Resistance to Fluorouracil in *Candida utilis*: Effects on the Uptake of Pyrimidines and Amino Acids

By Donald G. Wild* and Geoffrey I. Wilson

Microbiology Unit, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, U.K.

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5-Fluorouracil powerfully inhibits growth of *Candida utilis*. Isolates that are resistant to fluorouracil all have a reduced ability to transport uracil but most also have other defects. Their capacity to take up a wide range of amino acids is greatly reduced, as is their ability to alter rates of amino acid transport during nitrogen starvation. These isolates may be defective in the coupling of energy generation to transport systems.

INTRODUCTION

The yeast *Candida utilis* grows readily in a minimal medium with ammonium as nitrogen source. The rate of uptake of an amino acid is often increased after growth with another amino acid added to the medium (Jones & Wild, 1973): for example, lysine transport increases after growth with added leucine or aspartate. These effects are not restricted to amino acid transport. The rate of uptake of uracil also increases after growth with one of a diverse collection (e.g. leucine, methionine, aspartic acid, phenylalanine) of amino acids. Although transport in *C. utilis* involves a number of separate permeases, these may share a common element that can limit rates of uptake and be regulated by constituents of the medium.

Increased uptake of a compound after growth with an amino acid or, more generally, with a different nitrogen source, or after nitrogen starvation can involve increased synthesis and/or activity of a pre-existing transport system or expression of a second system that is synthesized or becomes active under the new conditions. Thus, in *Saccharomyces cerevisiae*, arginine transport in ammonium-grown organisms is by a specific permease; with proline as nitrogen source, or in nitrogen-starved organisms, uptake also involves a 'general' amino acid permease (Grenson et al., 1966, 1970). In *S. cerevisiae*, the study of these multiple transport systems has involved mutants resistant to toxic amino acid analogues because of defective transport. Thus, canavanine-resistant mutants fail to take up arginine when grown on ammonium; with glutamate or proline as nitrogen source, sensitivity is restored (Grenson et al., 1966).

These considerations prompted this study of fluorouracil-resistant isolates of *C. utilis*. Uracil transport can be increased by growth of this yeast with amino acids, by changes in the nitrogen source or by nitrogen starvation. The response of ammonium-grown organisms with defects in uracil transport might throw light on the system(s) and mechanism(s) involved. The results are unexpected. Most resistant isolates show reduced transport not only of uracil but also of a range of amino acids. Responses to nitrogen starvation are also different from those of the parent organism. The results demonstrate further the interrelatedness of transport systems in this yeast.

METHODS

Organisms and cultural conditions. The yeasts were *Candida utilis* NCYC 321 and fluorouracil-resistant isolates derived from it (see Results). Organisms were grown at 30 °C in shaking incubators in a minimal medium.
plus ammonium (ammonium-MM) pH 6·7, containing (per litre): glucose, 10 g; KH₂PO₄, 2 g; K₂HPO₄, 1·5 g; NH₄Cl, 1 g; MgCl₂, 6H₂O, 0·5 g; biotin, 5 µg. Solidified medium contained 20 g Oxoid L28 agar 1⁻¹. In the minimal medium plus glutamate (glutamate-MM). NH₄Cl was replaced by 3·7 g monosodium glutamate 1⁻¹. Nitrogen-free medium lacked both monosodium glutamate and NH₄Cl.

Cultures were adapted to minimal medium by growth for at least eight generations before use as inocula for experiments. Absorbances of cultures at 450 nm (A₄₅₀) were corrected, when necessary, for deviations from Beer’s Law. Resuspension in nitrogen-free medium was carried out by filtering up to 100 ml of an exponentially growing culture through an Oxoid membrane filter (6 cm diam.). Filtered cells were then washed with 250 ml nitrogen-free medium at 30 °C and resuspended in the original volume of fresh medium, also at 30 °C.

Rates of uptake. The uptake of pyrimidines and L-amino acids was measured essentially as described by Grenson (1966). A portion (usually 15 ml) of an exponentially growing culture was added to radioactive solution (0·3 ml) and shaken at 30 °C. Samples (2·0 ml) removed after 15 s and at 45 s intervals thereafter, were immediately filtered through Whatman GF/C glass-fibre discs (2·1 cm diam.). The filters were each washed for 30 s with about 1 ml of water at 0 °C, then placed in sample tubes and dried; scintillation fluid (3 ml: 5 g 2-(4'·tert-butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) per litre toluene) was added and radioactivity was measured. Radioactivity plotted against time gave initial rates of uptake in units (c.p.m. min⁻¹) that were satisfactory for many comparisons. For absolute determinations, absorbance was related to dry wt ml⁻¹ and radioactivity incorporated was converted to mol substrate transported.

For the determination of Michaelis constants and maximum velocities of uptake by exponentially growing or starved organisms a culture was chilled to 0 °C; at intervals, samples were incubated at 30 °C for 4 min (their temperature was then 29·5–30 °C) and uptake was measured at different substrate concentrations.


**RESULTS**

**General characteristics of uracil uptake**

Uptake of 0·18 mm-uracil by *C. utilis* during exponential growth in ammonium-MM or glutamate-MM, or by organisms starved of nitrogen, was linear for at least 5 min. Uptake by ammonium-grown organisms (A₄₅₀ 0·4) was abolished by prior incubation for 5 min with 1 mm-N-sodium azide or 4 mm-2,4-dinitrophenol and reduced about 90% by incubation for 5 min with 1 mm-N-ethylmaleimide. Uptake of uracil by glutamate-grown or starved organisms was inhibited similarly. Rates of uracil uptake in ammonium-MM or glutamate-MM were proportional to the absorbances of cultures; the rate of uptake by glutamate-grown organisms was about twice that of ammonium-grown cells at the same absorbance. Uptake by ammonium-grown organisms at a range of uracil concentrations showed simple Michaelis kinetics; the Michaelis constant (Kₘ) was 5 µM and the maximum velocity (V_max) was 0·9 nmol min⁻¹ (mg dry wt⁻¹). For glutamate-grown cells V_max doubled to 1·80 nmol min⁻¹ (mg dry wt⁻¹) while the Kₘ (3 µM) was not significantly different. When organisms were transferred from ammonium medium to medium without a nitrogen source, the transport capacity increased considerably. After 180 min the rate of uptake per ml culture could be 20 times that before the onset of starvation. In an experiment similar to that of Fig. 3, organisms were starved for 90 min and uptake was measured at a range of uracil concentrations. V_max [4·4 nmol min⁻¹ (mg dry wt⁻¹)] was about five times that of ammonium-grown organisms, while Kₘ (4 µM) was similar.

**Effect of 5-fluorouracil**

Fluorouracil competitively inhibited uracil uptake by ammonium-grown organisms; Kᵢ, the dissociation constant of fluorouracil from the transport system, was 22 µM. This agrees well with values of 17 and 22 µM for the Kₘ for [³H]fluorouracil uptake obtained in two experiments that measured uptake at a range of fluorouracil concentrations. Growth in ammonium-MM was slowed appreciably by the addition of 0·1 µg fluorouracil ml⁻¹; higher
Fluorouracil resistance in \textit{C. utilis}

Fig. 1. Growth with fluorouracil. Organisms from exponentially growing cultures (\(A_{490}\) about 0.5) of the parent organism (a) and isolate FU7 (b) were inoculated into portions (50 ml) of ammonium-MM containing fluorouracil at the concentrations (\(\mu\)g ml\(^{-1}\)) shown in parentheses. For clarity, the growth curves in each panel are staggered by 1 h.

concentrations had a progressively greater effect (Fig. 1a). These experiments suggested that fluorouracil-resistant organisms might be isolated and that resistance might sometimes arise through changes in the activity or synthesis of the uracil transport system.

Isolation of organisms resistant to 5-fluorouracil

About \(10^5\) to \(10^6\) organisms grown in ammonium-MM were spread on solidified medium containing 1 to 10 \(\mu\)g fluorouracil ml\(^{-1}\). 'Resistant' colonies emerged after 2–3 d from a film of background growth. The final number of colonies (about 25) did not obviously depend on the number of organisms spread or the concentration of fluorouracil. Colonies were picked, streaked on plates containing fluorouracil at the concentration originally used and single colonies were isolated. In a series of separate experiments, 47 fluorouracil-resistant isolates (FU1 to FU47) were obtained.

Properties of the fluorouracil-resistant organisms

\textit{Growth.} Each isolate was grown in ammonium-MM in the absence and presence of fluorouracil. Figure 1 (b) shows growth curves that were typical in that (i) the growth rate in the absence of fluorouracil of all but three of the isolates was similar to that of the parent and (ii) the isolates grew readily, albeit sometimes at reduced rates, in concentrations of the drug up to at least 5 \(\mu\)g ml\(^{-1}\).

\textit{Uracil transport.} Each isolate was grown in ammonium-MM to an \(A_{490}\) of 0.4 and its rate of uptake of 0.18 mm-uracil was compared with that of the parent strain. Two isolates which showed non-linear uptake were not considered further. The results, summarized in Fig. 2(a), showed a spectrum of behaviour. All 45 isolates had reduced rates of uptake. Thirty-nine took up uracil at less than half the normal rate. Five had rates less than 10\% of that of the parent and four of these scarcely took up uracil: three of the latter were the slow-growing isolates mentioned above but the fourth (FU9) grew at a normal rate.

Uptake of uracil at a range of concentrations was measured for five of the isolates; in each case, uptake showed simple Michaelis kinetics. Values of \(K_m\) were: FU15, 7 \(\mu\)M; FU20, 14 \(\mu\)M; FU21, 8 \(\mu\)M; FU27, 3 \(\mu\)M; FU47, 6 \(\mu\)M. Measurements of \(K_m\) in isolates with reduced rates of uptake are less accurate than with the parent; the affinity of uracil for the residual
urate uptake system in the isolates is probably not significantly different from that in the parent strain. Certainly the differences between the isolates and the parent as measured (e.g. Fig. 2a) with 0·18 mM-uracil must be completely due to differences in $V_{\text{max}}$.

Lysine uptake. About 30 of the isolates were examined for their ability to transport lysine, which is also taken up by a high-affinity system ($K_m$ about 8 μM; Jones & Wild, 1973). The rate of uptake of 5 μM-uracil by ammonium-grown organisms was the same in the absence and presence of excess (200 μM) lysine, so the uracil and lysine transport systems must be separate. Surprisingly, most of the isolates (20/29) transported lysine poorly, and often not detectably (Fig. 2b). At the other extreme, isolate FU9, with its exceptionally low rate of uracil uptake, had near normal lysine transport capacity.

Uptake of other amino acids. Thus the majority of fluorouracil-resistant isolates had a reduced capacity to take up uracil coupled with a virtual inability to take up lysine. Nine isolates of this phenotype were examined for their ability to take up other amino acids, and cytosine, from the medium. The ‘atypical’ FU9 was included in the survey (Table 1). Isolate FU9 transported each of the 12 amino acids tested (and cytosine) at rates that approached those in the parent. Isolate FU7, on the other hand, transported all amino acids at rates that were at most 10% of those of the parent strain. The other isolates had the phenotype of FU7 with a greatly reduced capacity to transport aspartate, a range of neutral amino acids and lysine. Glutamate was also transported poorly by FU7; since FU7, FU14, FU31 and FU39 failed to grow in glutamate-MM, glutamate transport may also be generally defective. The uptake of cytosine was not greatly reduced in any of the isolates. A separate experiment with the parent strain showed that the rate of uptake of 9 μM-uracil was not decreased in the presence of 180 μM-cytosine, so uracil and cytosine must be transported separately.

Uptake of uracil and amino acids during nitrogen starvation. Figure 3 shows increased uracil transport during nitrogen starvation of the parent strain. A parallel experiment with isolate FU9 (Fig. 3a) confirmed that in ammonium-MM this organism failed to take up uracil. After starvation, transport capacity rapidly appeared; at 150 min this was about half
Table 1. Transport of amino acids and pyrimidines by fluorouracil-resistant isolates

In a series of experiments, the parent strain and resistant isolates were grown in portions (50 ml) of ammonium-MM. At an A_{450} of 0-4, samples were taken to measure the rate of uptake of 0-10 mM ^14C-labelled substrate (0-18 mM in the case of uracil). For each substrate, the rates of uptake are expressed as percentages of the rate of uptake by the parent.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>FU7</th>
<th>FU14</th>
<th>FU15</th>
<th>FU20</th>
<th>FU21</th>
<th>FU27</th>
<th>FU31</th>
<th>FU39</th>
<th>FU47</th>
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<td>1</td>
<td>0</td>
<td>2</td>
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<td>18.5</td>
<td>90</td>
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Fig. 3. Uracil transport during nitrogen starvation: (a) the parent strain and isolate FU9; (b) the parent strain and isolate FU14. Organisms were grown in ammonium-MM. At an A_{450} of 0-4 (a) or 0-5 (b), a portion of each culture was filtered and the organisms were transferred to nitrogen-free medium. Uptake of 0-18 mM uracil was measured at intervals: numbers in parentheses are times (min) after the onset of starvation. Parent strain in ammonium-MM (O) and nitrogen-free medium (●); isolates in ammonium-MM (△, □) and nitrogen-free medium (▲, ■).

that of the parent starved for the same time. The other isolates tested also showed increased rates of uracil transport during starvation. Before starvation, isolate FU47 took up uracil at a rate about 15% of that of the parent. The rate after starvation for 30 min was similar to that of the (starved) parent and after 90 min was about half that of the parent (Fig. 3b). Other fluorouracil-resistant isolates behaved like FU47 (Table 2); uracil transport could increase considerably to rates comparable with that of the starved parent.

During nitrogen starvation, the ability of C. utilis to take up amino acids also increased considerably. This is shown for lysine in Fig. 4(a). Before starvation, the parent strain (A_{450} 0-4) took up 2 nmol lysine min^{-1} ml^{-1}. After starvation for 180 min the rate was 29 nmol lysine min^{-1} ml^{-1}. Isolate FU9 behaved similarly (Table 2) but the isolates with multiple
Table 2. Uracil and lysine transport after nitrogen starvation

Isolates and the parent organisms were grown to an $A_{450}$ of 0.45–0.50 in ammonium-MM. A portion of each culture was filtered and the organisms were transferred to nitrogen-free medium. The rate of uptake of 0.18 mM-uracil or 0.10 mM-lysine was measured immediately before filtration and after starvation for 105 min.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Before starvation</th>
<th>After starvation</th>
<th>Before starvation</th>
<th>After starvation</th>
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<td>Parent</td>
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<td>0.90</td>
<td>2.5</td>
<td>23.4</td>
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<td>FU7</td>
<td>0.05</td>
<td>0.33</td>
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<td>0.55</td>
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<tr>
<td>FU9</td>
<td>See Fig. 3 (a)</td>
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<td>2.5</td>
<td>21.3</td>
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<tr>
<td>FU14</td>
<td>0.09</td>
<td>0.90</td>
<td>0.06</td>
<td>0.55</td>
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<tr>
<td>FU31</td>
<td>0.09</td>
<td>0.80</td>
<td>0.05</td>
<td>0.30</td>
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<tr>
<td>FU39</td>
<td>0.14</td>
<td>0.80</td>
<td></td>
<td>See Fig. 4 (a)</td>
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</table>

Fig. 4. Lysine and leucine transport during nitrogen starvation: (a) lysine transport by the parent strain and isolate FU39; (b) leucine transport by the parent strain and isolate FU7. Organisms were grown in ammonium-MM. At an $A_{450}$ of 0.4, a portion of each culture was filtered and the organisms were transferred to nitrogen-free medium. Uptake of 0.1 mM-lysine (a) or 0.1 mM-leucine (b) was measured at intervals; numbers in parentheses are times (min) after the onset of starvation. Parent strain in ammonium-MM (○) and nitrogen-free medium (▲); isolates in ammonium-MM (□) and nitrogen-free medium (●).

defects in transport did not. Isolate FU39 (Fig. 4 a) transported lysine at a rate about 1% of that of the parent in ammonium-MM. The rate increased during starvation but the maximum was less than that of the unstarved parent. Other isolates tested behaved like FU39 (Table 2). For example, isolate FU7 increased lysine uptake about 10-fold in 105 min but to a value that was still only 20% of that of the unstarved ammonium-grown parent. Figure 4 (b) shows the drastically altered response of isolate FU7 in respect of leucine uptake. The capacity of the parent to transport leucine increased very considerably during 150 min starvation; leucine transport by FU7 was virtually undetectable before and after starvation. Isolate FU14 behaved similarly. This took up leucine before starvation at a rate about 15% of that of the parent at the same absorbance; after starvation for 90 min, leucine uptake was not detectable.

'Reversion'. The 10 isolates of Table 1 grew at rates no different from the parent strain in liquid or on solidified ammonium-MM. However, all the isolates except FU9 formed rather
smaller colonies than the parent on a rich medium (MGYP agar; Jones & Wild, 1973). Sometimes, a few larger colonies were visible among the smaller. In three cases (FU15, FU20, FU47) the ability of a large colony to take up lysine and uracil was measured after growth in ammonium-MM. The rates of uptake of both substrates were indistinguishable from those of the parent. Thus, fluorouracil-resistant organisms can ‘revert’ and reversion is accompanied by the simultaneous loss of at least two transport defects.

**DISCUSSION**

Fluorouracil is the only purine, pyrimidine or amino acid analogue that we (in unpublished work) have found to have more than a transient effect on growth of *C. utilis*. In particular, canavanine and thiosine, which powerfully inhibit *S. cerevisiae* (Grenson, 1966; Grenson et al., 1966), have little effect on *C. utilis* even though the ‘natural’ amino acids, arginine and lysine, are taken up by active high-affinity systems. This indicates differences in uptake and/or metabolism between the two yeasts. On the other hand, *C. utilis* is particularly sensitive to fluorouracil which inhibits growth at a concentration (1 μM) appreciably less than the *Kₘ* for uptake (about 20 μM). This is rather surprising because *C. utilis* incorporates exogenous uracil into RNA poorly (Jones & Wild, 1973) and should incorporate fluorouracil even less well. Ribosome maturation in *C. utilis* may be particularly sensitive to the fluoropyrimidine, or at low concentrations, effects may be primarily on messenger RNA, or DNA, synthesis (Heidelberger, 1965).

We have refrained from calling the fluorouracil-resistant organisms ‘mutants’ although the alterations are stable. Moreover, when reversion did occur, both defects investigated returned to normal. However, genetic crosses cannot be done with *C. utilis* and it is puzzling that resistance arose at high frequency with a low concentration of fluorouracil and without a preliminary mutagenesis such as that used by Jund & Lacroute (1970) in the isolation of fluoropyrimidine-resistant strains of *S. cerevisiae*. Fluorouracil may itself be mutagenic, as is suggested by its induction of petite mutants in *S. cerevisiae* (Whittaker et al., 1972).

Resistance to fluorouracil can arise in *S. cerevisiae* in different ways, not all of which are explained (Jund & Lacroute, 1970). Loss of the uracil permease confers resistance. So does desensitization of aspartate transcarbamylase and carbamylphosphate synthetase to feedback inhibition by UTP: this increases production of endogenous pyrimidines so that exogenous uracil, and fluorouracil, compete less favourably for incorporation into RNA. Resistance also arises by reduced conversion of uracil to UMP. Isolate FU9 takes up uracil so poorly that kinetic parameters could not be determined. Uptake of other compounds is scarcely affected and this isolate may well be defective in a uracil permease. If so, the greatly increased transport of uracil during nitrogen starvation may be due to synthesis of a second permease under these conditions. The majority of isolates have very different properties from FU9. They transport uracil at a range of reduced rates, due to lower maximum velocities of uptake unaccompanied by significant alterations in affinity for uracil. This could arise by reduced synthesis of a normal uracil transport system, or from a system with altered ability to unload substrate internally. However, these possibilities do not explain the poor transport of all amino acids tested.

Uracil transport is inhibited by dinitrophenol and so is energy-dependent. As with cytosine permeation in *S. cerevisiae* (Chevallier et al., 1975), uptake may be described by a mobile carrier model in which the initial rate of entry \( V = V_{\text{max}} \cdot S \cdot Q/(K_m + S) \), where \( S \) is the external uracil concentration and \( Q \) is the efficiency of the energy coupling mechanism. This mechanism might be defective in this category of isolate.

The uptake of purines and pyrimidines by *Escherichia coli* was thought to be mediated by membrane-bound phosphoribosyltransferases (Hochstadt-Ozer, 1972), but recent work (Roy-Burman & Visser, 1975; Burton, 1977) suggests that transport depends on a proton-motive force. This is also likely in *S. cerevisiae*, where cytosine, adenine and
hypoxanthine share a transport system distinct from that for uracil (Polak & Grenson, 1973). Uracil uptake does not involve uracil phosphoribosyltransferase because a mutant strain unable to convert uracil to UMP takes up the pyrimidine normally (Jund et al., 1977). Hypoxanthine uptake is accompanied by the absorption of protons and ejection of K\(^+\) ions with a 1:1 stoichiometry (Reichert & Forêt, 1977). The uptake of uracil and of cytosine are both inhibited by 2,4-dinitrophenol but inhibitors of membrane ATPases affect transport of the two pyrimidines differently. Thus, in S. cerevisiae uptake may not be energized by precisely similar mechanisms (Losson et al., 1978).

Similar considerations, applied to C. utilis, might explain why the rate of cytosine uptake by fluorouracil-resistant isolates is much less reduced than uracil uptake; a less specific and somewhat different cytosine permeation system may not be so susceptible to uncoupling from its energetic component. Defective energy coupling also explains why fluorouracil-resistant organisms may be virtually unable to take up a wide range of amino acids. In yeasts, the uptake of amino acids is also generally accompanied by an inflow of protons coupled to an efflux of K\(^+\) ions with the subsequent ejection of protons depending on energy metabolism (Eddy & Nowacki, 1971; Cockburn et al., 1975). The fluorouracil-resistant isolates consistently show almost negligible uptake of lysine (and methionine in those cases examined), whereas uptake of most neutral amino acids is significant, although greatly reduced. This again suggests that different permeases may not be linked to their energy supply in precisely the same way. During nitrogen starvation of C. utilis, the rates of uptake of lysine and leucine increase considerably; the failure of the major category of isolates to increase these rates appreciably emphasizes the wide ranging alterations that accompany fluorouracil-resistance. That these isolates do increase uracil uptake during nitrogen starvation may be a further example of the variable integration of energy production with transport.

Mutants of S. cerevisiae that lack the general amino acid permease (Grenson et al., 1970) show reduced uptake of a range of amino acids only when nitrogen-starved or grown on sources other than ammonium. They thus differ from the fluorouracil-resistant isolates of C. utilis. The latter are more akin to the amino acid permease (aap\(^+\)) and the allelic amino acid permease factor (aap\(^-\)) mutants of S. cerevisiae in which the activities of both specific and general amino acid permeases are depressed (Surdin et al., 1965; Grenson & Hennaut, 1971). However, the aap\(^-\) mutation does not confer resistance to 5-fluorouracil; nor are uptakes of uracil or cytosine affected. There are no reports of which we are aware of reduced rates of pyrimidine transport in yeasts defective in amino acid uptake nor do fluoropyrimidine-resistant isolates seem to have been screened for altered amino acid transport. Our results confirm and extend the previous suggestion (Jones & Wild, 1973) that the multiple transport systems of C. utilis have elements in common; the fluorouracil-resistant isolates are an approach to the test of our hypothesis as to what one of these elements might be.

The work in this paper was done during tenure of a S.R.C. Studentship by G.I.W.

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