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Potentiation by Purines of the Growth-inhibitory Effects of Sulphonamides on *Escherichia coli* K12 and the Location of the Gene which Mediates this Effect

By I. BRUCE,† JUDITH HARDY AND K. A. STACEY*
Biological Laboratory, The University, Canterbury, Kent CT2 7NJ, UK

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The increased toxicity of sulphonamides for *Escherichia coli* in the presence of low concentrations (50-100 μM) of purines or purine nucleosides has been confirmed and investigated further. The potentiating effect of a purine was dependent upon the activity of the appropriate phosphoribosyl transferase; a *gpt* mutant strain was not potentiated by guanine but remained fully sensitive to the addition of adenine. Mutants resistant to the potentiating effect of all purines have been isolated and partially characterized. The site of these mutations has been located in the region between *oriC* and *asnA* at minute 83 on the *E. coli* chromosome map. It is suggested that this locus be temporarily designated *psp* (potentiation of sulphonamides by purines) because these mutants have unaltered sensitivities to sulphonamides acting alone. Mutations in *purA*, *purR* and *folB* did not affect the potentiation of sulphonamides by purines. Hypoxanthine-insensitive strains harbouring *λasn20* were as sensitive as the wild-type to the potentiating effect. This result suggests that these lysogens are heterozygous for *psp* and that the wild-type allele is dominant. It is probable that *psp* is a regulatory gene, affecting some rate-limiting step in the biosynthesis of methionine.

INTRODUCTION

Purines have been implicated in the action of anti-folate inhibitors on *Escherichia coli*, both as agents which alleviate their effects and as agents which increase their toxicity. The effects of purines on the toxicity of sulphonamides appear to depend upon whether or not methionine is added to the medium. The first report (Harris & Kohn, 1941) emphasized that in the absence of methionine the addition of a purine (10^{-4} M) caused significantly greater inhibition of growth by sulphanilamide. Shive & Roberts (1946) did not find this effect, but did find that in the presence of methionine the addition of a purine lowered still further the growth inhibition by sulphanilamide and Winkler & de Hann (1948) confirmed the protective effect of xanthine when added together with methionine. Breeze (1972) showed that in the presence of guanine, hypoxanthine or inosine (100 μM) the minimum inhibitory concentration of trimethoprim for a sensitive strain of *E. coli* K12 was one-half to one-third that found in its absence, and for trimethoprim-resistant mutants derived from it, only one-eighth. Then & Anghern (1973, 1974) showed that combinations of trimethoprim and sulphamethoxazole were bactericidal, not merely bacteriostatic, for *E. coli* in the presence of methionine and a purine, because under these conditions the cells suffered 'thymineless death'.

It is, at first sight, paradoxical that the addition to the medium of low concentrations of a product of folate metabolism should increase rather than reduce the toxicity of anti-folate drugs.

For the isolation of sulphonamide-resistant mutants the addition of hypoxanthine is valuable, because it increases the toxicity of the sulphonamide. We have confirmed for our strains that this effect is a general one for purines and is distinct from the adenine-sensitivity studied by

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† Present address: Department of Biochemistry, University College, Gower Street, London WC1E 6BT, UK.

Dalal *et al.* (1966) in certain mutants of *Salmonella typhimurium*. Purines also potentiate the effects of trimethoprim on our strains.

The present paper reports some preliminary observations on the purine potentiation of the action of sulphonamides and ways in which it can be reduced, in particular by mutation. A new genetic locus has been identified and located at minute 83 on the *E. coli* chromosome map by the isolation of mutants which are less sensitive than the wild-type to sulphonamides when low concentrations of a purine are added to the medium.

METHODS

Bacterial strains. All strains were derivatives of *E. coli* K12 (Table 1). The bacteriophage P1 used was a laboratory strain. P1$_{c1}$. *λasn20* was given to us by Dr M. Masters (Dept of Molecular Biology, Edinburgh University, UK), and *λasn89* and *λasn212* by Professor K. von Meyenburg (Dept of Microbiology, Technical University of Denmark, Lyngby-Copenhagen, Denmark).

Media. Defined salts medium (minimal medium) was prepared according to Clowes & Hayes (1968) and solidified with 1% (w/v) Oxoid agar. Where necessary, amino acids (20 μg ml^{-1}) and vitamins (0·1 μg ml^{-1}) were added. To test for the Bgl phenotype, arbutin (4-hydroxyphenyl-*β*-D-glucopyranoside) or salicin (2-hydroxymethylphenyl-*β*-D-glucopyranoside) were substituted for glucose.

Chemicals. Arbutin, salicin, sulphanilamide, sulphadiazine and sulphathiazole were obtained from Sigma, streptomycin from Glaxo and spectinomycin from Upjohn.

Measurements of minimal inhibitory concentrations (MICs). All the tests involved the formation of single colonies on solid minimal media. They were quicker to perform and less ambiguous than tests in liquid media in which the inoculum size is important. The formation of single colonies was scored either on streak plates or by plating 0·01 ml drops of a series of tenfold dilutions of an overnight culture in minimal medium with the appropriate supplements.

Isolation of Psp mutants. Mutants of strain AB1157 able to form colonies on supplemented minimal medium containing 10 μg sulphanilamide ml^{-1} and 50 μg hypoxanthine ml^{-1} were obtained by spreading approximately 10^7 bacteria per plate. Colonies were picked, re-streaked on the same medium and tested for sulphonamide resistance. Those clones which were still as sensitive as the parent to sulphanilamide alone were tested for resistance to the potentiation by adenine and guanine. The majority of the mutants growing on the selective plates proved to be

Table 1. *E. coli strains used*

Strain	Relevant phenotype	Source*
AB1157		P. Howard-Flanders[1]
IB1	as AB1157 but sulphonamide-resistant	This work
IB3	as AB1157 but Psp$^-$	
TL505-6	*gpt*$^+$ *hpt purR*$^+$ *met*	M. Taylor[2]
CSH26	*gpt hpt*$^+$ *purR*$^+$ *met*	
TL462	*gpt*$^+$ *hpt*$^+$ *purR*	
TL505-M	as TL505-6 but *met*$^+$	This work (P1 transductants)
CSH26-M	as CSH26 but *met*$^+$	
JF448	*asnA31 asnB32 bglR13 rbs-4*	B. J. Bachmann[3]
AT2465	*guaA21*	
PC0950	*purA54*	
G2	as AT2465 but Psp$^-$	This work
G10	as G2	
G16	as G2	
A2	as PC0950 but Psp$^-$	
A4	as A2	
A16	as A2	
KL16 *recA*	*srl*::Tn*10 recA1*	P. Oliver[4]
RH64	F$^-$ *thi asnB32 asnA34*::Tn*5*	R. D. Simoni[5]
RH64-1	as RH64 but *psp*	This work (P1 transduction from IB3)
RH64 *recA psp*	as RH64-1 but *recA1*	This work (P1 transduction from KL16 *recA*)
RH64 *recA*	as RH64 but *recA1*	

* Addresses of donors: 1, Dept of Molecular Biology, Yale University, New Haven, Conn., USA; 2, Dept of Biology, University of Indiana, Bloomington, Ind., USA; 3, Dept of Microbiology, Yale University Medical School, New Haven, Conn., USA; 4, Dept of Genetics, Downing Street, Cambridge, UK; 5, Dept of Biological Sciences, Stamford University, Stamford, Calif., USA.

Psp⁻. One clone, IB3, has been studied in detail. Similar mutants of the purine-requiring strains PC0590 and AT2465 were easily obtained because these strains proved particularly sensitive to sulphanilamide.

Genetic location of psp. Standard methods of conjugation and P1-mediated transduction were used (Stacey & Lloyd, 1976). The conjugal crosses were carried out in nutrient broth. Phage P1 was grown through two cycles on plates with the appropriate donor strains. Linkage of unselected markers was scored using 200 recombinants for each selected marker. The BglR phenotype was scored on arbutin plates; growth was taken to indicate BglR .

RESULTS

Potentiation of sulphonamide toxicity by purines and the sparing effect of vitamin B_{12}

The MIC of sulphanilamide for strain AB1157 was lowered by the addition of purines or their ribonucleosides (Table 2). The addition of hypoxanthine also increased the toxicity of sulphanilamide for a sulphonamide-resistant mutant of AB1857, IB1, which probably owes its resistance to overproduction of *p*-aminobenzoate, the MIC being reduced from 160 to 40 μg ml^{-1} by 10 μg hypoxanthine ml^{-1} (Bruce, 1981). None of the *E. coli* strains used in this study was purine-sensitive in the absence of sulphonamide. Similar results were obtained for the much more toxic sulphonamides, sulphadiazine and sulphathiazole (J. Hardy, unpublished results).

Of the metabolites whose biosynthesis is limited by sulphonamides, only methionine is an effective antagonist at low concentrations (Harris & Kohn, 1941; Shive & Roberts, 1946) and we found vitamin B_{12}, although it induces an alternative transmethylation pathway, had no significant effect (Table 2). However vitamin B_{12} was as effective as methionine in 'sparing' the combined effects of sulphanilamide and hypoxanthine; both compounds raised the MIC of sulphanilamide fourfold in the presence of 10 μg hypoxanthine ml^{-1}.

Resistant mutants

Two classes were studied. It was expected that mutants deficient in the uptake of a purine, because of a mutation in the gene which encodes the relevant phosphoribosyltransferase, would not be made more sensitive to sulphonamides by the presence of that purine although they should continue to be affected by purines taken up by the other phosphoribosyltransferases. So it proved; a *gpt* mutant, strain CSH26, grew in the presence of 50 μg guanine ml^{-1} plus 20 μg sulphanilamide ml^{-1} but was sensitive to the addition of 10 μg hypoxanthine ml^{-1}. Strain AB1157, which is known to be defective for guanine uptake (Hoeckstra & Vis, 1977), behaved similarly: it was resistant to guanosine (50 μg ml^{-1}) but still sensitive to adenosine (10 μg ml^{-1}) in the presence of 10 μg sulphanilamide ml^{-1}. The *hpt* mutant, TL505M, was however still partially sensitive to hypoxanthine because guanine phosphoribosyltransferase is slightly active towards this base (Jochimsen *et al.*, 1975; Holden *et al.*, 1976). Thus the efficiency of plating was reduced to about 1% by the addition of 10 μg hypoxanthine ml^{-1} plus 20 μg sulphanilamide ml^{-1} but not abolished. It was, however, more sensitive to the addition of inosine. The *purR* mutation (in strain TL462) had no effect on the potentiation by purines nor did a mutation in *folB* which causes overproduction of dihydrofolate reductase.

The second class of mutants was obtained by selection (see Methods). The majority of clones, isolated by their ability to grow in the presence of 50 μg hypoxanthine ml^{-1} plus 10 μg sulphanilamide ml^{-1}, were not sulphonamide-resistant in that they proved to be just as sensitive as the parent strain to sulphonamides acting alone. Nor were they simply hypoxanthine-uptake mutants because they also grew in the presence of combinations of sulphonamide and adenine or guanine which were inhibitory for the parent strain. One such mutant strain, IB3, has been studied in detail (Table 2) and the mutation shown to be in a hitherto unknown gene which, it is proposed, should be tentatively designated *psp* (*p*otentiation of *s*ulphonamides by *p*urines).

Location of psp

The approximate location of the mutation in IB3 which confers partial resistance to purine potentiation was obtained by scoring the ability to form colonies on sulphanilamide plus hypoxanthine plates of recombinants from crosses with various Hfr donor strains. Analysis of

Table 2. *Effect of additives upon the toxicity of sulphanilamide to* Psp^+ *and* Psp^- *strains of E. coli in minimal medium*

Purine (μg ml^{-1})	Other compounds (μg ml^{-1})	MIC of sulphanilamide (μg ml^{-1})	
		AB1157 psp^+	AB1157 *psp* (IB3)
None	None	80	80
Hypoxanthine (10)	None	10	40
Hypoxanthine (50)	None	10	20
None	Methionine (0·1 and 0·5)	320	NT
Hypoxanthine (10)	Methionine (0·5)	40	NT
None	Vitamin B_{12} (10^{-2})	80	80
Hypoxanthine (10)	Vitamin B_{12} (10^{-2})	40	NT
Inosine (20, 50 and 100)	None	10	20
Adenine (10)	None	10	40
Adenine (50)	None	10	20
Adenosine (100)	None	10	20

NT, Not tested.

Table 3. *P1-mediated transductants of JF448*

P1 (IB3 rbs^+ $asnA^+$ *psp* $bglR^+$) × JF448 (*rbs asnA* psp^+ *bglR*)

Selected for Asn^+								
rbs	+	+	−	−	+	+	−	−
psp	−	−	−	−	+	+	+	+
bglR	+	−	+	−	+	−	+	−
Percentage of 200 recombinants:	26	47·5	4·5	11	2·5	5·5	1	2
Selected for Rbs^+								
asnA	+	+	−	−	+	+	−	−
psp	−	−	−	−	+	+	+	+
bglR	+	−	+	−	+	−	+	−
Percentage of 200 recombinants:	38	58	0	0·5	1·5	1·5	0	0

these recombinants suggested that the *psp* mutation lay between *xyl* and *argE*. It was located more accurately by phage P1-mediated transduction. Co-transduction of *psp* and *bglR* with either *asnA* or *rbsK* was measured with strain JF448 as the recipient in recombinants selected for either asparagine independence or ribose utilization. The results suggest that the *psp* mutation in IB3 is close to *asnA* (Table 3). Although the absolute value of the linkage of *psp* to *rbs* was greater than that to *asnA*, the data are only consistent with the order *bglR–psp–asnA–rbs* (see Bachmann & Low, 1980). For Rbs^+ recombinants, co-transduction of *asnA* was 99%, of *psp* 97% and of *bglR* 40%; for $AsnA^+$ recombinants, co-transduction of *psp* was 89%, of *rbs* 81% and of *bglR* 34%. Other alleles of *psp* (see below) yielded rather similar results. This location has been confirmed (in *recA* derivatives) by lysogenization with transducing phage, *λasn* (von Meyenburg *et al.*, 1978). Hypoxanthine-resistant strains made lysogenic for either *λasn20* or *λasn212* were sensitive to purine potentiation while those harbouring *λasn89* were not. These results place *psp* in the 1 kb segment of the chromosome between *oriC* and *asnA* (von Meyenburg & Hansen, 1980). Further experiments (J. Hardy, unpublished results) with *λasn* derivatives confirm this allocation but the interpretation of the results is complicated by incompatibility effects (Yamaguchi *et al.*, 1982) and it has not yet been possible to identify *psp* unambiguously with either of the two proteins encoded by this segment of the chromosome (Hansen *et al.*, 1981).

Purine auxotrophs

An attempt was made, using purine auxotrophs, to determine which purine nucleotide might be involved in the sensitization to sulphonamides, but both the adenine-requiring (*purA*) strain, PC0950, and the guanine-requiring (*guaA*) strain, AT2465, tested in preliminary experiments, were especially sensitive to sulphonamides. Strain AT2465 was unable to form colonies on media containing 10 μg sulphanilamide ml^{-1}, even at guanine concentrations as low as 1 μg ml^{-1}. However, both strains readily threw off mutants which could grow under these conditions, and three such mutants derived from each strain were transduced (with phage P1 grown on CR63) to purine independence. The transductants showed resistance to hypoxanthine and sulphonamide. One mutant from each strain (A4 from PC0950 and G16 from AT2465) was then used as the donor in phage P1-mediated crosses with JF448 as recipient. Resistance to the purine effect was scored as an unselected marker and it showed in both crosses the high level of linkage to the markers *asnA* and *rbs* that was found for *psp* in the crosses discussed earlier. It was assumed that these mutants were allelic with those selected in strains prototrophic for purines.

These findings suggested that the sensitization by purines might be indirect and due to a limitation in the supply of pyrimidine nucleotides by competition for and inhibition of phosphoribosyl pyrophosphate synthetase. However, hypoxanthine and sulphanilamide were just as inhibitory when the medium contained ribose (as carbon source), histidine, tryptophan and uridine (100 μg ml^{-1}) as when the medium contained only glucose.

Dominance of psp⁺

Attempts to isolate stable F-prime merodiploids of RH64 *recA psp* were not successful, but with phage *λasn20* (von Meyenburg *et al.*, 1978) lysogens sufficiently stable to test were obtained. The lysogenic strains proved as sensitive as the wild-type to the presence of hypoxanthine when tested at 30 °C on plates containing 20 μg sulphanilamide ml^{-1}. This phage has a temperature-sensitive repressor (CI_{857}) and incubation at 42 °C readily yielded asparagine-requiring, λ-sensitive (cured) clones which proved to be once more insensitive to the purine effect. These results imply the existence of a *trans*-active dominant gene encoded by part of the segment of the chromosome carried by *λasn20*.

Lysogens of the wild-type (hypoxanthine-sensitive) strain RH64 *recA* harbouring either *λasn20* or *λasn212* made only tiny colonies at concentrations of hypoxanthine and sulphanilamide which permitted normal growth of RH64 *recA* (*λasn89*). Thus the presence of extra copies of *psp*, carried by *λasn20* and *λasn212* but not by *λasn89*, made growth more difficult for Psp^+ cells.

DISCUSSION

Our results confirm the observations of Harris & Kohn (1941) that lower concentrations of sulphonamides were required to inhibit growth of *E. coli* in the presence of low concentrations (approx 0·1 mM) of a purine than when acting alone (Table 2). Usually the MIC was reduced to about one-quarter of the value obtained when only the sulphonamide was present. The same effect was also seen for a sulphonamide-resistant mutant (Bruce, 1981).

The active inhibitory compound must be a purine nucleotide or a related metabolite, because a purine whose uptake is substantially reduced by a mutation in the gene for the relevant phosphoribosyltransferase did not exert any potentiation although other purines retained their effectiveness. It has been suggested that the hypoxanthine present in urine may be responsible, because of this potentiation, for the efficacy of sulphonamides in the treatment of urinary infections (J. T. Smith, personal communication).

It is possible that the growth inhibition by mixtures of sulphonamide and hypoxanthine is due, like that by sulphonamides alone, to the limitation in the biosynthesis of methionine. Vitamin B_{12}, while it does not affect the MIC of sulphanilamide acting alone, does reduce the potentiating effect of hypoxanthine (Table 2).

The final stage of methionine biosynthesis involves the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine. *Escherichia coli* possesses two alternative mechanisms for this transmethylation. In minimal media the transmethylase is provided by the *metE* gene but in the presence of vitamin B_{12} *metE* is repressed and the *metH* gene is induced (or derepressed). The *metH* gene product, the B_{12}-dependent transmethylase, is a more efficient enzyme; it has a higher turnover number and a lower K_m for 5-methyltetrahydrofolate, and is synthesized in lower amounts than the *metE* gene product (Flavin, 1975). The action of vitamin B_{12} in lowering the effect of hypoxanthine suggests that potentiation of sulphonamides by purines is brought about by an additional limitation of the pool of 5-methyltetrahydrofolate to a level such that only the more efficient B_{12}-dependent enzyme can sustain methionine biosynthesis.

The effects of hypoxanthine can also be reduced by mutations in what appears to be a single gene. Evidence for this gene was obtained in two ways. Mutants which were less sensitive to purine potentiation represented the majority of mutants able to grow on sulphanilamide plus hypoxanthine plates. These mutants were no more resistant than the parent strain to sulphonamides acting alone, nor were they merely defective for hypoxanthine uptake, because they were equally resistant to the addition of all the other purines and purine nucleosides tested. The mutation responsible has been located by its linkage as an unselected marker in phage P1-mediated crosses to *asnA* and *rbs* (Table 3). The data suggest that the order is *bglR–psp–asnA–rbs*. This result was confirmed by the finding that lysogens of Psp$^-$ strains harbouring *λasn20* were sensitive to potentiation by purines. The *psp* and *asnA* genes must lie, therefore, within the approximately 1 kb segment of DNA close to *oriC* at minute 83 on the *E. coli* map (von Meyenburg & Hansen, 1980). This result also suggests that purine sensitivity is dominant and therefore due to the action of a *trans*-active gene product. The action of the *psp* gene product appears to be quantitative rather than qualitative because the wild-type was made more sensitive to hypoxanthine if it harboured a *λasn* carrying *psp*.

Purine auxotrophs proved to be especially sensitive to sulphonamides and mutants able to grow at low sulphonamide concentrations were, therefore, easily isolated. Of the two mutants tested, both were shown to be allelic with the *psp* mutation obtained in a purine-independent strain. Since these Psp$^-$ strains continued to be dependent for growth upon external sources of adenine or guanine, resistance to the purine potentiation cannot be due to substantial changes in the metabolic mobilization of the purines.

If purines exert their influence through a reduction in the rate of synthesis of methionine which is already low because of the action of the sulphonamide, a possible role of the *psp* gene product (when activated by a purine metabolite) is in the regulation of the pool size of tetrahydrofolate co-factors. Little is known about the regulation of folate metabolism. Methionine and, surprisingly, vitamin B_{12} repress the formation of 5,10-methylenetetrahydrofolate reductase (Katzen & Buchanan, 1965; Greene *et al.*, 1973) and purines repress the synthesis of 5,10-methylenetetrahydrofolate dehydrogenase (Taylor *et al.*, 1966) although, at most, by only 40%. The latter enzyme is also inhibited at physiological concentrations by purine nucleoside triphosphates (Dalal & Gots, 1966). It is likely that these effects and that of *psp* relate to a more complicated set of regulatory mechanisms which prevent wasteful trapping of tetrahydrofolate co-factors in forms not needed by the cell when exogenous sources of methionine and purines are available, a form of economy which becomes suicidal when the synthesis of tetrahydrofolate is limited by sulphonamides.

That other regulatory mechanisms remain to be discovered is shown by the fact that the gene product which makes *E. coli* more sensitive to trimethoprim when purines are added to the medium (Breeze, 1972) is not the same as that encoded by *psp*. Both Psp$^+$ and Psp$^-$ strains are equally sensitive to combinations of purines and trimethoprim, and mutants resistant to these combinations are just as sensitive to combinations of sulphonamide and hypoxanthine as their parent strains. Moreover, in contrast to the results obtained with *λasn20*, merodiploids heterozygous for the equivalent gene affecting purine potentiation of trimethoprim are as resistant as the haploid (J. Hardy, unpublished results). The mutation which eliminates the sensitivity to purines is either *cis*-dominant or a mutation in a gene for a positive control element which is thereby rendered insensitive to the level of exogenous purines.

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