}	36	60.0	40.0
1 year male	none	33	−0.6	29.3
	8×10^{-5}	33	−2.3	24.5
	4×10^{-4}	33	−7.7	22.2
	2×10^{-3}	33	−4.7	22.0
1 year female	none	36	−4.1	25.9
	8×10^{-5}	36	−5.6	26.7
	4×10^{-4}	36	−4.4	25.9
	2×10^{-3}	36	−4.2	23.9

* In each case one animal died during the period of treatment.

difference between the two groups can be attributed to these early deaths rather than the overall shortening of lifespans.

Results of the same experiment with female mice are also shown in table II. In this case there are no significant differences between the longevities of controls and any of the treated groups.

1-year-old animals were also treated with the same concentrations of pFPA for the same time period. The intake of liquid was about 60% of that in the experiments with young mice, and there was a slight weight decrease (table I). With females, the intermediate analogue concentration reduced the subsequent average lifespan by 77 days (table II) and a t test (table III) showed that this effect was significant ($p = 0.02–0.05$). The survival curves for the untreated and 4×10^{-4} *M* pFPA-treated females are shown in figure 2. As in the experiment in figure 1, the curves suggest that the reduction in lifespans is due to a proportion of early deaths. If mice are divided into those living less than 450 days after treatment and those living longer, then a 2×2 $\chi_1^2 = 4.92$ ($p = 0.02–0.05$). With 1-year-old males, there were no significant differences in the longevities of the control and the three treated groups (table II).

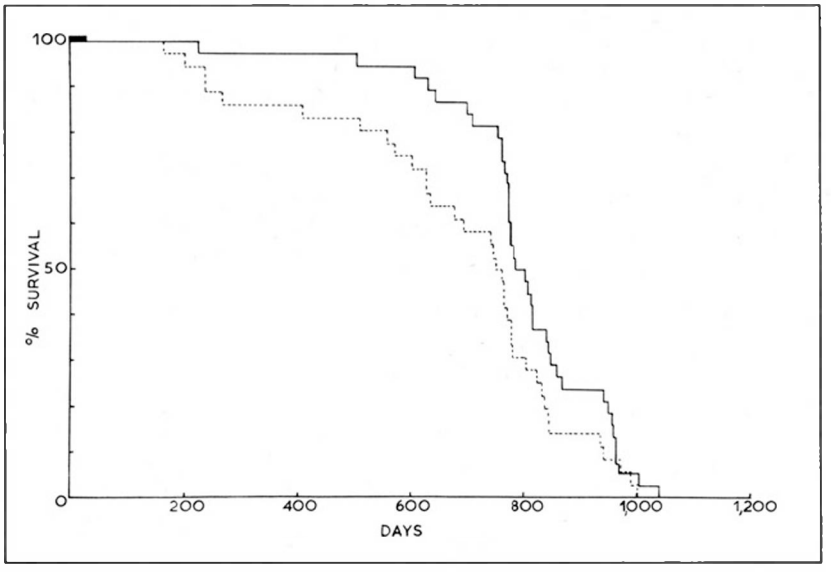

Fig. 1. The effect of pFPA on the lifespan of male CBA mice: ——= control, - - - - - = treated with 4×10^{-4} *M* pFPA. The black bar indicates the period of treatment.

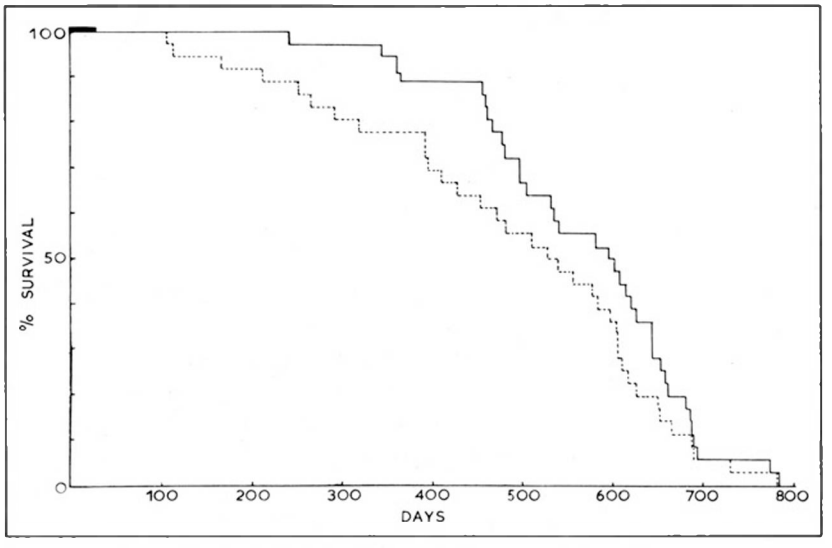

Fig. 2. The effect of pFPA on the lifespan of 1-year-old female CBA mice: —— = control, - - - - - = treated with 4×10^{-4} *M* pFPA. The black bar indicates the period of treatment and the scale on the abscissa the subsequent lifespan.

Table II. Results of the longevity experiments

Animals age and sex	pFPA treatment *M*	Mean lifespan days from start of treatment	SD
3–4 weeks male	none	797	149
	8×10^{-5}	753	164
	4×10^{-4}	674	228
	2×10^{-3}	717	224
3–4 weeks female	none	901	157
	8×10^{-5}	876	204
	4×10^{-4}	877	231
	2×10^{-3}	842	270
1 year male	none	420	90
	8×10^{-5}	387	101
	4×10^{-4}	422	74
	2×10^{-3}	434	88
1 year female	none	565	124
	8×10^{-5}	565	124
	4×10^{-4}	488	179
	2×10^{-3}	538	144

Table III. Student's t tests on control and pFPA treated animals

Animals age and sex	t tests	Probability
3–4 weeks male	control V 8×10^{-5} M: $t_{72} = 1.20$	0.2
	control V 4×10^{-4} M: $t_{72} = 2.75$	*<0.01*
	control V 2×10^{-3} M: $t_{71} = 1.80$	0.05–0.1
3–4 weeks female	control V 8×10^{-5} M: $t_{68} = 0.57$	0.6
	control V 4×10^{-4} M: $t_{68} = 0.51$	0.6
	control V 2×10^{-3} M: $t_{69} = 1.15$	0.2
1 year male	control V 8×10^{-5} M: $t_{64} = 1.38$	0.1–0.2
	control V 4×10^{-4} M: $t_{64} = 0.10$	0.9
	control V 2×10^{-3} M: $t_{64} = 0.08$	0.9
1 year female	control V 8×10^{-5} M: $t_{70} = 0$	–
	control V 4×10^{-4} M: $t_{70} = 2.13$	*0.02–0.05*
	control V 2×10^{-3} M: $t_{70} = 0.79$	0.4

It was important to determine whether there was any cage to cage variation in longevity, which might be attributed to deaths from infection or any other environmental cause. This was done by two factor analysis of variance on the mean lifespans of the different treatment/cage groups in each experiment. There was no significant cage to cage variation in the experiments in which 3- to 4-week-old mice were treated, nor for the 1-year-old females. In the experiments with 1-year-old mice there was a significant cage to cage variation ($p = 0.05$). Since this experiment in any case gave a negative result, we do not need to consider the importance of a possible environmental effect on lifespan.

Discussion

In these experiments the mice receiving different pFPA treatments were marked by ear punch and mixed in cages. It is therefore extremely unlikely that any significant effects we saw on lifespan could be attributed to death by infection or to any unknown environmental cause. We observed that 3- to 4-week-old males and 1-year-old females treated with 4×10^{-4} *M* pFPA had significantly reduced lifespans and that five-fold lower and five-fold higher doses had less or no effect. Also 3- to 4-week-old females and 1-year-old males were not significantly affected.

Our experiments show that the effect of pFPA on longevity depends not only on the dose given, but also the age at which it is administered and the sex of the recipient. Moreover, where a reduction in lifespan was seen, it appeared that this was largely due to an 'all or none' response, that is, a proportion of the animals had quite severely diminished longevity, whereas the rest were unaffected.

It is well-known that the pFPA can be incorporated in place of phenylalanine in proteins (e.g. *Kruh and Rosa,* 1959; *Westhead and Boyer,* 1961), but the expectation that there might be a simple relationship between analogue substitution, induction of errors in protein synthesis and ageing, is, of course, a naive one. For instance, it is now known that analogue-containing proteins turn over more rapidly than normal proteins (*Goldberg and Dice,* 1974; *Shakespeare and Buchanan,* 1976) and it is possible that a given level of altered protein is necessary to induce a protease scavenging mechanism. Also, elaborate detoxification mechanisms exist in mammals and it is known that free fluoride is formed from pFPA administered to rats (*Armstrong and Lewis,* 1951). Some enzymes involved in detoxification are inducible (*Parke,* 1975) and it is therefore possible that the synthesis of an enzyme which degrades pFPA only occurs above a certain dose threshold. It is also conceivable that cells containing abnormal proteins on their surface might provoke an immune response. This may occur

only if the effect on proteins is quite severe. For all these reasons, we would not expect the response of animals to analogue treatment to be a simple one.

One conclusion that we can draw is that the effect on longevity is not related directly to toxicity, since the highest concentration did not significantly alter lifespan. It is therefore unlikely that the premature ageing can be due to the killing of cells essential for prolonged survival or to the uptake of analogue into long-lived proteins. Although amino acid analogues can be mutagenic (*Lewis and Tarrant,* 1971; *Talmud and Lewis,* 1974) it is unlikely that pFPA is acting as radiomimetic agent. Radiation reduces lifespan to an extent which is dependent on dose; yet in these experiments no direct relationship between dose and life shortening is seen.

Previously it was shown that treatment of *Drosophila* larvae with amino acid analogue reduced the lifespan of the adult flies (*Harrison and Holliday,* 1967). This effect could have been due to an increase in error levels in protein synthesis, or to the incorporation of analogue into the protein of adult flies. Adults themselves synthesise very little protein and treatment of flies with pFPA does not affect their longevity (*Maynard Smith et al.,* 1970; *Dingley and Maynard Smith,* 1969). pFPA also has no effect on the longevity of cultured human fibroblasts (*Ryan et al.*, 1974; *Holliday and Tarrant,* unpublished), whereas the lifespan of the fungus *Podospora* is reduced by amino acid analogue treatments (*Holliday,* 1969). In fact, the original prediction by *Orgel* (1963) that amino acid analogues should shorten lifespans turns out to be an oversimplification, at least in the case of dividing cells. A theoretical analysis of the structure of populations of cultured human cells has shown that a rapid build up of a lethal level of errors in certain cell lineages could either have the effect of increasing or decreasing the lifespan of the overall population (*Kirkwood and Holliday,* 1975). It is also clear that parallel populations of cells grown under identical conditions have very variable lifespans and this is attributed to the stochastic fluctuations and final loss of a small number of immortal cells (*Holliday et al.*, 1977). A similar situation may apply to an essential stem-line population in mice and this could explain the variability which is always seen in lifespan, even if the animals are genetically identical (e.g. fig. 1, 2). The 'all or none' effect of pFPA on lifespan could then depend on the stochastic nature of the loss of these essential cells.

Although experiments with amino acid analogues cannot any longer be regarded as an adequate test of the protein error theory of ageing, nevertheless a demonstrated effect of an analogue on longevity certainly adds weight to the general hypothesis that ageing is in some way related to altered protein structure or function. There is now a large body of evidence that aged tissues or cells accumulate significant amounts of altered or inactive enzyme (e.g. *Gershon and Gershon,* 1973a, b; *Reiss and Gershon,* 1976; *Reiss and Rothstein,* 1975; *Anderson,* 1974; *Holliday and Tarrant,* 1972; *Lewis and Tarrant,* 1972; *Holliday*

et al., 1974; *Goldstein and Moerman*, 1976; *Wulff and Cutler*, 1975). It is not yet known whether these altered proteins are due mainly to errors in synthesis or to post-synthetic modifications. So far only one enzyme actually involved in information transfer between macromolecules has been examined in detail. *Linn et al.* (1976) demonstrated that the fidelity of DNA polymerase was reduced in senescent human fibroblasts and *Barton et al.* (1974) reported similar preliminary results for DNA polymerase from liver tissue of young and old mice.

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R. Holliday, FRS, National Institute for Medical Research, The Ridgeway, Mill Hill, *London NW7 1AA* (England)