Genet. Res., Camb. (1970), **15**, *pp.* 7–17
With 1 *text-figure*
Printed in Great Britain

Purine-excreting mutants of *Saccharomyces cerevisiae*

I. Isolation and genetic analysis

BY SUSAN ARMITT* AND ROBIN A. WOODS
*Department of Genetics, The University of Sheffield, Sheffield S*10 2*TN*

(*Received* 27 *February* 1969)

SUMMARY

Purine-excreting mutants of yeast have been obtained by selecting for (*a*) adenine-insensitive pigment accumulation in a strain carrying *ad-2* and (*b*) by isolating prototrophic mutants which crossfeed to an adenine-requiring indicator strain. The first method yielded both auxotrophic and prototrophic mutants. The auxotrophs were assigned to *ad-12* which specifies adenylosuccinate synthetase. The prototrophs were affected by a gene, *su-pur*, which prevented purine excretion, and the majority of them showed anomalous behaviour of mating-type *a* associated with the mutations causing excretion. Mutants obtained by the second method were allocated to six unlinked genes. Those assigned to *pur-1* to *pur-5* are recessive and affected by *su-pur*. *pur-6* is a complex locus with both dominant and recessive alleles; it is closely linked or allelic to *su-pur* and *ad-4*. Functional tests involving mutants of *pur-3* and *pur-6* in combination with *pur-1* and *pur-4* did not show normal intergenic complementation. The suppressor shows gene-specific dominance/recessiveness. Some metabolic lesions which could give rise to purine excretion are discussed.

1. INTRODUCTION

Regulation of purine biosynthesis was first encountered by Gots (1950) when he observed that exogenous purines inhibited the formation of an early purine precursor in sulphonamide-inhibited bacteria. Further study showed that the site of this inhibition was apparently prior to the formation of the imidazole ring but after the completion of the ribose moiety of the purine molecule (Gots, 1957). The specific enzyme involved in this inhibition was identified by Wyngaarden & Ashton (1959) working with pigeon liver. They showed that the enzyme glutamine phosphoribosylpyrophosphate amidotransferase (PRPP amidotransferase), which catalyses the first reaction specific to purine biosynthesis was inhibited by a number of purine nucleotides. Later work indicated that this enzyme, in common with many other regulatory enzymes, has separate binding sites for substrates and inhibitors (Caskey, Ashton & Wyngaarden, 1964). These workers also showed that mixtures of 6-hydroxy and 6-amino purine nucleotides resulted in co-operative feedback inhibition. Evidence for the inhibition of this enzyme by purine nucleo-

* Present address: Department of Genetics, The University of Leicester.

https://doi.org/10.1017/S0016672300001324 Published online by Cambridge University Press

tides has been found in a number of other organisms, e.g. *Aerobacter aerogenes* (Nierlich & Magasanik, 1965), mammalian tumour tissue (McFall & Magasanik, 1960; Henderson & Khoo, 1965) and *Schizosaccharomyces pombe* (Heslot, Nagy & Whitehead, 1966).

Regulation by a feedback mechanism is also found at the level of purine uptake (Berlin & Stadtman, 1966) and the interconversion of purine nucleotides (Mager

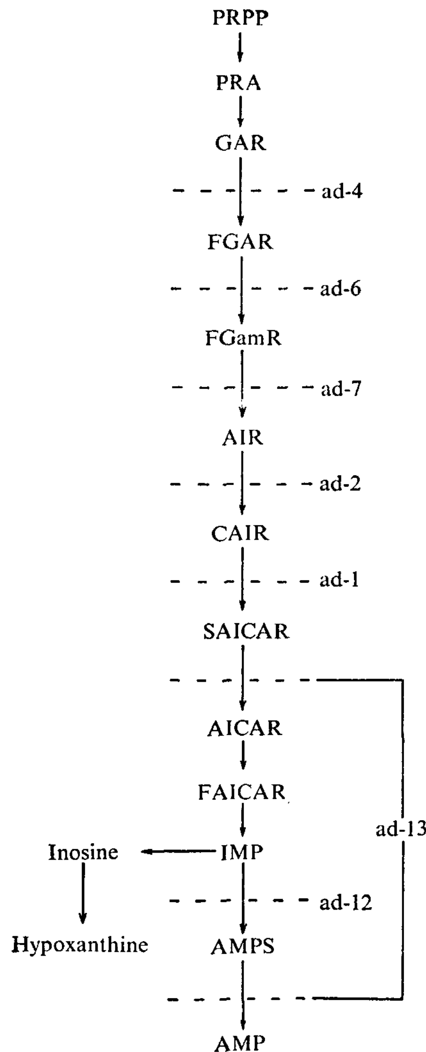

Fig. 1. Metabolic lesions in purine biosynthesis. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formyl glycinamide ribonucleotide; FGamR, formyl glycinamidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-amino-4-imidazolecarboxylic acid ribonucleotide; SAICAR, 5-amino-4-imidazole-N-succinocarboxamide ribonucleotide; AICAR, 5-amino-4-imidazolecarboxamide ribonucleotide; FAICAR, 5-formamido-4-imidazolecarboxamide ribonucleotide; IMP, inosinic acid; AMPS, adenylosuccinic acid; AMP, adenylic acid.

https://doi.org/10.1017/S0016672300001324 Published online by Cambridge University Press

& Magasanik, 1960; Wyngaarden & Greenland, 1963). In addition to these metabolic controls the enzymes of the pathway are subject to genetic repression in *Escherichia coli* (Love & Remy, 1966; Momose, Nishikawa & Shiio, 1966).

In *Saccharomyces cerevisiae* the pathway of purine biosynthesis has been studied extensively from both the genetic and biochemical points of view. Mutants causing a requirement for purine have been allocated to 13 loci (Yeast Genetics Supplement to Microbial Genetics Bulletin, **25**, 1966) and the majority of the biochemical lesions have been identified (Jones & Magasanik, 1967; Silver & Eaton, 1968; Dorfman, 1969; Fisher, 1969; Silver & Eaton, 1969). The locations of the lesions for *ad-1*, *ad-2*, *ad-4*, *ad-6*, *ad-7*, *ad-12* and *ad-13* are shown in Fig. 1. Mutants of *ad-3* require adenine and histidine for growth and have been shown to be defective in the synthesis of N^{10} formyltetrahydrofolic acid (Jones & Magasanik, 1967). The blocks in *ad-5*, *ad-8* and *ad-9* have not yet been identified with certainty (Silver & Eaton, 1968).

Mutants at the *ad-1* and *ad-2* loci are characterized by the accumulation of an intracellular red pigment. Burns (1964) found that the accumulation of pigment and aminoimidazole was reduced by exogenous adenine. He attempted to correlate changes in aminoimidazole with changes in the intracellular levels of purine nucleotides and deduced that the feedback inhibitor controlling purine synthesis *in vivo* was inosine monophosphate (IMP).

We reasoned that mutants defective in the regulation of *de novo* synthesis would continue to accumulate pigment in the presence of exogenous adenine. We were able to isolate mutants of this kind; some were auxotrophic and allelic to *ad-12* while others were prototrophic and excreted purine into the medium. Unfortunately most of the prototrophic purine-excreting mutants showed an anomaly of mating type and were consequently unsuitable for genetic analysis. We then isolated another series of prototrophic mutants by a more direct method and found that they could be allocated to six unlinked loci, *pur-1* to *pur-6*. The genetic analysis of these various mutant strains is presented in this paper.

2. MATERIALS AND METHODS

The complete, minimal and sporulation media have been described previously (Cox & Bevan, 1962; Ahmed & Woods, 1967). We shall refer to them as YCM, YMM and YSM respectively. Genetic techniques such as mating and ascus dissection were also as described by Ahmed & Woods (1967).

Mutants were induced by ultraviolet irradiation or treatment with ethylmethane sulphonate (EMS). The ultraviolet dose was such as to give approximately 1% survival; EMS was added to cell suspensions to give a final concentration of 1%, and incubation of the treated suspensions in a shaking water bath at 28 °C for 2 h resulted in about 50% survival.

Purine excretion was detected by the ability of mutant strains to crossfeed to a diploid homozygous for *ad-2*. This indicator strain was either spread as a lawn on the surface of YMM or incorporated into the medium as a cell suspension. The

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YMM used for these crossfeeding tests was supplemented with 2 % YCM to initiate growth of the indicator strain.

The derivation of the various standard strains used in the experiments was as follows: *ad-1* and *ad-2* (mutant *R2*) provided by R. A. Woods (Woods & Bevan, 1966); *ad-3* to *ad-8* were derived from cultures obtained from Dr R. K. Mortimer; *ad-12* (AW217/2A) and *ad-13* (A473/4C) were obtained from Dr B. Dorfman; wild-types *a* and *α* were those described by Ahmed & Woods (1967). Stock cultures were maintained on silica gel (Brockman & de Serres, 1962).

The genetic symbols used are as follows: *ad*, *his* and *ser* denote requirements for adenine, histidine and serine respectively; *pur* refers to the ability to excrete purine and *su-pur* is a genetic factor which prevents the expression of the purine-excreting phenotype. Adenine-insensitive mutants are named according to the medium from which they were isolated, e.g. *C1* and *C2* from YCM; *M1* and *M2* from YMM.

3. RESULTS

(i) *The isolation and characterization of adenine-insensitive mutants*

The purine requirement of *ad-2* can be satisfied only by adenine or hypoxanthine, both of which are effective inhibitors of pigment formation (Burns, 1964). We found that both mating types of *R2* were white on YCM containing 75 μg of adenine/ml and on YMM containing 50 μg/ml and we decided that 50 μg/ml would be a suitable supplement for both media. An irradiated suspension of *R2*, mating type *a*, was accordingly plated on to both YCM and YMM plus adenine, and colonies

Table 1. *Classification of mutants causing adenine-insensitive pigment accumulation*

Phenotype	No. of mutants
Auxotrophic	
(*a*) Responding to adenine only, crossfeeding to *ad-2*	16
(*b*) Growth on YCM only, crossfeeding to *ad-2*	2
Prototrophic	
(*a*) Recessive, crossfeeding to *ad-2*	24
(*b*) Dominant, crossfeeding to *ad-2*	1
(*c*) Non-crossfeeding	37

which were more deeply pigmented than the background of *R2* were isolated for further investigation. These presumptive mutants were retested on media supplemented with a range of concentrations of adenine and hypoxanthine; 80 were retained for further analysis. These adenine-insensitive mutants were crossed to wild-type *α* and the segregants from each cross tested for pigment accumulation and ability to crossfeed. The results of this analysis are shown in Table 1. We continued investigation of the auxotrophic and prototrophic crossfeeding mutants.

(*a*) *Auxotrophic crossfeeding mutants.* The 16 adenine-specific mutants were all isolated from YMM plus adenine. We were unable to identify the requirement of

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the other two auxotrophic mutants, both of which were isolated from YCM plus adenine. These two mutants crossfed weakly and reverted frequently.

Functional tests for allelism between these mutants were carried out by replica-plating (Woods & Bevan, 1966). The results indicated that all of the adenine-specific mutants could be allocated to a single gene with one pair of alleles showing inter-allelic complementation. The other two mutants were allelic to each other but complemented all of the adenine-specific mutants. The results of these functional tests were confirmed by tetrad analysis.

Mutants at the *ad-12* and *ad-13* loci are adenine-specific and can be obtained by selecting for constitutive pigment formation in strains carrying *ad-2* (Dorfman, 1969). Our adenine-specific mutants did not complement Dorfman's *ad-12* whereas the two mutants with unidentified requirements complemented both *ad-12* and *ad-13*. Unfortunately all of these crosses gave very low spore viability and we were unable to confirm these results by tetrad analysis. However, we feel justified in assigning our adenine-specific mutants to *ad-12*.

(*b*) *Linkage relationships of* ad-2, ad-12 *and mating type.* The data obtained from the classification of the crosses of the original adenine-insensitive mutants (*ad-2*, *ad-12*) to wild-type indicated linkage between *ad-2* and *ad-12* and also between *ad-12* and the mating-type locus. Considering *ad-2* and *ad-12*, the total numbers of parental ditype (PD), non-parental ditype (NPD) and tetratype asci (TT) were 52, 10 and 82 respectively. The deviation of the PD:NPD ratio from 1:1 is highly significant ($\chi^2 = 28{\cdot}45$, $P < 0{\cdot}0001$), indicating linkage (Perkins, 1953). Single strand analysis gives a recombination frequency of 34·5 % between *ad-2* and *ad-12*. With respect to *ad-12* and the mating-type locus the PD:NPD:TT ratio was 33:16:95; again the deviation of the PD:NPD ratio from 1:1 is significant ($\chi^2 = 5{\cdot}9$, $P < 0{\cdot}015$) and single strand analysis gives 44·1 % recombination. We were unable to detect linkage of *ad-12* to either *his-8* or *ser-1*, both of which are centromere distal with respect to *ad-2* (Mortimer & Hawthorne, 1966). These results suggest that Linkage Fragment I, which carries *ad-2*, *his-8* and *ser-1*, may be part of chromosome II, which carries the mating-type locus (Mortimer & Hawthorne, 1966).

Dorfman (1969) has carried out an extensive analysis of the linkage relationships of his *ad-12* mutants to a number of marker genes. His results do not suggest linkage of *ad-12* to either *ad-2* or the mating-type locus. We cannot, at present, offer any explanation for this disagreement.

(*c*) *Prototrophic purine-excreting mutants.* Thirteen of these mutants were isolated from YCM plus adenine and 12 from YMM. Designating the new mutations *pur* we expected the following segregants from the crosses to wild-type α: adenine-insensitive (*ad-2 pur*), adenine-sensitive (*ad-2 pur*$^+$), prototrophic crossfeeding (*ad-2*$^+$ *pur*), prototrophic non-crossfeeding (*ad-2*$^+$ *pur*$^+$) in the ratio 1:1:1:1. In fact we found a deficiency of adenine insensitives and prototrophic crossfeeders such that the observed ratio was 1:3:1:3. This discrepancy between the observed and expected results cannot be due to linkage, as one of the deficient classes, adenine-insensitive, is parental rather than recombinant. The results can be accounted for

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if we assume that a genetic factor which suppresses the *pur* mutations is segregating in all of the crosses. We presumed that this factor, *su-pur*, was carried by wild-type α and segregates independently of *ad-2* and *pur*. We set up crosses to test our hypothesis and were able to show that *su-pur* was present in both of our standard wild-type strains. The single dominant mutant listed in Table 1 was apparently not affected by the suppressor.

During this analysis we found that some of the purine-excreting segregants derived from 19 of the recessive mutants failed to mate to either *a* or α tester strains. Tetrad analysis showed that all of these non-mating segregants should have been of mating-type *a*. This mating anomaly seemed to be a secondary consequence of the mutations causing purine excretion, since no excreters of mating-type *a* have been recovered in extensive analysis of the anomaly. The suppressor did not affect the expression of the mating anomaly.

This association between purine excretion and the mating anomaly was found to affect all but five of the recessive mutants and meant that genetic analysis could only be attempted on these. In view of this we decided to obtain phenotypically similar mutants by a more direct method.

(ii) *The isolation and characterization of prototrophic purine-excreting mutants*

We were able to identify prototrophic strains which did not carry *su-pur* in the course of the analysis described in the previous section. It was felt that the use of such a strain in mating-type *a* for mutant isolation would allow the expression of a greater number of mutant genes and also permit immediate recognition of mutants exhibiting the mating anomaly. Following mutagenic treatment by ultraviolet irradiation or EMS the cell suspension was plated on to YMM seeded with

Table 2. *Phenotypic classification of prototrophic purine-excreting mutants*

Group	Dominant or recessive	Effect of *su-pur* on excretion	No. of mutants
A	Recessive	Suppressed	13
B	Recessive	None	5
C	Dominant	None	9
D	Recessive	Reduced	6

the indicator *ad-2* diploid. Crossfeeding colonies were isolated, purified and crossed to wild-type α. Fourteen mutants, numbered between *P1* and *P19*, were induced by ultraviolet irradiation and 18, *P20* to *P40*, by EMS. Three of the mutants induced by ultraviolet irradiation failed to mate to wild-type α and were discarded. The remainder of the mutants, together with those salvaged from the previous experiments, were classified into four groups, A–D, according to their response to *su-pur* and whether they were dominant or recessive. Mutants which were recessive and affected by *su-pur* were allocated to group A; these mutants are phenotypically similar to those described in the previous section. The mutants assigned to the next two groups, B and C, segregated two crossfeeders in each tetrad and were

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presumed not to be affected by the suppressor. Mutants in group B were recessive whereas those in group C were dominant. Group D mutants also segregated two crossfeeders per tetrad but these showed two levels of excretion; half crossfed as strongly as the parental mutants but the remainder crossfed weakly.

All of the mutants were crossed to a strain which did not carry *su-pur* and, as expected, each tetrad contained two crossfeeding segregants. These crosses also showed that the dominance versus recessivity of the various mutant groups was not affected by the absence of *su-pur*. Only strong crossfeeders were recovered from the mutants allocated to group D, suggesting that the reduced excretion observed among the progeny of the crosses to wild-type α was caused by *su-pur*. This supposition was confirmed by a complete analysis of two tetrads from a cross of one group D mutant to wild-type α. We have termed these mutants in which excretion is reduced by *su-pur* 'partially-suppressible'. The complete classification of the four groups of mutants is listed in Table 2.

The apparent insensitivity of groups B and C to the suppressor was checked by mating the non-crossfeeding segregants from a number of asci to a suppressible mutant from group A. Thirty-four segregants from 17 tetrads were all shown to carry *su-pur* by the deficiency of crossfeeders among the progeny of these crosses. Since the mutants of these two groups proved to be allelic or closely linked (see next section) we can infer that the gene to which we allocated them, *pur-6*, is also allelic or closely linked to *su-pur*.

(iii) *Tests for allelism*

We assumed that allelic mutants would give a purine-excreting diploid whereas mutants of different genes would be complementary. However, since this simple functional criterion could not be applied to tests involving the dominant mutants of group C, all diploids were checked by tetrad analysis to detect recombination.

Table 3. *Allocation of purine-excreting mutants to the genes* pur-1 *to* pur-6

Gene	Mutants	Group	Total
pur-1	*P 3, P6, P17, P29, P32, P34, M11*	A	8
pur-1(ps)*	*P 5, P10, P23, P26, P37, P 30*	D	6
pur-2	*P12, P13*	A	2
pur-3	*C8*	A	1
pur-4	*C 3*	A	1
pur-5	*P1*	A	1
pur-6	*P14, P22, P28, P36, P39*	B	5
Pur-6	*P7, P19, P24, P25, P27, P31, P33, P40, M 5*	C	9

* Partially suppressible.

The results of the dual test for genetic identity allowed allocation of the mutants to six unlinked genes, *pur-1* to *pur-6*, as shown in Table 3.

Two of the genes are complex: *pur-1* has alleles which differ in their response to the suppressor whilst *pur-6* includes the dominant and recessive mutants which are closely linked or allelic to *su-pur*. One pair of mutants allocated to *pur-1*, *P17* and *P32*, complemented each other to form a non-crossfeeding diploid.

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Several pairs of mutants which formed purine-excreting diploids turned out to be non-allelic by tetrad analysis. In the absence of the genetic test these mutants would have been allocated to the same genes. These anomalous intergenic functional tests were obtained with the mutant *C8* (*pur-3*) and the recessive mutants of *pur-6* in combination with mutants of *pur-1*, *pur-4* and each other.

During the tests for allelism the various double mutants, most readily identified in non-parental ditype asci, were isolated and compared with the mutants from which they were derived. The results indicated that all of the double mutants, with the possible exceptions of *Pur-6 pur-2* and *Pur-6 pur-5*, crossfed more strongly than their parental single mutants. This means that most of the *pur* genes are additive in their effects.

(iv) *Effects of the suppressor in diploids*

Whilst investigating the *pur-6* mutants we found that diploids which were homozygous for a dominant mutant and heterozygous for *su-pur* crossfed less strongly than those which did not carry the suppressor. To see if this partial dominance was a general property of *su-pur* we synthesized diploids of the genotype *pur su-pur*/*pur su-pur*$^+$ with representative mutants of each of the genes *pur-1* to *pur-5* and tested them for their ability to crossfeed. The extent of excretion compared with that of strains which did not carry *su-pur* is shown in Table 4. The suppressor is thus dominant with respect to *pur-1*, *pur-2* and *pur-4*; partially dominant over *pur-3* and *pur-5* and recessive in combination with *pur-1*(*ps*).

Table 4. *Expression of* su-pur *in diploids homozygous for mutants of* pur-1 *to* pur-6

Gene	Purine excretion by diploid	Expression of *su-pur*
pur-1	None	Dominant
pur-1(*ps*)	Strong	Recessive
pur-2	None	Dominant
pur-3	Weak	Partially dominant
pur-4	None	Dominant
pur-5	Weak	Partially dominant

(v) *Tests for allelism between the* pur *genes and* ad-1 *to* ad-8

Representative mutants of *pur-1* to *pur-6* were mated to strains carrying *ad-1* to *ad-8*. The diploids were tested for purine excretion and sporulated to allow genetic analysis. None of the intergenic matings gave crossfeeding diploids except those involving *Pur-6*; however, this could have been due to the suppressor which was shown to be present in the strains carrying *ad-1*, *ad-3*, *ad-5*, *ad-7* and *ad-8*. No recombinants were recovered from the crosses of the dominant and recessive mutants of *pur-6* to *ad-4*, indicating that these two loci are closely linked. Since the diploid formed by *ad-4* and a recessive mutant of *pur-6* did not crossfeed we think it unlikely that they are allelic. One intergenic cross, *pur-5* by *ad-8*, indicated linkage between the two genes; the tetrad ratios were PD 21:NPD 0:TT 6, single

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strand analysis giving 11·1 % recombination. The remainder of the crosses showed free recombination between the *ad* and *pur* genes.

Since we had originally obtained purine-excreting mutants by selecting for adenine-insensitivity, we tested the segregants from the *pur* × *ad-2* crosses on YMM supplemented with 50 μg/ml of hypoxanthine. As might have been expected, the double mutants derived from *pur-1*, *pur-3*, *pur-4* and *Pur-6*, to which we had allocated four of the mutants isolated on the basis of adenine-insensitivity, were deeply pigmented on this medium. However, the mutants of *pur-1(ps)* and *pur-5* gave double mutants which were as sensitive as *ad-2 pur*$^{+}$, whilst *pur-2* gave a double mutant which was hypersensitive.

We concluded from the genetic analysis that with the possible exception of *ad-4* and *pur-6* there were no instances of allelism between the *ad* and *pur* loci tested. We can also be reasonably certain that none of the *pur* genes are allelic to *ad-9*, since this locus is only some 10 map units from *ad-2* (Mortimer & Hawthorne, 1966).

4. DISCUSSION

The aim of this investigation was to isolate and characterize mutants defective in the regulation of purine biosynthesis. The mutants that we have described crossfed to *ad-2* and therefore must be excreting adenine or hypoxanthine. It can be assumed that they are defective in some aspect of the regulation of purine metabolism. For one class of mutants, those allocated to *ad-12*, we can infer the metabolic lesion giving rise to the mutant phenotype. Mutants at this locus contain large amounts of intracellular inosine (Dorfman, 1969) and excrete inosine and hypoxanthine (R. A. Woods & S. Armitt, in preparation). Dorfman concludes that the mutants lack the enzyme adenylosuccinate synthetase and in consequence accumulate IMP. The IMP can then be degraded to inosine by a 5′-nucleotide phosphohydrolase (Demain & Hendlin, 1967) or converted to hypoxanthine by hypoxanthine:guanine phosphoribosyltransferase. The double mutant *ad-2ad-12* must utilize exogenous adenine for the provision of guanine nucleotides; this will involve the deamination of AMP to IMP. Burns (1964) has furnished evidence that the feedback inhibitor active *in vivo* is IMP, yet the double mutant *ad-2ad-12*, which will presumably contain a high level of this compound, continues to synthesize purine precursors. It has been suggested (Dorfman, 1969) that the protein specified by *ad-12* has both catalytic and regulatory functions and might act as an aporepressor for enzymes earlier in the pathway. If this is so then a mutation affecting its regulatory but not its catalytic activity could lead to overproduction of purine.

At present we know nothing about the biochemical lesions causing purine excretion by mutants of *pur-1* to *pur-6*. Heslot *et al.* (1966) have isolated mutants of *Schizosaccharomyces pombe* resistant to 8-azaguanine which are prototrophic and excrete hypoxanthine. These mutants, allocated to a gene *aza-1*, were found to be allelic to *ad-4*, the structural gene for PRPP amidotransferase in this organism. We have found no such correlation between our presumed regulatory mutants and any of the loci specifying steps in purine synthesis with the exception of the close

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linkage between *pur-6* and *ad-4*. Silver & Eaton (1968) have shown that *ad-4* probably specifies the enzyme catalysing the conversion of glycinamide ribonucleotide to formylglycinamide ribonucleotide. This is the third step of purine biosynthesis and has not yet been shown to be subject to either feedback inhibition or genetic repression, so that allelism between *pur-6* and *ad-4* seems unlikely. The structural gene for PRPP amidotransferase has not yet been identified in yeast, but the biochemical evidence presented by Silver & Eaton (1968) suggests that it is probably *ad-5*, *ad-8* or *ad-9*, none of which are allelic to any of the *pur* genes.

Excessive purine synthesis is characteristic of the Lesch–Nyhan syndrome, a sex-linked neurological disorder, and it has been shown by Seegmiller, Rosenbloom & Kelley (1967) that the apparent cause is a deficiency of the enzyme hypoxanthine:guanine phosphoribosyl transferase. If this metabolic lesion has the same phenotypic effect in yeast it could account for one of the *pur* genes; a possibility that we are currently investigating.

The interactions of the *pur* genes with each other and with *su-pur* indicate a complex system of metabolic regulation. Unfortunately our genetic studies have not allowed us to identify any of these 'regulatory' mutants with genes known to specify steps in purine biosynthesis. We hope that the biochemical and physiological studies we are currently undertaking in this laboratory will allow us to allocate functions to some, if not all, of the *pur* loci.

We are indebted to the Science Research Council for a Research Studentship (S.A.) and Research Grant no. B/SR/2969 (R.A.W.). We would like to thank Drs B. Dorfman and M. Grindle and Mr C. A. Lomax for their comments on the manuscript.

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https://doi.org/10.1017/S0016672300001324 Published online by Cambridge University Press