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A genetic study of primary and secondary reversions of some *tryptophanA* auxotrophs of *Salmonella typhimurium*

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1. INTRODUCTION

It was customary at the time this work was started to score reversions of an auxotroph of *Salmonella typhimurium* by their rate of growth on minimal medium and so separate probable back-mutations from probable suppressor mutations. These latter were sometimes further analysed to discover whether they were site-specific or not. The aim of the present study was to investigate more fully the range of genetic changes that could result in reversions. We were particularly interested to discover whether some of the reversions were due to further changes in the gene, these further changes complementing or suppressing the original mutant site. We chose the *tryA* gene, and genetically analysed the reversions of a number of independently isolated mutants. The *tryA* gene controls a step prior to anthranilic acid in the synthesis of tryptophan; it is probably responsible for the production of anthranilate synthetase (Doy, 1966). All the structural genes mediating the pathway of tryptophan synthesis, together with the gene *cysB*, are carried on the same transducing fragment in the *Salmonella*-PLT-22 system; that is, they are linked in the terminology of transduction.

2. MATERIALS AND METHODS

The mutant strains *tryA-8*, *tryA-47*, *tryA-50*, *tryA-52*, *tryA-56*, *tryB-4*†, *tryB-13*, *tryC-44*, *tryD-6*, *cysB-12*, *tryA-8 tryB-4*, *tryA-56 cysB-12* and the wild-type strain of *S. typhimurium* known as LT-2 were obtained from the collection of the Carnegie Department of Genetics, Cold Spring Harbor, Long Island, New York. The double mutants *tryA-52 cysB-12*, *tryA-8 cysB-12*, *tryA-50 cysB-12*, and *tryA-47 cysB-12* were obtained by transducing each *tryA* mutant to the recipient *tryB-4 cysB-12* and selecting the desired recombinants on enriched minimal medium plus anthranilic acid and cysteine.

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† On the basis of biochemical examination of extracts, Blume & Balbinder (1966) have recently recognized two genes, *tryB and tryE*, in the region originally called *tryB*. Throughout this paper *tryB* is used in its old sense.

The phage PLT-22 (Zinder, 1955) was used in all the transduction experiments. When a phage-sensitive transductant was required, the donor phage was prepared using mutant H_4 of PLT-22 (termed v_1 by Zinder, 1958). One or two transductants were then streaked for single colonies and these were tested for their sensitivity to wild-type phage.

Anthranilic acid and amino acids were added to the medium to a final concentration of 0·002%. Cysteine was added to nutrient broth or nutrient agar whenever a cysteine-requiring strain was cultured.

The system of nomenclature, composition of media, procedures for phage preparation, storage and transduction were essentially similar to those described by Smith-Keary (1960). The only abbreviations that we have used are MM, for minimal medium, and EMM, for minimal medium enriched with 0·01% dehydrated Difco nutrient broth.

3. PRELIMINARY STUDY

(i) *Genetic analysis of the* tryA *auxotrophs*

Three-point transductions were used to study the positions of the mutant sites in *tryA-8*, *tryA-50*, *tryA-52*, and *tryA-56* relative to that in the *tryA-47* strain. For example, the relative position of the *A-8* site was obtained from these two transductions:

(*a*) *tryA-47 cysB-12* (×) *tryA-8*
(*b*) *tryA-8 cysB-12* (×) *tryA-47*.

The *cysB* locus is on the same transduced fragment as the *tryA* locus. *cysB-12* was used in all the transductions. cys^+ transductants were selected on EMM medium and include both large (try^+cys^+) and small (try^-cys^+) colonies. The latter are pinpoint colonies, each surrounded by a halo of growth of the cys^- recipient cells. It appears as if mutual feeding takes place between cys^- and $tryA^-$ cells; the effect occurs with all the *tryA* mutants that we have studied. If the order is *A-8—A-47—cysB-12* a much higher proportion of try^+cys^+ transductants will be recovered from transduction (*a*) than from transduction (*b*). If the order is *A-47—A-8—cysB-12* the reverse will be found (Fig. 1). The data from all the transductions is set out in Table 1 and from these the most probable order appears to be *A-8—A-47—*(*A-56—A-52*)*—cysB-12*.

The data from the transductions that involve *tryA-50* are curious in that about the same frequency of wild-type transductants is found in both transductions. No wild-type recombinants are produced by homologous transductions of *tryA-50* so the strain is not a selfer (Demerec, 1963). We considered the possibility that cells with both *A-47* and *A-50* mutant sites in the *tryA* gene have a phenotype indistinguishable from that of wild-type. If this is the correct explanation the majority of the wild-type colonies in one of the two transductions should be of this genotype. We tested this by transducing two such colonies from each transduction to *cysB-12* and selecting cys^+ transductants on MM + anthranilic acid medium. If the donor

strain were *tryA-47-50* a few of these transductants would arise by recombination between *A-47* and *A-50* sites and would be phenotypically tryA⁻. Two hundred clones from each of the four transductions were scored and none were tryA⁻. We can conclude either that *A-47* and *A-50* do not interact, which leaves the behaviour

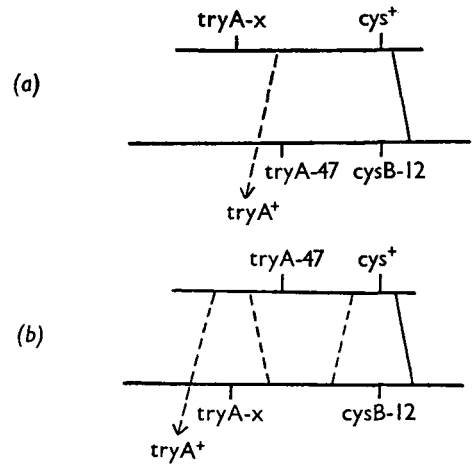

Fig. 1. Transductions to locate an unknown *tryA-x* site relative to *tryA-47*.

(*a*) *tryA-47 cysB-12* (×) *tryA-x cys*⁺. Selection for cys⁺ transductants: tryA⁺ transductants arise by double crossovers when *tryA-x* is to the left of *tryA-47*.

(*b*) *tryA-x cysB-12* (×) *tryA-47 cys*⁺. Selection for cys⁺ transductants: rare tryA⁺ transductants arise by quadruple crossovers when *tryA-x* is to the left of *tryA-47*.

When *tryA-x* is to the right of *tryA-47*, tryA⁺ transductants arise by double crossovers in transduction (*b*), and are rarer and arise by quadruple crossovers in transduction (*a*).

of *A-50* unexplained, or that when the *A-47* and *A-50* sites are in the same gene they not only interact but also suppress recombination between them. This second alternative might be tested by an analysis of the enzyme in the prototrophs from the two transductions.

Table 1. *Transductions of the types described in Fig. 1, to distinguish the order of five sites in the* tryA *gene*

	Proportion of cys⁺ transductants that were tryA⁺ in transductions	
tryA-x	*tryA-47 cysB-12* (×) *tryA-x cys*⁺	*tryA-x cysB-12* (×) *tryA-47 cys*⁺
tryA-8	34/1317	0/164
tryA-47	0/1000	0/361
tryA-50	17/962	10/870
tryA-52	6/1624	49/3683
tryA-56	0/325	32/2457

(ii) *The classes of reversions and their frequency*

(*a*) *Primary reversions*

The types of reversions of each mutant, and their relative frequencies, were examined in the following way. A number of independent broth cultures of each mutant, started from small inocula, were aerated for between 16 and 18 hours at 37°C. and 0·1 ml. samples transferred to two plates of EMM. After the plates had been incubated for 5 days the number of colonies were scored. Some of these colonies from different plates, derived from as many independent cultures as possible, were examined for their growth characteristics. The results are summarized in Table 2.

Table 2. *Frequencies of reversions, and classes of reversions, of* tryA *auxotrophs*

Auxotroph	Number of independent cultures	Average number of reversions/10^8 bacteria	Number examined for rate of growth	Classes		
				Fast (F)	Semi-fast (SF)	Slow (S)
tryA-8	5	8·53	30	4	1	25
tryA-47	20	5·60	30	23	2	5
tryA-50	8	6·62	24	5	—	19
tryA-52	10	0	—	—	—	—
tryA-56	20	0·68	24	6	10	8

With the exception of *tryA-52*, all the mutants produce reversions. *tryA-47*, *tryA-50* and *tryA-8* have similar frequencies of reversions; *tryA-56* has a lower frequency. The reversions could be divided into three classes: fast (F), semi-fast (SF) and slow (S). All those which were either indistinguishable from wild-type or had only a slightly lower growth rate were included in the first class. Those which required at least 48 hours incubation before they formed visible colonies were called slow-growing reversions. The semi-fast class included all reversions with rates of growth between these two extremes. The reversions of *tryA-47* that grow slower than wild-type have a wide range of growth rates, the faster falling into the semi-fast class and the slower into the slow-growing class. As their range of growth rates shows no sharp discontinuity between these two classes, all of them will be referred to as slow-growing (S) reversions in the subsequent account. With the exception of reversions of *tryA-8* and *tryA-50*, the frequencies of the different classes varied widely from one mutant to another.

(*b*) *Secondary reversions*

Secondary reversions are phenotypic variants of primary reversions. Two types of secondary reversions were found in the course of experiments with the S reversions of *tryA-47*. One has a very slow rate of growth (VS). The other has a similar or

higher rate of growth to that of the faster growing S reversions; to clearly distinguish them from these primary reversions they are called intermediate (I).

The VS reversions first arose in transduction of S reversions of *tryA-47* to *tryA-47 cysB-12*. They are very unstable in that they have a high rate of reversion to the original S type.

The I reversions were isolated by plating saturated cultures of S reversions of *tryA-47* on minimal medium. After incubation, small papillae formed on the surface of the confluent growth and from these the I reversions were obtained. Although a wide range of growth rates was found among twenty-one independently isolated I reversions none grew quite as fast as wild-type.

4. GENETIC ANALYSIS OF THE REVERSIONS

To distinguish those reversions that had arisen by a mutation at a suppressor locus that was not linked to *tryA*, each reversion was transduced to a strain of its original auxotroph which was also *cysB-12*. cys$^+$ transductants were selected and scored for their ability to grow in the absence of anthranilic acid. If all the transductants were tryA$^-$ the reversion contained a mutation at an unlinked suppressor.

Table 3. *Transductions between reversions of* tryA *auxotrophs, all of which were* cys$^+$, *as donors and* tryA-x cysB-12 *as recipient: selection for cys$^+$ transductants*

	Reversions used as donors			Results*	
Recipients	Source of reversions	Class of reversions	Number of reversions tested	Number of reversions due to mutation at linked site	Number of reversions due to mutation at unlinked suppressors
tryA-8 cysB-12	*tryA-8*	Fast	20	14	6
		Semi-fast	7	5	2
		Slow	7	—	7
tryA-47 cysB-12	*tryA-47*	Fast	19	19	—
		Slow	13	13	—
tryA-50 cysB-12	*tryA-50*	Fast	10	6	4
		Slow	5	—	5
tryA-56 cysB-12	*tryA-56*	Fast	7	7	—
		Semi-fast	3	3	—
		Slow	2	—	2

*If all transductants were tryA$^-$ the reversion was scored as unlinked suppressor; if not all were tryA$^-$ the reversion was scored as mutation at a site linked to, or within, the *tryA* gene.

Of ninety-three primary reversions analysed in this way twenty-six were of this type (Table 3) and included a substantial proportion of the fast-growing reversions of *tryA-8* and *tryA-50*, some of the semi-fast reversions of *tryA-8* and all the slow-growing reversions of *tryA-8*, *tryA-50*, *tryA-56*. The transductions of the remaining

sixty-seven reversions yielded between 17% and 47% of transductants which would grow in the absence of anthranilic acid; the genetic change was therefore within the limits of the *try-cys* transduction fragment.

(i) *Unlinked suppressor mutations*

The only unlinked suppressor mutations that we analysed further were those of *tryA-8* which had fast-growing phenotypes. We found that they included at least two types: one that suppresses both *tryA-8* and *tryB-4* and another that suppresses mutations only in the *tryA* locus.

(*a*) *Suppression of* tryA *and* tryB

When a fast-growing reversion (F_9) of *tryA-8* was transduced to *tryA-8 tryB-4* and transductants selected on MM + anthranilic acid it was reasonable to expect that all the transductants would have arisen by incorporation of the *tryB*$^+$ gene of the donor strain into the genome of the recipient. As both donor and recipient carry *tryA-8* all these transductants should be tryA$^-$. However, out of 504 tryB$^+$ transductants, 76 had tryA$^+$ phenotypes. No tryA$^+$ transductants were recovered when the auxotrophic *tryA-8* was used as donor. When the former transduction was repeated using three other independently isolated F reversions (F_{10}, F_{13}, F_{16}) of *tryA-8* as donors the numbers of tryA$^+$ phenotypes/total number of tryB$^+$ transductants scored were 45/368, 24/355 and 35/380. It seems that the data combine two distinct transductions: the transduction of the *tryA-8 tryB*$^+$ fragment leading to the production of tryA$^-$ tryB$^+$ transductants and the transduction of the fragment carrying the suppressor mutation which appears to suppress not only *tryA-8* but also *tryB-4* and so gives clones which have tryA$^+$ tryB$^+$ phenotypes. This explanation implies that a single suppressor mutation was responsible for the changed expression of the *tryA* and *tryB* genes; the *tryB* had not been structurally changed. This was confirmed by using ten of these tryA$^+$ tryB$^+$ transductants, chosen from transductions involving all four F reversions, as donors in transductions to *tryB-4*. tryB$^+$ phenotypes were selected by plating on MM + anthranilic acid. Had the *tryB* gene been structurally changed most of the tryB$^+$ transductants would have been tryA$^-$ by incorporation of that part of the fragment that carries these adjacent tryptophan loci. If the tryB$^+$ transductants arose by transduction of the unlinked suppressor there would be no possibility of their being also tryA$^-$. 854 transductants were tested, none were tryA$^-$ and so the action of the suppressor in suppressing both *tryA-8* and *tryB-4* was confirmed.

(*b*) *Suppression confined to* tryA

One fast-growing reversion (F_{20}) of *tryA-8* which had an unlinked suppressor was transduced to four different *tryA* mutants. If the number of fast-growing transductants was much greater than when the auxotrophic *tryA-8* was used as donor we concluded that the suppressor could suppress the mutation in the recipient strain. There were between 10- and 100-fold differences when *tryA-47*, *tryA-50* and *tryA-56* were used as recipients but no difference when *tryA-52* was used.

tryA-52 does not produce reversions spontaneously. There was also no difference when *tryB-4*, *tryC-44* and *tryD-6* were used as recipients. We conclude that this unlinked suppressor suppresses four out of five apparent site mutations of the *tryA* gene; unlike those described in the previous section, it does not suppress *tryB-4*.

(ii) *Genetic changes inseparable from original mutant site*

Of the sixty-seven reversions in which the genetic change had been shown to be within the limits of the *try-cys* transduction fragment, fifty-five were further studied to locate the site of this change. These include F, SF and S reversions. Each

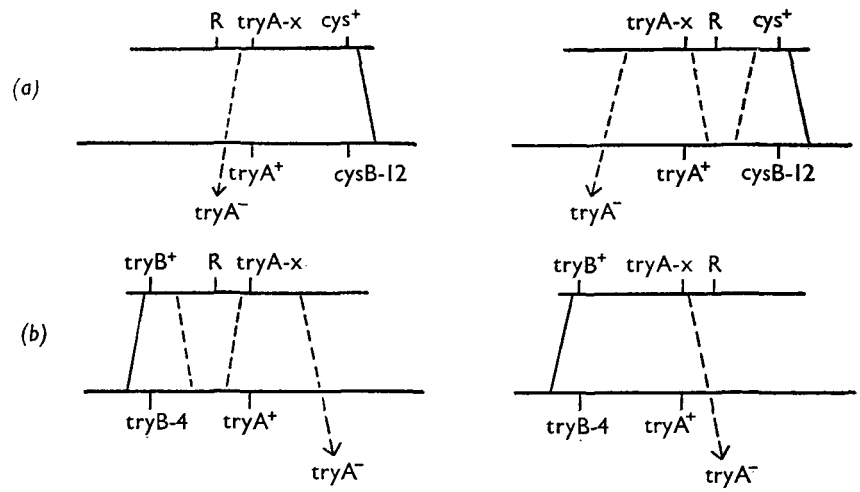

Fig. 2. Transductions to distinguish whether the genetic change (*R*) in reversion is to the left or right of the mutant site of the auxotroph.

(*a*) *tryA*$^+$ *cysB-12* (×) reversion of *tryA-x cys*$^+$. Selection for cys$^+$ transductants: tryA$^-$ transductants arise by double crossovers when *R* is to the left of *tryA-x*, and are rarer and arise by quadruple crossovers when *R* is to the right of *tryA-x*.

(*b*) *tryA*$^+$ *tryB-4* (×) reversion of *tryA-x tryB*$^+$. Selection for tryB$^+$ transductants: tryA$^-$ transductants arise by double crossovers when *R* is to the right of *tryA-x*, and are rarer and arise by quadruple crossovers when *R* is to the left of *tryA-x*.

reversion, in combination with *cys*$^+$ was transduced to *cysB-12* and cys$^+$ recombinants selected on MM + anthranilic acid. If the site of the suppressor is to the left of the *tryA* mutant site, some of these transductants will be tryA$^-$; if it is between the *tryA*$^-$ site and *cysB-12* only tryA$^+$ transductants are expected from double crossovers although some tryA$^-$ transductants may arise from quadruple crossovers (Fig. 2*a*). The results of these transductions are summarized in Table 4 and clearly demonstrate that none of these reversions have suppressors located to the left of the *tryA*$^-$ site. The possibility that the location of the suppressor is very close to the site of *cysB-12*, which would also explain these results, is excluded by the transductions of each reversion to a strain of its parent auxotroph, with selection for cys$^+$, always yielding a majority of tryA$^-$ transductants (first paragraph of Section 4, above).

Table 4. *Transductions between reversions of* tryA *auxotrophs, all of which were* cys$^+$, *as donors and* cysB-12 *as recipient: selection for cys$^+$ transductants*

	Reversions used as donors			Results (Number of tryA$^-$ transductants/total number of cys$^+$ transductants scored)
Recipients	Source of reversions	Class of reversions	Number of reversions tested	
tryA$^+$ cysB-12	*tryA-8*	Fast	14	0/11,279
		Semi-fast	5	0/2413
tryA$^+$ cysB-12	*tryA-47*	Fast	13	0/4840
		Slow	7	0/8338
tryA$^+$ cysB-12	*tryA-50*	Fast	6	0/4000
tryA$^+$ ysB-12	*tryA-56*	Fast	7	0/2996
		Semi-fast	3	0/900

Forty-nine of these reversions were then transduced to *tryB-4* and transductants selected on MM + anthranilic acid. If the site of the suppressor is to the right of the *tryA$^-$* site some of the transductants arising by double crossovers will be tryA$^-$ (Fig. 2*b*). The results are summarized in Table 5 and clearly demonstrate the tight linkage of the suppressor site with the *tryA$^-$* site or the identity of these two sites.

Table 5. *Transductions between reversions of* tryA *auxotrophs, all of which were* tryB$^+$, *as donors and* tryB-4 *as recipient: selection for tryB$^+$ transductants*

	Reversions used as donors			Results (Number of tryA$^-$ transductants/total number of tryB$^+$ transductants scored)
Recipients	Source of reversions	Class of reversions	Number of reversions tested	
tryB-4	*tryA-8*	Fast	14	0/8993
		Semi-fast	5	0/1688
tryB-4	*tryA-47*	Fast	7	0/3663
		Slow	7	1/7924
tryB-4	*tryA-50*	Fast	6	0/3700
tryB-4	*tryA-56*	Fast	7	0/2731
		Semi-fast	3	0/921

The presence of the single tryA$^-$ transductant is hardly sufficient to rule out the possibility of identity as it arose in a transduction in which the donor was later shown to produce very slow-growing colonies which were unstable; it could have been one of these very slow-growing colonies.

It will be demonstrated in the next section that some of the secondary reversions of the S reversions of *tryA-47* that have an intermediate growth rate between slow-growing and wild-type are due to unlinked modifiers. These provide the possibility

of searching for differences in response to a particular modifier mutation of independently isolated S reversions of *tryA-47*. A difference in the response of two S reversions would imply that they arose by different genetic changes. One secondary reversion with intermediate growth rate was transduced to four independently isolated S reversions and I transductants were recovered in all four transductions. No I transductants were found when the recipient was the auxotroph *tryA-47*. Thus either the modifier is unspecific for different S mutations or the different S mutations involve the same genetic change.

(iii) *The secondary reversions*

The genotype of the slow-growing reversions is designated *tryA-47S* in which *47S* represents a mutant site that is inseparable by recombination from the *47* site. Of the secondary reversions of *tryA-47S*, the VS type has not been further investigated. This account will deal only with the I type reversions.

If the second genetic change in I type reversions is in that part of the genome included in the *try-cys* transduction fragment it will be transduced linked to the *cys*

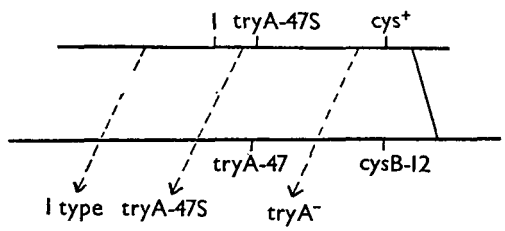

Fig. 3. Transduction: *tryA-47 cysB-12* (×) I type reversion (derived from an S reversion of *tryA-47*) *cys*+. Selection for cys+: *tryA-47S* transductants arise by double crossovers when site of *I* is to left of the *tryA-47S* site.

locus. If it is not in this region of the genome, selection for cys+, using an I type reversion as donor, will not give any I type transductants. Sixteen independent I type reversions of a single S reversion were transduced to *tryA-47 cysB-12* and about 500 cys+ transductants from each transduction were selected on EMM. Six of the I type reversions gave many I type transductants; ten gave no I type transductants. In identical transductions with five independent I type reversions of a different S reversion, four gave I type transductants and one gave none. So in some of these reversions the second genetic change is a mutation at one or more unlinked modifiers; in others the mutation is within the limits of the *try-cys* transduction fragment. Further study was confined to this latter class.

The genetic change in the *try-cys* region of the genome that results in S reversions becoming I type reversions will be designated *I*. If the site of *I* is to the left of *A-47S* the above transductions should produce S transductants when recombination occurs between *I* and *A-47S* (Fig. 3). These transductants are found in all but one of the transductions and their frequency is represented by the data of Table 6.

To be sure that they arise by this recombination and so demonstrate that the site of *I* is to the left of *A-47S* it is necessary to eliminate three other possible explanations of their origin.

(*a*) The site of *I* is to the right of *A-47S* and S transductants arise by quadruple crossovers.

(*b*) The site of *I* is to the right of *A-47S* and S transductants have the genotype of *A-47-I*.

Table 6. *Transductions between I-type reversions of* tryA-47S, *all of which were* cys$^+$, *as donors and* tryA-47 cysB-12 *as recipient: selection for cys$^+$ transductants*

Independently isolated I type reversions of *tryA-47S* used as donors	Total number of cys$^+$ transductants scored	Number of cys$^+$ transductants with slow-growing tryA phenotypes
I_1	424	2
I_{10}	880	2
I_{11}	380	2
I_{13}	372	3
I_{14}	292	1
I_{16}	2800	0
I_{21}	768	4
I_{22}	Not scored	Some
I_{23}	580	1
I_{24}	560	1

If either of these models is correct as high or higher frequency of S transductants should be found when a marker to the left of *tryA* is selected than in the above transduction where *cys*$^+$ to the right of *tryA* was selected. Three I type reversions I_{11}, I_{16} and I_{21}, were transduced to *tryC-44* and transductants selected on MM + anthranilic acid. Out of a total of 1331 transductants no S clones were found. These two models are therefore unlikely but the result is that expected if *I* is to the left of *A-47S*. The third possible explanation is:

(*c*) *I* is a further mutation at, or extremely close to *A-47S* and the S transductants arose not by recombination but by a relatively high rate of back-mutation of *I*.

This possible instability of *I* was tested by plating I type reversions and testing individual colonies to try to discover S reversions. 8350 colonies were tested from nine different I type reversions and no S reversions were found. This stability is also true for those I type reversions due to unlinked modifiers; 7800 colonies of eight such reversions included no S reversions.

We conclude that the nine I type reversions that gave some S transductants in the original transductions to *tryA-47 cysB-12* had their genetic change slightly to the left of *A-47S*. While the similar frequency of S transductants from each of these transductions (Table 6) suggests that the sites are in one small region they are unlikely to be identical mutations as the different reversions vary in their growth rates

on MM and EMM. The one reversion that gave no S transductants among the very large number, 2800, tested probably has the site of *I* very close indeed to *A-47S*.

Lastly we considered whether the site of *I*, when it was to the left of *A-47S*, was within the limits of the *tryA* locus. The first experiment consisted of transducing two I type reversions to *tryB-13* and selecting for tryB$^+$ transductants. If the order of sites were *tryB-13—I—A-47S* all the transductants would have either wild-type or I type phenotypes. If the order were *I—tryB-13—A-47S* the transductants would include wild-type, I type and slow-growing clones. 500 transductants were tested from each donor; all were either wild-type or I type. The most probable order is therefore *tryB-13—I—A-47S*. As no tryA$^-$ clones appeared it is likely that the few transductants with the mutant *I* site, but without the mutant *A-47S* site, that would be expected in an experiment on this scale are neither auxotrophic nor slow growing.

The second experiment was to transduce the same two I type reversions to *tryA-8*, which maps to the left of *tryA-47*. tryA$^+$ transductants were selected and 1000 examined from each transduction to discover whether or not they included slow-growing clones. None were found in excess of the controls and so the order of sites was concluded to be *tryA-8—I—A-47S*, thus establishing the site of *I* within the limits of the *tryA* locus. *I* can therefore be termed an inter-site suppressor of *A-47S*.

5. DISCUSSION

The aim of the present study was to survey the range of genetic changes that could alter the phenotypes of five tryptophan-requiring strains of *S. typhimurium* to that of wild-type or in the direction of wild-type. The five strains were arbitrarily chosen; one was early shown to be a completely stable strain and so all the further work was on the other four.

These four strains produced reversions that were phenotypically and genetically indistinguishable from what would be expected by true back mutation of the original mutant site. Two of the four auxotrophs, *tryA-8* and *tryA-50*, also produced reversions which were phenotypically indistinguishable from wild-type yet were due to mutations at unlinked suppressor loci. Five of the mutations that suppressed *tryA-8* were analysed further and four were found to suppress *tryB-4* as well as *tryA-8*; the other suppressed *tryA-47*, *tryA-50* and *tryA-56* as well as *tryA-8* but did not suppress *tryB-4*, nor did it suppress the completely stable mutant *tryA-52*. There are therefore two types of unlinked suppressors of *tryA-8*; super-suppressors that suppress mutants in *tryA* and also *tryB-4* and those that suppress mutants in *tryA* but do not suppress *tryB-4*. That the latter type does not open up an alternative metabolic pathway to that facilitated by *tryA* is demonstrated by its failure to suppress *tryA-52*. About one-third of the fast-growing reversions of these two auxotrophs were due to unlinked suppressor mutations; fast-growing reversions cannot be assumed to be due to true back-mutations of the original mutant site. It seems that it will usually be necessary to carry out a preliminary genetic analysis of

the reversions of any auxotroph of *S. typhimurium* that is to be used in a study of mutagenesis to discover the extent to which their phenotypes distinguish between different genetic changes.

Similarly the data demonstrate that not all slow-growing reversions are due to mutations at suppressor loci. While all the slow-growing reversions of *tryA-8*, *tryA-50* and *tryA-56* were due to unlinked suppressor mutations, all the slow-growing reversions of *tryA-47* were due to changes that were inseparable from the site of the original mutation in the *tryA* gene. While some of the semi-fast reversions of *tryA-8* were due to mutations at unlinked suppressors the majority were due to mutation at, or close to, the site of the original mutation, as were the semi-fast reversions of *tryA-56*. In these four auxotrophs, all reversions were due either to changes in genes not linked to *tryA* or to changes inseparable from the original mutant site; none were due to mutations elsewhere in the tryptophan operon or in other regions of the transduction fragment that includes this operon. This contrasts with the analysis of reversions of a leucine-requiring strain, *leu-151*, where all the slow-growing reversions were due to genetic changes in a region adjacent to the leucine complementation groups (Smith-Keary, 1960). This region appears to be the same as, or very close to, that identified as the operator of the leucine operon (Margolin, 1963).

In an attempt to find recombinants between the site of the reversion and the original mutant site, in those reversions not due to unlinked suppressors, very large numbers of transductants were scored. In studying the fast-growing reversions 23,115 transductants were scored relevant to recovering the original mutant if the reversion site were to the left of the original mutant site; 19,087 were scored relevant to recovering the original mutant if the reversion site were to the right of the original mutant site. The corresponding numbers of transductants scored in experiments with slow-growing and semi-fast reversions were 11,651 and 10,533. Only one transductant with a tryA$^-$ phenotype was found and its identification is open to serious doubt. As a minimum estimate of the length of the tryptophan-cysteine transduction fragment has been calculated as nineteen times the average length of the *tryA*, *B* and *C* genes (Dawson, 1963), and as we might reasonably guess that each of the polypeptides coded by these genes contains about 250 amino acids, there are likely to be at least 14,250 base pairs in this fragment of the genome. Even in the unlikely event of the fragment being twice as long as this, the scale of the experiments is such as to distinguish genetic changes occurring within a few base pairs of each other. Assuming that the changes do not appreciably suppress recombination in the adjacent regions, the reversion sites are likely to be either in the same codon as the original mutant site or in the adjacent codons.

Some of the reversions of *tryA-8*, *tryA-50* and *tryA-56* were shown to be due to unlinked suppressors. Although we detected no reversion of *tryA-47* that was due to an unlinked suppressor we did find an unlinked suppressor of *tryA-8* that also suppressed *tryA-47*. Each of these strains is therefore unlikely to be either an addition or deletion mutant. They are more likely to be mutant in a single base pair such that the mutant codon specifies a different amino acid or is the suppressible

chain terminating triplet UAG (Weigert & Garen, 1965; Brenner, Stretton & Kaplan, 1965; Garen, Garen & Wilhelm, 1965). The unlinked suppressors that result in the partial, or apparent complete, restoration of the wild-type phenotype then would be mutations in genes that code the transfer RNA molecules or in those that code the amino acid activating enzymes (Yanofsky, Helinski & Maling, 1961; Garen & Siddiqi, 1962) or are mutations that so change the environment of the polypeptide that it regains enzymic activity.

The only secondary reversions that were studied were those of the slow-growing reversions of *tryA-47*. They have phenotypes that are intermediate between the slow-growing reversions and wild-type. Some of these are due to mutation at one or more unlinked modifying genes while others are due to mutation within the limits of the *try-cys* transduction fragment. Ten of these latter reversions were analysed genetically and nine were shown to be due to a genetic change slightly to the left of the *47S* site; one had a genetic change that was inseparable from the *47S* site. Those to the left of the *47S* site were clearly shown to be well within the limits of the tryA gene. It is remarkable that no secondary reversions were found that were due to changes to the right of the *47S* site. This could be because the structure of the polypeptide is such that only changes close to, or to the left of, the *47S* site result in a polypeptide of enhanced catalytic activity. Alternatively it could be that mutations occur more readily to the left, than to the right, of the *47S* site. That this second explanation is at least as likely as the first is indicated by the fact that the overwhelming majority of tryA$^-$ spontaneous mutants selected in the *tryA-47S* strain have their mutant sites to the left of *47S*; this will be described fully in a separate paper.

When the genetic changes that result in these secondary reversions are present without *47S*, the phenotypes appear to be indistinguishable from wild-type. This seems to exclude the possibility of them being addition or deletion mutants. They are probably base-pair changes that result in the substitution of one amino acid for another, a substitution that has little effect on the otherwise wild-type polypeptide but enhances the catalytic activity of that coded by *tryA-47S*. As different secondary reversions have different growth rates it is likely that there are a number of possible substitutions that are expressed in this way. The genetic changes that result in the secondary reversions can be most appropriately called inter-site suppressors.

SUMMARY

1. The linkage order of four *tryA* mutants of *S. typhimurium*, and *cysB-12*, is:

tryA-8—tryA-47—(tryA-56—tryA-52)—cysB-12.

Attempts to plot the position of *tryA-50* were unsuccessful.

2. Some of the reversions of *tryA-8*, *tryA-47*, *tryA-56* and *tryA-50* were analysed genetically; *tryA-52* does not revert. All four auxotrophs gave reversions that were phenotypically and genetically indistinguishable from that expected by back-mutation of the original mutant site.

3. Both *tryA-8* and *tryA-50* produced reversions that grew as wild-type but were due to unlinked suppressor mutations. Some of these were super-suppressors in that they suppressed both *tryA-8* and *tryB-4*; others suppressed many site mutants in the *tryA* gene but did not suppress *tryB-4*.

4. All the slow-growing reversions of *tryA-8*, *tryA-50* and *tryA-56*, and a minority of the semi-fast reversions of *tryA-8*, were due to unlinked suppressors.

5. All the slow-growing reversions of *tryA-47*, the semi-fast reversions of *tryA-56* and the majority of the semi-fast reversions of *tryA-8* were due to genetic changes that were inseparable, in very extensive experiments, from their original mutant site.

6. Slow-growing reversions of *tryA-47* produced faster growing mutants. Some of these were due to mutation in unlinked modifying genes and in others the genetic change was within the *tryA* gene. Nine of the latter had the genetic change just to the left of the *47S* site; in one the change was inseparable from the *47S* site. None had this further change to the right of the *47S* site. These further changes, in the absence of the *47S* site, gave prototrophic phenotypes; they are inter-site suppressors.

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