CHEMICALLY INDUCED REVERSIONS IN THE *cysC* REGION OF SALMONELLA TYPHIMURIUM

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WHEN a mutation occurs, one would like to know the precise molecular alteration that has been produced in the genome. The most obvious way to detect such an alteration would be to compare nucleotide sequences in the DNA of a mutant and a wild-type organism. Since this is not yet possible, one must look for alternative approaches. The observation that each site has a specific set of responses to chemical mutagens may permit partial characterization in molecular terms of these genetic sites, especially since some of the mutagens have restricted chemical actions. This rationale has been used in the analysis of mutational sites in phages (CHAMPE and BENZER 1962; FREESE 1963) and in bacteria (KIRCHNER 1960; MARGOLIN and MUKAI 1961; BALBINDER 1962), and is the basis for conclusions in this report.

A large number of mutations within the *cysC* region of *Salmonella typhimurium* LT-2 are available in the collection of mutants kept at Brookhaven National Laboratory. DEMEREC, GILLESPIE, and MIZOBUCHI (1963) have presented a detailed study, including a genetic map, of the mutational sites in this region. In the present investigation, about 200 of the suitable mutants were analyzed with regard to reversion to wild-type phenotype on treatment with chemical mutagens. The data were examined to determine whether information might be obtained concerning the differences in properties of these numerous genetic sites within the *cysC* region.

MATERIALS AND METHODS

Most of the procedures used in this study have been described by IYER and SZYBALSKI (1958), KIRCHNER (1960) and BALBINDER (1962). The methods provide a rapid means of testing a large number of strains for mutability by certain chemicals. In brief, the auxotrophs (ca. 2×10^8 cells per plate) are spread on plates containing minimal agar enriched with 0.02 percent nutrient

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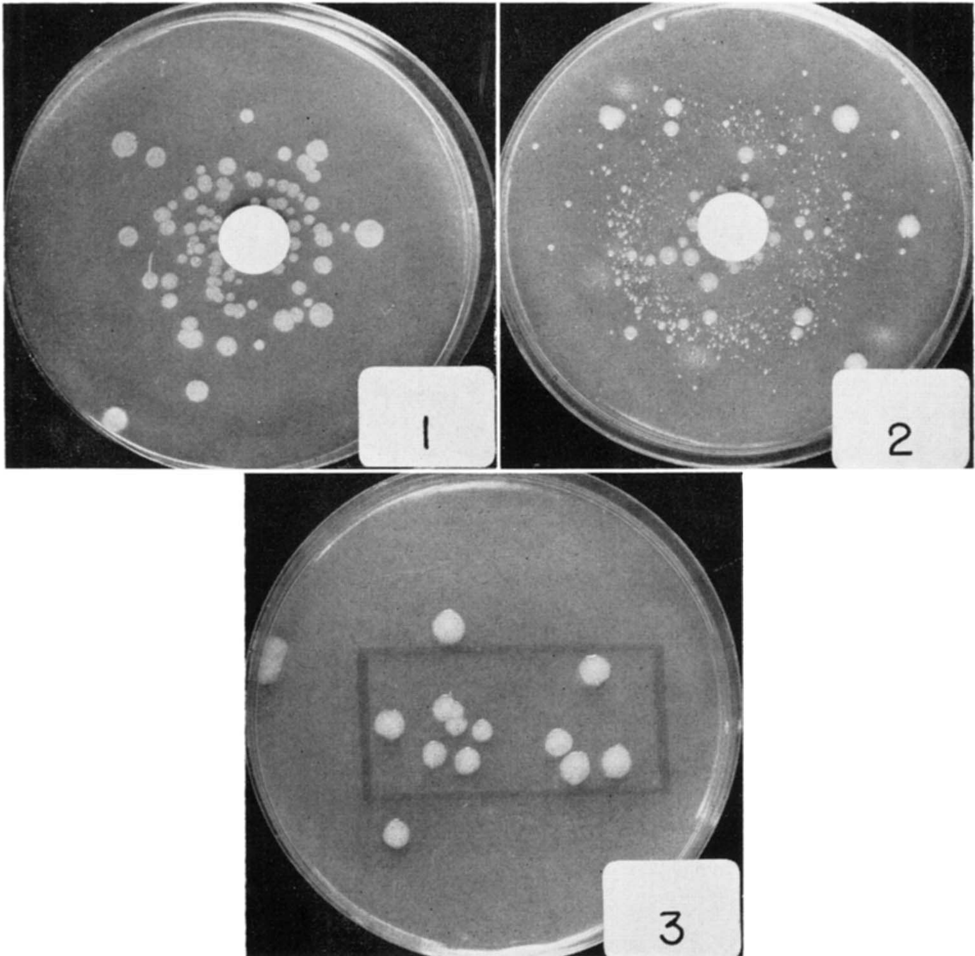
broth powder. The medium permits only a few divisions of the auxotrophs but allows revertants to grow to full size colonies. The mutagen is added to the inoculated plate. After suitable incubation, the number and distribution of colonies on the treated plates are compared with those on control plates, thus giving a semi-quantitative determination of each mutagen's effect. In some cases, the mutagen action was so great that only estimates could be made of revertant numbers. It should be emphasized that, since the mutagen is added at a particular position on the petri plate (Figure 1), the orientation of the prototroph colonies to the mutagen is important in scoring of results and leaves little question about the cause-and-effect relationship of the mutagen.

Auxotrophic cultures: Cultures were started by inoculating 3 ml of cysteine-supplemented nutrient broth (2×10^{-4} moles cysteine per ml) with a loopful of bacteria. After shaking overnight at 37°C, cultures contained about 2×10^9 cells per ml. Often, when the number of revert-



FIGURES 1–3.—FIGURE 1.—Reversions produced by a mutagen (DES). The plate was spread with ca, 2×10^8 mutant cells. 0.05 ml of mutagen was placed on the filter paper pad. Note orientation of revertants around filter pad. FIGURE 2.—Nonwild-type colonies arising upon treatment with mutagen. When small colonies are picked and restreaked, only small colonies arise. FIGURE 3.—Instead of using a filter paper pad, AP is spread over a protion of petri dish, as marked. This area is inspected for revertants.

ants within a mutant culture was high or unknown, a subculture with a known low number of revertants was used to facilitate scoring.

Plating of auxotrophs: Cells were spread on enriched minimal agar plates; 0.1 ml of culture was used per plate. Initially, four plates were used for the control and four plates for each of the mutagens. However, it was later found that the action of certain mutagens was so decisive (e.g., ethyl methanesulfonate, diethyl sulfate) that two plates for each would suffice.

Mutagens: Diethyl sulfate. (Eastman Organic Chemicals, Rochester, N.Y. Used full strength, nondistilled.) Ethyl methanesulfonate. (Eastman. Used full strength, nondistilled.) Beta-propiolactone. ("Betaprone" #608, 99 percent pure. Testagar & Co., Detroit, Michigan. Used full strength.) 2-aminopurine nitrate. (California Corporation for Biochemical Research, Los Angeles. Grade B purity. Used as a saturated solution.) Sodium nitrite. (Crystals. 98.1 percent purity. Nitrous acid was prepared with an acetate buffer of pH 4.1. The buffer consisted of 5 parts of 0.2M acetic acid and 1 part of 0.2M sodium acetate. Before being used as a mutagen, 1 part acetate buffer was mixed with 1 part of 10M sodium nitrite solution, giving a 5M NO_2 solution of pH 4.5 to 4.6. Since pH is critical, it was checked before being added to petri plates.) 5-bromodeoxyuridine. (California Corporation. A grade. Used as saturated solution.) Aminopterin. (California Corporation. C grade. Used as saturated solution.) Proflavine. (National Aniline Division, Allied Chemical Corporation, N.Y. Used as saturated solution or as crystals.) Hydroxylamine. (The method of FREESE, BAUTZ, and FREESE [1961] was used.)

Nomenclature: The following notations are used: "*cysC* region" designates linked loci involved in cysteine metabolism. From the studies of MIZOBUCHI, DEMEREC and GILLESPIE (1962) it is now known that this particular region controls the synthesis of five different enzymes in the cysteine pathway (see also DREYFUSS and MONTY 1963). The five linked loci that control these are designated *cysC*, *cysD*, *cysH*, *cysI*, *cysJ*, and are found in that order in the *cysC* region. A detailed map of the sites in this region may be found in DEMEREC *et al.*, 1963. The position of a single nucleotide pair is referred to as a *site*.

Abbreviations are: 2-aminopurine (AP), 5-bromodeoxyuridine (BDU), diethyl sulfate (DES), ethyl methanesulfonate (EMS), 5-fluorouracil (5FU), beta-propiolactone (BPL), X rays (X), neutrons (N), ultraviolet light (UV), nitrous acid (NA), proflavin (PRO), spontaneous (SP), adenine (A), thymine (T), guanine (G), cytosine (C). When a mutant number is followed by an abbreviation in parenthesis, this designates the mutagen used in producing the forward mutation; e.g., *cysH*229(AP). The possibility must be kept in mind that a mutant recovered after treatment with a particular agent could have been spontaneous rather than induced.

Mutagen tests: For the mutagens DES, EMS, and BPL, a drop of undiluted mutagen was placed on a sterile filter paper pad on the surface of the inoculated plate (Figure 1). The purpose of the filter pad was to keep the mutagen from "running" and to mark the point of mutagen addition. More recently tests have been performed satisfactorily by simply adding mutagen directly onto plates without pads. Since AP does not diffuse readily through the agar, 0.1 ml was added to the surface of plates and spread over rectangular areas covering about 14 cm^2 (Figure 3). Almost all colonies were found within the rectangle.

The results with BDU were less clear-cut than those with other mutagens, and spot tests did not permit decisive scoring. Instead, the procedure was to spread 0.1 ml of a saturated solution over the surface of each of four inoculated plates. Positive scores were given when the number of revertants on the BDU plates was significantly higher than on the control plates; this increase was usually about 2 to 10-fold. In many tests, cells were treated with aminopterin before adding BDU, but it is uncertain whether this pretreatment is necessary.

RESULTS AND DISCUSSION

Throughout this report, a simple assumption is made: in point mutation, the final result is the substitution of one base pair for another base pair, and reversion constitutes the restoration of the original nucleotides. It is obvious that this is vast oversimplification, but to operate on more complex assumptions would

involve a severely difficult testing scheme. In the present approach, the following complexities were not considered: (a) Neighboring nucleotides might influence reversion patterns. (b) The substitution of another base pair (but not the original) at the mutated site might still restore enzyme activity. (c) Enzyme activity might be fully restored by a second mutation at a different site, whether it be neighboring or distant (HELINSKI and YANOFSKY 1963). (d) Cellular, nongenetic factors might influence the reversion patterns. (e) Instead of restoring the absolute, initial nucleotide sequence, reversion may merely represent the restoration of the original "frame of reference" (CRICK, BARNETT, BRENNER, and WATTS-TOBIN 1961). (f) Reversion might represent a change in a "rate control" region, rather than restoration of the initial base pair (PARDEE and BECKWITH 1962).

Despite these complexities, chemical mutagens do show distinct differences in mutational patterns, and thus permit categorization of mutant sites and speculation as to the mode of action of each mutagen (FREESE 1963; KREIG 1963).

Influence of forward mutation induction method on reversion pattern: The mutants examined were induced in a number of different ways. Since the various mutagens might differ as to kinds of molecular alterations each could inflict on the genome of the organism, it would be anticipated that the choice of mutagen

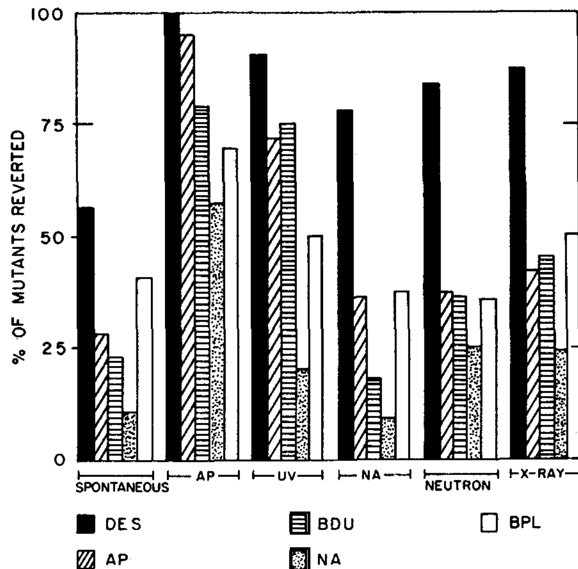


FIGURE 4.—Percentage of mutants in *cysC* region that revert upon treatment with chemical mutagens. The agent used to induce forward mutation is given along the bottom of the figure (i.e., "spontaneous" means that these *cys* mutants were selected without the use of any mutagenic agent). The height of each bar indicates the percentage of the mutants that could be reverted by each agent (e.g., among the *cys* auxotrophs obtained after treatment with AP, 100 percent could be reverted with DES, and 97 percent with AP). The actual number of *cysC* auxotrophs that were tested include 54 that were obtained spontaneously, 48 that were obtained after treatment with AP, 11 afer UV, 37 after NA, 24 after neutrons, and 32 after X rays.

used to obtain auxotrophs would influence reversion patterns. This is precisely the case, as may be seen from Figure 4, which is a summary of the numerous mutagenesis tests performed.

It is obvious that a large majority of AP mutants are reverted with AP, and every one of the 48 are reverted with DES. In contrast, mutants obtained spontaneously and by treatment with X rays, neutrons and nitrous acid are far from unanimous in their response to AP; in fact, a large number of them fail to respond to DES. There was no clear-cut case in which DES was nonmutagenic and a base analogue was mutagenic.

The results in Figure 4 may be interpreted as follows: based on the assumptions discussed by FREESE (1963), the AP mutants resulted from transitions only; i.e., $\begin{smallmatrix}A\\T\end{smallmatrix} \longleftrightarrow \begin{smallmatrix}G\\C\end{smallmatrix}$. Changes from $\begin{smallmatrix}A\\T\end{smallmatrix} \longleftrightarrow \begin{smallmatrix}T\\A\end{smallmatrix}$, $\begin{smallmatrix}A\\T\end{smallmatrix} \longleftrightarrow \begin{smallmatrix}C\\G\end{smallmatrix}$ or $\begin{smallmatrix}G\\C\end{smallmatrix} \longleftrightarrow \begin{smallmatrix}C\\G\end{smallmatrix}$ (transversions) apparently did not occur. On the other hand, mutants produced spontaneously and by X rays, neutrons, and nitrous acid apparently are the result of both transitions and nontransitions, as well as multi-site changes. Until verified by more direct means, the following assumption may be made: mutants that failed to respond to base analogues were the result of nontransitional changes.

The results with the nitrous acid mutants raise questions with regard to the kinds of mutations that this chemical can produce. It is apparent that the nitrous acid is not limited to the production of transitions in bacteria. Otherwise, the results in Figure 4 for nitrous acid should be about the same as for AP mutants; i.e., each NA mutant should be capable of reversion by AP. Further, it should be emphasized that if the only other action of NA were production of transversions, DES should be effective in restoring wild-type colonies. In several mutants, even DES failed to produce reversions. Obviously, nitrous acid may have effects beyond point mutation (i.e., alteration of single nucleotide pair). This conclusion receives its strongest support from the work of DEMEREC (cf. SHULL 1962) who found that NA is an effective agent for the production of deletions in the *cysC* region. Also, TESSMAN (1962) has reported large deletions in phage produced with NA.

Ethyl methanesulfonate was used in the screening, and the information obtained was merely additive inasmuch as it seemed to act much as did DES. There were a few cases with differences in response, but these exceptions cannot be explained.

Mode of action of base analogues: Although mutagenesis with AP may be restricted to transitions, apparently the change can be either in the direction of GC to AT or AT to GC. CHAMPE and BENZER (1962) distinguished between the two on the hypothesis that, if a mutant reverts with both AP and with BDU, this suggests that the mutant base pair is GC; if it does not revert with BDU, the mutant base pair is AT. In our tests, BDU was present through several cell divisions, and transitions might not be restricted to the one direction of GC to AT (TERZAGHI, STREISINGER and STAHL 1962). Nevertheless, only half of the mutants in the *cysC* region that were reverted with AP could also be reverted with BDU, giving two distinct groups of mutants. If the rule of CHAMPE and

BENZER applies to these mutants of *cysC* region, a division of the two types of transitions might be made: Group A would be examples of GC sites that revert to AT, and Group B, examples of AT sites that revert to GC.

Group A (BDU positive, AP positive): *cysC*200(AP), 211(AP), 436(X); *cysD*207(AP), 216(AP), 234(AP), 412(SP); *cysH*229(AP); *cysI*158(SP), 206(AP), 222(AP), 238(AP), 239(AP), 250(AP), 270(AP), 367(X), 404(X); *cysJ*204(AP), 245(AP), 372(X), 402(X).

Group B (BDU negative, AP positive): *cysC*428(X); *cysD*220(AP), 378(X), 516(NA); *cysH*224(AP), 241(AP), 271(AP); *cysJ*223(AP), 230(AP), 266(AP), 429(X).

The decision of CHAMPE and BENZER (1962) on the direction of transition produced by BDU was based on the correlation of results with hydroxylamine, a mutagen whose chemical activities can be examined. Unfortunately, the same correlation could not be established with *cysC* mutants; hydroxylamine failed to give consistent mutagenesis in our hands, possibly because it is easily metabolized before it attacks the genome of the bacterial organism.

Mutants that are spontaneously revertible, but mutagen stable: A number of mutants (Table 1) were found to revert spontaneously, but not to respond to any of the mutagens tested. Whether these are single site substitutions or alterations of a grosser nature (i.e., small deletions and insertions) can not be ascertained from this study. Apparently, the latter is a possibility, since CRICK *et al.* (1961) found that proflavine, an agent that may add or subtract small numbers of nucleotides, was capable of reverting phage mutants; such mutants also had spontaneous reversion rates. In examining whether any of the mutants in Table 1 are the result of single nucleotide pair substitutions, the critical mutagen was DES, since it has the broadest spectrum and it may be capable of producing all

TABLE 1

Mutants of cysC *region found to be spontaneously revertible, but mutagen stable*

Mutant No.	Spontaneous reversions*
*cysC*514 (NA)†	5/10
*cysC*573 (NA)	39/4
*cysC*583 (NA)	7/4
*cysC*599 (NA)	2/4
*cysD*109-LT-7 (SP)	8/8
*cysD*490 (N)	7/8
*cysH*28 (UV)	17/4
*cysH*74 (SP)	1/8
*cysH*180 (SP)	5/5
*cysI*184 (SP)	8/9
*cysI*413 (SP)	6/5
*cysI*570 (NA)	3/4
*cysI*577 (NA)	9/4

* 5/10 means that a total of five revertants were found on ten plates. Each plate had about 10^9 cells; therefore, a rough estimate of spontaneous revertants would be 5 per 10×10^9 bacterial cells.

† The mutagen originally used in obtaining each mutant is indicated in parentheses. NA, nitrous acid; SP, spontaneous; UV, ultraviolet light; N, neutrons.

six types of the possible alterations (two transitions plus four transversions). If DES has a strong preference for acting on GC, perhaps the two transversions of AT do not take place readily. Some insight might be gained by examining auxotrophic mutants produced by DES. If DES has a strong preference for GC, it would be predicted that not all DES induced mutants would be revertible by DES, unless the mutation was *always* from GC to CG (an unlikely possibility). The few DES mutants available for this study did not permit adequate analyses.

The main point is that one is not absolutely certain that the mutants in this spontaneously-revertible-mutagen-stable group represent single-site substitutions until it is known that the mutagens used induce all six kinds of transitions and transversions. This should be kept in mind in the search for new mutagens; especially useful would be a mutagen that preferentially produces transversions on AT.

Mutants that do not revert spontaneously: Several mutants of *cysC* region show no spontaneous reversion to wild type. While it may be assumed that these are multi-site changes, one would like to be reassured that these are not single-site mutations with extremely low reversion frequency. A means of reducing this uncertainty is to test with mutagens. The results of such a test (Table 2) show that such organisms are indeed mutagen-stable, as well as spontaneously stable.

One can only speculate that the molecular alteration that accounts for this stability involves a deletion or addition of a small number of nucleotides. Large deletions could be detected by transduction tests. The accumulation of mutants at additional sites in the *cysC* region might indicate the size of the deletion involved.

Possible sub-classes of response to DES: Predictions of nucleotide pair alterations may be made with some confidence from the results with base analogues; however, one is less confident of the significance of results with other mutagens whose modes of actions are less specific. Although one of these mutagens might be capable of inducing several different kinds of base-pair changes, it may do so

TABLE 2

Mutants of cysC region with no spontaneous reversions, but with no evidence of deletions

Mutant	Spontaneous reversions*	Revertants after treatment with several mutagens
*cysC*520 (NA)† (deletion?)	1/37	1/71
*cysH*433 (X)	0/37	0/108
*cysJ*168 (SP)	0/4	0/17
*cys*531 (NA) (locus not identified)	0/20	1/78
*cys*552 (NA) (locus not identified)	0/4	0/12

* See Table 1 for explanation of numbers. The three colonies found on a total of 388 plates probably represent contaminants.
† The mutagen originally used in obtaining each mutant is indicated in parentheses. NA, nitrous acid; X, X rays; SP, spontaneous.

with sharply different probability and thus permit a decision on the basis of *quantitative* response. DES was considered as a candidate for such a mutagen. According to FREESE (1963), the main mutagenic effect of ethylation is removal of ethylated guanine; therefore, GC pairs would be attacked preferentially. In our testing, DES usually resulted in an "explosive" response (see Figure 1); often the revertants were so numerous that counting was difficult. However, in some cases, a definite positive response was observed but the increase in revertants was small. The number of spontaneous reversions was comparable for the two groups of mutants. Although there was usually a sharp division between high and low reversion number, a few mutants defied categorization into either the high or low group. Table 3 lists mutants that gave low, but positive, responses to DES. Mutant *cysC*220 is included in the table as an example of high reversion rate; it should be compared with mutant *cysC*355 which has about the same spontaneous reversion rate but does not respond so readily to DES. Because neighboring nucleotide pairs or other factors might play an important role, the decision that this represents a difference in nucleotide pair at the two mutant sites cannot be made.

Reversion by beta-propiolactone, manganese chloride, and other mutagens: To

TABLE 3

Mutants of cysC *region with low reversion frequency with DES*

Mutant	Spontaneous reversions*	DES rate	Reversion with AP	Reversion with BPL
*cysC*78 (SP)†	18/4	33/2	—	—
*cysC*362 (X)	8/17	28/1	+	+
*cysD*215 (AP)	60/2	139/2	+	+
*cysD*559 (X)	76/4	232/4	+	+
*cysH*7 (UV)	3/7	44/6	—	—
*cysH*38 (SP)	27/8	22/4	—	—
*cysH*58 (SP)	70/8	56/4	—	—
*cysH*112 (SP)	24/4	282/4	—	—
*cysH*146 (SP)	7/4	47/2	+	+
*cysH*194 (SP)	5/4	29/2	—	+
*cysH*229 (AP)	17/4	95/4	+	+
*cysH*237 (AP)	2/4	6/2	+	—
*cysH*241 (AP)	2/6	32/6	+	+
*cysH*530 (X)	5/3	3/1	—	—
*cysI*186 (SP)	37/2	170/2	—	—
*cysI*387 (X)	6/5	10/4	+	—
*cysJ*98 (SP)	280/1	500/1	—	—
*cysJ*123 (SP)	300/3	1200/3	—	—
*cysJ*355 (SP)	4/4	11/2	—	—
*cysJ*391 (X)	2/1	7/1	—	—
*cysJ*429 (X)	7/2	13/3	+	—
*cysD*220‡ (AP)	7/7	1000/4	+	—

* 18/4 indicates that 18 reversions occurred on a total of four petri plates examined, as explained in Table 1. In addition to actual numbers of observed reversions, the decision to place mutants in this category was based on orientation of colonies to mutagen.

† The mutagen originally used in obtaining each mutant is indicated in parentheses. SP, spontaneous; X, X rays; AP, 2-aminopurine; UV, ultraviolet light.

‡ Mutant *cysD*220, which does not belong in this group, is listed to show the contrast to the other mutants.

establish additional classes of responses, other mutagens were used throughout this study, even though knowledge of their modes of action is obscure.

A question arose whether beta-propiolactone might be used to establish an additional class, or classes. Although it would be premature to attempt any explanation without more knowledge about the mode of action of BPL, it can be stated that at least four different kinds of mutants can be identified on the basis of BPL reaction. Examples for each of these catagories are as follows:

Group A (DES +, BPL —, AP —): *cys*D466(N), 313(UV); *cysH7*(UV), 112(SP); *cysI*123(SP), 397(X).
Group B (DES +, BPL —, AP +): *cysD*216(AP), 220(AP); *cysC*646(AP); *cysH*224(AP), 271(AP), 455(N); *cysI*239(AP); *cysJ*429(X).
Group C (DES +, BPL +, AP —): *cysC*80(SP); *cysJ*148(SP), 367(X), 468(N), 596(NA), 630(SP).
Group D (DES +, BPL +, AP +): *cysC*200(AP), 211(AP), 218(AP); *cysD*207(AP), 234(AP); *cysH*73(SP), 146(SP); *cysI*206(AP); *cysJ*204(AP).

A small number of mutants responded to proflavin, and a few responded to manganese chloride, but the small numbers preclude any explanation, on a molecular basis, for specificity of mode of action. Proflavin mutants were *cysD*246(AP), *cysH*146(SP), *cysI*238(AP), *cysJ*115(SP) and 262(AP). Mutants that responded to manganese chloride were *cysC*507(N), *cysJ*402(X) and 414(X). They are listed merely for identification for possible future experiments.

Nitrous acid gave a positive response with many mutants, but the results failed to indicate the kind of molecular alteration that might have taken place. Many of the responses with NA were so weak that they left some doubt regarding the method of testing and scoring, especially when compared with the more decisive results with AP. Perhaps reduction in buffering action, both intracellular and extracellular, would increase the mutagenic activity of NA.

Apparent reversions, but not to wild type: The simplest assumption is that the growth of a prototrophic colony represents a return to the identical base pair that existed in the wild type. Obviously, this need not be the case, although results with mutagens support this view for the majority of sites tested. First, if one assumes that a site represents a single nucleotide pair, four different pairs are possible. Perhaps any one of three of these pairs will suffice to produce a prototroph (degenerate code) and only one results in an auxotroph. The return to prototrophy, on reversion, might not correspond to a restoration of the initial base pair. Another complicating factor might exist in another degeneracy of the code in the nucleotide triplet that specifies a particular amino acid; i.e., return to prototrophy could result from a change in either of the other two base pairs within the triplet. In addition, return to prototroph phenotype could result from mutation at an entirely different locus (i.e., a suppressor locus), or might result from mutations at sites adjacent to or in the gene that control enzyme levels (PARDEE and BECKWITH 1962).

In our experiments, at least 36 of the *cysC* mutants gave revertants by mutagens, but the revertants did not represent restoration to wild type. These could be recognized by the fact that some colonies, even after four days incubation,

were small (Figure 2). Isolation and replating of colonies from each of these mutants demonstrated that the small size was a stable characteristic. There were representatives of each of the loci within *cysC* region, with the exception of *cysJ*, in this group of 36 mutants. The experience of others (YURA 1956; HOWARTH 1958) indicates that these small colonies may result from mutations at a suppressor locus. Typical examples of mutants in this category are: *cysH7*(UV), *cysC*200(AP), *cysD*496(N), and *cysD*378(X).

Comparison of mutants that cannot be distinguished by transduction tests: It would be particularly useful if mutagens could be employed to distinguish between sites that cannot be resolved by transduction tests. It is possible that two mutant sites might be so close together that recombination frequencies, when each is crossed against the other by transduction, would not rise significantly above the background reversion rate or even give rise to abortive transduction. Mutagens, however, might be able to distinguish differences in the two sites, even if these differences were in neighboring nucleotides. Mutagens might even be used to distinguish mutational events at identical sites but with different alteration; i.e., one mutant could have resulted from a transition and the other from a tranversion perhaps even *at the identical site.* BALBINDER (1962) considered this problem with regard to differences between *tryD*10 and *tryD*11 mutants.

Mutants *cysH*229 and 370 do not recombine measurably by transduction tests; however, the response to mutagens are not identical for the two mutants, indicating that *cysH*370 is not merely a repeat of the mutational event of *cysH*229. Mutant *cysH*229 is reverted by AP, BDU, and BPL, whereas *cysH*370 is not.

Orientation: One would like to know whether a particular nucleotide appears in rather random distribution along the chromosome, or whether one segment might be particularly rich in one base pair (e.g., repeats of AT). Mutagens might be a way of testing this, provided enough mutant sites were known within a very small segment of the genome. Although the available mutants within the *cysC* region are far too few to saturate the genetic map, the reversion patterns were examined for possible indication of nonrandomness. Consideration of the mutants (Table 4) according to the five loci (DEMEREC *et al.*, 1963) that comprise this region indicates no striking orientation.

Response of mutants containing mutator gene: KIRCHNER (1960) found that base analogues increased reversions in *S. typhimurium* LT-7 mutants that possessed a mutator gene and that had a high frequency of spontaneous reversions.

TABLE 4

Reversion patterns according to loci within cysC *region*

Locus	Number of mutants		
	DES positive AP negative	DES positive AP positive	Spontaneous positive Mutagen negative
cysC	11	18	6
cysD	7	13	2
cysJ	18	28	0
cysI	9	10	6
cysH	19	17	5

He concluded that the mutator gene is responsible for the synthesis of a compound that induces exclusively transition-type mutations, since he found only four mutants of 80 tested that did not respond to a base analogue. That some exceptions exist, even small in numbers, leaves the possibility that the mutator gene may induce changes other than transitions. This possibility was examined with regard to our mutants isolated from *S. typhimurium* LT-7, which harbors *mut*⁻. Of 20 mutants tested, all with high spontaneous reversion frequencies, some failed to respond to AP (*cysD*132, 162; *cysH*102, 112; *cysI*123; *cysJ*148). Even more interesting, one did not respond to DES (*cysH*102). These exceptions raise the question whether a transition-producing substance accounts for the high frequency of spontaneous reversions in these particular strains. It should be kept in mind, however, that the mutator gene may have been lost in some of these strains and that we observed only reversions that arose from the normal background mutational pattern rather than those that resulted from the presence of the mutator gene. Another possibility is that a second, unrelated, mutator gene may be present which acts differently.

Influence of lysogeny on mutagenic action: The rationale for checking the influence of prophage was that, if positive, this would be a clue that the prophage site was in or near the *cysC* region. If it were some distance from this region, it is unlikely that it would be effective in influencing the action of the mutagen.

To test this, lysogenic and nonlysogenic cultures of a number of mutants were tested as to reversion patterns with mutagens; the mutants tested were *cysC*532; *cysD*213, 216, 505; *cysH7*, 530; *cysI*206; *cysJ*204. The results show that there was no observed protection of any site by the prophage since there was no difference in reversion pattern, either qualitatively or quantitatively.

Influence of additional mutation on reversion pattern: The assumption has been that mutation and reversion are confined to the molecular alteration of a single nucleotide pair without any influence of the remainder of the genome. One method of examining this assumption would be to introduce a second mutation at a different locus to see if it alters the mutational pattern in any way. To test this possibility, reversion patterns in two double mutants (*cysI*46-*tryD*2, and *cysI*51-*tryD*2) were compared with those in the appropriate single mutant (*cysI*46, *cysI*51 and *tryD*2). Cysteine or tryptophan was added to the medium in reversion tests for each site in the double mutants. The results showed that mutation at the *tryD*2 locus does not influence the reversion pattern of either the *cysI*51 or *cysI*46 mutant. Also, neither of the mutations in *cysC* region influences the reversion pattern of the *tryD* mutant. This is another example of the independence of individual sites to mutagen action. There is little doubt that the basic assumption of the independence of the single base pair is oversimplified, if not naive; close neighbors are likely to influence mutational events (cf. CRICK *et. al.*, 1961, for an example of the influence of additional mutations). This problem would be clarified if selection methods could be developed to obtain double mutants involving *neighboring* sites.

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SUMMARY

Approximately 200 single-site mutants of the *cysC* region of *S. typhimurium* were examined for reversions on treatment with a series of chemical mutagens. The method of induction of forward mutation definitely influences whether a particular mutant can be reverted. Base analogues failed to produce reversions in numerous nitrous acid mutants; this indicates that the action of nitrous acid is not restricted to production of transitions in the DNA of bacteria. Several mutants were mutagen stable on rigorous testing. All of these showed spontaneous reversion. A few mutants, with no spontaneous reversion, but no evidence of deletion, were also mutagen stable. In several cases where diethyl sulfate was mutagenic, the number of reversions was much lower than found for the majority of mutant sites; this quantitative distinction might be useful in distinguishing between kinds of base pair substitutions produced by diethyl sulfate.

A number of colonies (apparent reversions) were tested to see if they showed any deviation from wild-type colonies in appearance; in 36 cases (about 18 percent) of the mutants tested, it could be shown that these were not wild-type, but possibly examples of mutation at another locus. In at least one case, mutants that cannot be distinguished by transduction test were shown to differ in mutagen response. Each class of mutant (based upon response to mutagen) was scattered throughout the five loci of the *cysC* region, with no orientation within any locus.

All mutants containing the mutator gene (LT-7 strains) and having a high spontaneous reversion frequency should be revertible with either AP or BDU, if the mutator gene acts by producing transitions. This was not the case among all of the mutants tested; some of them were mutagen-stable. It might be questioned whether mutator gene action is restricted to transitions. Reversion patterns were independent of lysogeny and of the presence of a second mutation within the strain tested.

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