SPONTANEOUS AND INDUCED COLOR-VARIATION OF THE HY STRAIN OF SERRATIA MARCESCENS[1]

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Since the elucidation of hereditary mechanisms is dependent largely on the observation of differences and similarities between parents and offspring, those species which exhibit conspicuous color-variations are often of great value in the perception and solution of genetic problems. For this reason bacteriologists have long recognized the usefulness of the fast growing, pigmented *Serratia*, and many observations have been made on spontaneous color changes in this species (reviewed by Bunting, 1946). The same properties make it potentially valuable for the investigation of induced mutations. Furthermore, since the organism grows readily on simple synthetic media and is capable of breaking down a very wide variety of organic compounds, it offers excellent material for genetic studies with biochemical mutants. Although neither sexual recombination nor transformation has yet been demonstrated in *Serratia*, the organism is a gram negative rod closely resembling the coliform bacteria morphologically and physiologically and may well exhibit comparable genetic behavior. It appears, therefore, to be a particularly useful species for the study of many of the genetic problems which are carried out to advantage with bacteria and which promise to shed light not only on microbial phenomena but also on basic genetic mechanisms.

Serratia marcescens is the type species in the genus *Serratia* (Breed *et al.*, 1948) and is reported to be the species used in most genetic studies made with *Serratia*. However, different strains in the American Type Culture Collection and in various laboratories throughout the country differ markedly in color, stability, morphology, and physiological reactions. At the present time the relationship between these strains, many isolated from widely different sources, is not clear. It is hoped that further knowledge, aided by genetic studies showing the range and pattern of variation characteristic of individual strains, will provide the information needed to decide which could have arisen as closely related variants and which probably had distant origins. Until this is evident it is essential to specify and preserve for later comparative studies the strains used in any experimental series.

The HY strain was selected for these studies because it appeared to be far more stable than strain 274 used in previous experiments. Most of the data presented in this paper were secured with the Yale culture which has been maintained in this laboratory since the early studies of Rettger and Sherrick (1911). All essential findings have been confirmed with strain 8195 obtained from the American Type Culture Collection in 1951.

Color-variation could not be detected by the microscopic appearance of individual cells but was determined by the color of colonies developing from cells spread on the surfaces of agar plates. The cells of the HY strain are small coccobacilli which occur singly or in very short chains so that most colonies represent clones developing from single cells and can be used to characterize the cells.

A synthetic ammonium-citrate-glycerol agar was preferred for plating because it gave good color-differentiation, was reproducible, and was so highly buffered that the appearance of colonies was not confused by changes in the pH which affect the pigment. It contained per liter of water: ammonium citrate, 5.0 g; glycerol, 10.0 ml; dipotassium phosphate, 10.0 g; magnesium sulfate, 0.5 g; ferric ammonium citrate, 0.05 g. Variants were observed also on a simple peptone (0.5 per cent) glycerol (1.0 per cent) agar which intensified pigmentation (Bunting *et al.*, 1949) and permitted the differentiation of certain paler

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types which could not be distinguished on the synthetic medium.

Part I. Spontaneous color-variation in the HY strain. It was not the purpose of this investigation to conduct a comprehensive survey of the array of color-variants which might be derived from cultures of the HY strain but rather to study the pattern of variation characteristic of the strain under standardized conditions similar to those we anticipated using in future experiments with mutagenic agents. Previous work with strain 274 had indicated (Bunting, 1946) that populations with relatively high proportions of red cells could be obtained from colonies grown on agar or from broth cultures maintained in the logarithmic phase. The amount of color-variation existing in similar HY colonies and broth cultures therefore was investigated.

When the HY stock cultures from the Yale collection and from the National Type Culture Collection were plated on agar, a few pink and white colonies were noted in addition to the typical red type. Upon subculture the pink and white variants proved to be very stable, but the red colonies contained an appreciable number of color-variants. A more extensive survey of spontaneous variation as it occurred in red colonies therefore was undertaken.

Three day old red colonies from synthetic agar plates were picked to water blanks, and aliquots from appropriate dilutions were spread over the surfaces of fresh synthetic plates. The numbers of daughter colonies of each color type were counted after the plates had been incubated for three days at 26 C. The results of an early analysis of this kind are presented in table 1. Bright red, pink, and white variant colonies were found on plates from many but not all of the red colonies analyzed. Speckled white-and-red colonies occurred more frequently and with surprising regularity. Colonies of this sort had not been noted in earlier studies (Bunting, 1950) in which the HY strain had been plated extensively on a peptone-glycerol-phosphate medium. The color-characteristics and stabilities of the different spontaneous variants were examined by replating representative colonies of each type on synthetic and on peptone-glycerol agar. Typical results are shown in table 2. Synthetic agar plates inoculated with bright red variants could not be distinguished from plates inoculated with the parent HY culture until they had been incubated for five or six days. On old synthetic plates some of the daughter colonies from the variants remained bright red instead of fading to the dull lavender typical of old red type colonies. Further selection did not stabilize the nonfading bright red variant.

When suspensions of cells from the large pink or from the large white colonies were inoculated on synthetic plates, the resulting colonies were very uniform in appearance. Occasional speckled pink-and-white colonies were seen on plates from the pink variants, and sometimes colonies of slightly different shades of pink were observed, but reversions to more highly pigmented forms were very rare. Some of the white variants were slightly pink when grown on peptone-glycerol agar, but these were no less stable than those which were incapable of producing detectable pigment even on the low-phosphate medium. When cells from the variant speckled colonies were plated on synthetic agar, two types of daughter colonies were found in varying proportions. There were red colonies which were indistinguishable in appearance and stability from the parent red type, and there were colonies which came up white but soon developed small red areas where the unstable colorless variants evidently had reverted to the pigmented form. No stable white or intermediate pink colonies were seen. Very different results were obtained when suspensions of cells from speckled colonies were plated on the peptone-glycerol agar, for on this medium all of the colonies were a uniform dark red. Quantitative platings then were made

TABLE 1

Spontaneous color-variation in three day old red colonies of the HY strain of Serratia marcescens

RED COLONY ANALYZED	AGE OF COLONY	NUMBER OF DAUGHTER COLONIES OF EACH COLOR-TYPE ON SYNTHETIC AGAR PLATES					PER CENT OF COLOR VARIANTS
		Red	Bright red	Pink	White	Speckled	All types
	days						
1	3	590	1	0	0	3	0.67
2	3	4,426	16	22	8	40	1.94
3	3	1,098	0	0	0	20	1.80
4	10	925	1	2	0	27	1.11
5	10	1,670	0	1	1	44	2.72
6	10	2,384	0	1	2	56	2.42

on synthetic agar plates and on synthetic agar with 0.5 peptone. Comparable total counts were found on the two media, but whereas the majority of colonies were speckled on the synthetic ammonium-citrate-glycerol agar, all were uniformly red when the medium had been supplemented with peptone. That the alteration in appearance of colonies arising from the colorless variants on the two media was not phenotypic but reflected a shift in the proportions of pigmented and colorless cells in the colonies was demonstrated readily by picking colonies and replating them on synthetic agar. Variants which gave speckled colonies containing high proportions of unstable colorless cells on synthetic agar gave red colonies with very few such cells when grown in the presence of peptone, yeast extract, or casein hydrolyzate.

It was of interest from theoretical as well as practical considerations that the incidence of most of the common variant types was reduced markedly by selecting red colonies through successive platings and reisolations. At three day intervals suspensions from red colonies were used to reinoculate fresh synthetic plates; after several weeks individual red colonies were analyzed with the results shown in table 3. Bright red, pink, and white variants arose far less frequently than had been true in colonies recently isolated from stock cultures (table 1), but selection had had no effect on the proportion of cells giving speckled colonies.

Observations were made then on the stability of the selected red line when maintained in the logarithmic growth phase in synthetic and in peptone-glycerol broth cultures. A modification of the method used with strain 274 (Bunting, 1946) was found to maintain cultures of strain HY in the logarithmic phase. Ten ml of culture medium in test tubes were inoculated with ap-

TABLE 2

Characterization of spontaneous color variants of the HY strain of Serratia marcescens

COLOR TYPE PICKED FOR ANALYSIS	NO. OF COLONIES COUNTED	PER CENT OF DAUGHTER COLONIES OF EACH COLOR TYPE ON SYNTHETIC AGAR					APPEARANCE OF COLONIES ON PEPTONE-GLYCEROL AGAR
		Red	Bright red*	Pink	White	Speckled	
Bright red	827	90.6	8.6	0	0	0.8	dark red
Bright red	598	82.7	16.6	0	0	0.8	dark red
Bright red	761	78.4	20.0	0	0	1.6	dark red
Pink	2,354	0	0	99.2	0	0.8	red
Pink	2,053	0	0	99.5	0	0.5	red
Pale pink	243	0	0	99.8	0	0.2	dark pink
White	ca 4,000	0	0	0	100	0	white
White	ca 4,000	0	0	0	100	0	white
White	ca 5,000	0	0	0	100	0	pink
Speckled	734	68	0	0	0	32	dark red
Speckled	700	9	0	0	0	91	dark red
Speckled	955	22	0	0	0	88	dark red

* Not evident until plates were 5 to 6 days old.

TABLE 3

Spontaneous color-variation in three day old colonies of a selected red line of HY strain of Serratia marcescens

RED COLONY ANALYZED	NUMBER OF DAUGHTER COLONIES OF EACH COLOR-TYPE ON SYNTHETIC AGAR PLATES				
	Red	Bright red	Pink	White	Speckled
1	1,191	0	0	0	56
2	1,208	0	0	0	21
3	1,623	0	0	0	13
4	1,200	1	0	0	10
5	1,021	0	0	0	27
6	7,789	0	1	0	210

proximately 10^3 cells per ml and incubated at 26 C until a slight turbidity indicated approximately 10^6 cells per ml. Then one ml was transferred to a 99 ml water blank from which 1.0 ml was taken to inoculate the next tube of medium. The turbid cultures were reincubated for later analyses of color-variation in aging broth cultures.

Stable pink and white variants were extremely rare on synthetic agar plates inoculated with cells from logarithmic or from aging cultures of the selected red line as shown in table 4. However, in the rapidly growing synthetic cultures the proportion of unstable colorless variants rose rapidly to approximately 25 per cent of the population. No such rise was seen in peptone cultures, and the proportion of variant cells fell promptly when the synthetic cultures matured beyond the logarithmic phase. In another similar experiment transfers were made with fewer cells (10) from the turbid cultures, and considerably higher proportions of colorless variants were obtained (up to 70 per cent). Evidently the conditions prevalent in synthetic cultures during the early stages of growth were conducive to the development of populations with large numbers of colorless variants. Whether the mechanism responsible for the shift was selection or some process of cell-conversion from the pigmented to the colorless form was not evident, but it was apparent that the number of variants giving speckled colonies on synthetic plates could be influenced markedly by cultural conditions and therefore could not be used as an index of mutagenic activity in the usual sense.

Additional evidence for a burst of colorless variants in young synthetic cultures was obtained by plating suspensions from 24 hour colonies picked from synthetic agar plates inoculated with red cells. Such pinpoint red colonies were found to contain from 10 to 20 per cent of variants of the unstable colorless type. As the colonies continued to develop the proportion of variants fell until at three days approximately 1 per cent remained. No such increase in colorless variants was found when young red colonies from peptone plates were analyzed.

In the course of these studies occasional color-variants of other types were encountered. Orange, gray, and purple types were isolated and found to be quite stable. Various mottled colonies also were seen. A number of small, pale colonies were found which later proved to be biochemical mutants requiring specific amino acids or purines for continued growth. Usually pigmentation was relatively poor even in the presence of the supplement, but reversion to nutritional independence was common and the revertants generally were well pigmented. Undoubtedly many other color-variants would have been encountered if a wider variety of cultural and plating methods had been used.

TABLE 4

Color variants in broth cultures inoculated from red colonies of the HY strain

DAYS MAINTAINED IN LOGARITHMIC GROWTH PHASE	DAYS PERMITTED TO AGE	SYNTHETIC BROTH		PEPTONE BROTH	
		Speckled colonies	Pink and white colonies	Speckled colonies	Pink and white colonies
		%	%	%	%
0	0	0.8	0.0	0.4	0.0
1	0	4.2	0.0	0.0	0.0
2	0	21.8	0.0	0.9	0.0
3	0	23.8	0.0	0.0	1.4
4	0	30.0	0.0	0.0	0.4
5	0	24.6	0.0	0.0	0.0
6	0	25.7	0.0	0.0	0.1
7	0	29.2	0.0	0.3	0.0
8	0	28.8	0.0	0.0	0.0
8	2	21.0	0.0	0.2	0.0
8	3	8.8	0.0	0.7	0.0
8	4	4.6	0.0	0.2	0.0
8	6	0.9	0.0	0.8	0.0
8	7	2.7	0.0	0.6	0.0
8	8	1.2	0.0	0.9	0.0
8	12	0.3	0.0	0.7	0.0
8	15	0.7	1.4	0.2	0.0
8	20	2.6	0.0		
8	25	0.4	0.0		
8	31	0.7	0.0		

The experiments described have shown that the HY strain of *S. marcescens* is capable of giving rise to spontaneous color-variants of different kinds. The commonest variant found in colonies and broth cultures was an unstable colorless type which gave characteristic speckled colonies on synthetic agar plates. The number of these variants was found to be influenced by cultural conditions in a manner which made them quite unsuitable for the detection of mutagenic activity. However, the HY strain also gave rise spontaneously but infrequently and irregularly to conspicuous, stable pink and white variants which offered promising material for the quantitative study of induced mutations. The presence of the

unstable colorless type could always be masked by adding peptone to the plating medium. Other variants were seen but were of rare occurrence.

Part II. The effect of mutagenic agents on color-variation in the HY strain.

Experiments with ultraviolet light. Three day old red colonies of the selected red line were picked to water blanks to give suspensions containing approximately 10^8 viable cells. One ml of the suspension was diluted and plated to determine the number of spontaneous variants in the populations. Eight ml were pipetted into a deep petri dish and agitated at a distance of 7 inches beneath a Westinghouse sterilamp with 95 per cent of its output at 2537 A. The intensity of the ultraviolet at this distance was approximately 37 ergs/mm-2/sec-1. After exposure the suspensions were diluted and plated in a room illuminated only with yellow light to avoid photoreactivation. Synthetic agar was used for both the control and experimental platings in order to obtain information on variations in the numbers of cells giving speckled colonies as well as other variant types. All of the plates were incubated in the dark for three days at 26 C before the colonies were counted and classified as to color.

TABLE 5

The effect of increasing doses of ultraviolet light on color-variation in the HY strain of Serratia marcescens

ULTRA-VIOLET DOSE	FRACTION SURVIVING	COLONIES COUNTED	PER CENT OF COLONIES		
			Pink	White	Speckled
sec					
0		1,385	0	0	0.9
10	3.0×10^{-1}	555	3.2	0.9	0.7
15	8.0×10^{-2}	1,483	4.7	2.4	1.8
20	2.5×10^{-2}	1,310	8.5	3.9	0.5
25	7.8×10^{-4}	916	14.8	4.0	0.3
30	1.3×10^{-4}	1,142	11.5	8.4	1.8
40	5.0×10^{-5}	1,876	7.8	4.2	0.6
50	1.8×10^{-5}	446	5.4	0.7	0.4

The number of color-variants on the plates inoculated with irradiated suspensions was very much higher than on the control plates. The colonies varied in color from an intense red through different shades of pink to solid white. Since it was sometimes difficult to differentiate the darker types from the parent red colonies, only the data for the conspicuous pink, white, and speckled variants are presented in table 5. The proportions of pink and white colonies increased with the dose of ultraviolet light to a maximum at 30 seconds, but there was no significant change in the relative number of cells giving speckled colonies. It was found later that considerable clumping occurred in water suspensions shaken under ultraviolet light for more than 30 seconds, which of course would interfere with the effectiveness of further irradiation and also could bring about a decrease in the apparent numbers of variants in the suspension since all colonies arising from clumps containing a large proportion of red cells probably would be classified as red and the presence of variants in the clumps would be masked.

Representative colonies of the different variant types found on the plates inoculated with irradiated cells were picked to water blanks and replated on synthetic and on peptone-glycerol agar. In general the induced variants proved similar in stability and color characteristics to spontaneous variants of the same appearance. However, a dark red type was found which proved to be quite stable and gave colonies which were pigmented more intensely at all ages than those of the parent red type. Stable dark red variants had not been noted on plates from untreated cells. Orange, purple, gray, and other variants were seen somewhat more frequently on plates from irradiated cells than on those inoculated with unirradiated suspensions, but they were far less common than the pink and white types. Irradiation also was observed to increase the relative numbers of biochemical mutants requiring nutritional supplements for good growth. In most cases the variants with nutritional deficiencies were less stable than those which showed only modifications in pigmentation.

Although the proportion of color-variants on plates from irradiated cells was very much higher than that observed in the controls, the increase was not sufficient to rule out entirely the possibility that the results had been due to selective killing of the red type. In order to check this possibility the lethal effects of irradiation were tested on various mixtures of stock red cells and color-variants obtained from plates inoculated with irradiated cells. The results of one such experiment performed with a mixture of red and stable white cells are presented in table 6. In this case irradiation of the mixture resulted in a large increase in the proportion of cells giving

red colonies. Evidently, the induced stable white type was more sensitive to ultraviolet light than was the original red parent. Similar results were obtained with a mixture of HY red and induced pink types although in this case the differential was not so great. These results indicated that the action of the ultraviolet light was mutagenic rather than selective.

The remarkable stability of the white variants used in these and in previous experiments (Bunting, 1950) suggested that the cells might have lost permanently the ability to produce pigment. Because of the theoretical importance of this possibility considerable effort was spent in attempting to obtain reversions to the pigmented type. Irradiations with ultraviolet light followed by extensive platings on synthetic medium were consistently negative. By working with artificial mixtures containing one red cell per million white it was found that although the presence of red cells seldom was revealed in heavily inoculated pour or surface-spread plates, it was demonstrated easily on streak plates. Evidently, the red type had some competitive advantage which was stifled under the urban conditions of an over-crowded smear or pour plate but which was expressed clearly at the margins of heavily populated streaks. Red marginal colonies developed regularly on plates streaked from mixtures containing one red cell per million white per ml. In spite of the use of streak plates, however, no pigmented colonies were detected when suspensions of cells of a stable white type were plated on synthetic medium before or after irradiation with ultraviolet light.

Eventually, by substituting peptone-glycerol agar for the synthetic medium, it was possible to demonstrate occasional reversions of the stable white type to pigmented forms. After irradiation and plating on peptone-glycerol agar, a few colonies were found which showed a trace of pink. When these were treated again with ultraviolet light, well-pigmented colonies appeared on both peptone-glycerol and synthetic plates. It was found also that by plating very old peptone broth cultures of white variants spontaneous reversions to pigmented forms sometimes were obtained.

The effects of irradiation were observed on a dark red strain and on a spontaneous pink type. Exposure of the dark red cells resulted in an array of red, pink, and white variants but again, no change in the proportion of cells giving speckled colonies. When the suspension of cells from the stable pink strain was irradiated, there was an increase in both white and red types. The increase in both darker and lighter variants provided additional evidence that the action of the ultraviolet light was mutagenic rather than selective.

Experiments then were carried out to observe the effect of photoreactivation on populations of cells which had been treated with ultraviolet light. By comparing the total numbers and distribution of color-variants on plates made before and after photoreactivation the ability of the light to reverse the lethal and the mutational effects of the ultraviolet treatment could be determined. Suspensions of cells from three day old red colonies were irradiated in the usual manner with doses varying from 10 to 50 seconds. They then were divided into two portions; one was kept in the dark at 37 C for one hour while the other was photoreactivated. For photoreactivation 2 ml aliquots of the irradiated suspension were pipetted into ½ inch glass tubes and placed in a glass-fronted water bath at 37 C. The reactivating light source was a 500 watt tungsten filament lamp in a projection lantern with the bellows fully contracted. A filter of 0.03 N aqueous CuCl, in a 3.5 cm deep cell, was used to absorb a large part of the infrared. The distance from the lens to the glass tube containing the suspension was 3 inches, and the period of illumination was one hour. To avoid unwanted photoreactivation during plating procedures the

TABLE 6

The effect of ultraviolet irradiation on red and white variants of the HY strain of Serratia marcescens

SUSPENSION MADE FROM:	EXPOSURE TO ULTRAVIOLET	VIABLE CELLS PER ML	PER CENT OF COLONIES		
			Red	White	Pink
	sec				
Red colonies	0	6,500,000	99.7	0.3	<1
	15	148,000	88	4	7
	30	240	79	8	12
White colonies	0	8,000,000	0	100	0
	30	830	0	100	0
Red and white colonies	0	36,000,000	22	78	<1
	30	2,400	53	42	5

work was done in a laboratory illuminated only by yellow light. The treated and untreated cellular suspensions were assayed by plating them quantitatively on synthetic agar in the usual manner. Ten plates were inoculated for each dilution.

TABLE 7

Fraction of cells surviving with and without photoreactivation in ultraviolet-irradiated suspensions of Serratia marcescens, HY strain

ULTRAVIOLET DOSE	NO. OF VIABLE CELLS/ML NONIRRADIATED	FRACTION SURVIVING	
		Dark	Light
sec			
10	3.1×10^8	3.0×10^{-1}	7.1×10^{-1}
20	3.1×10^8	2.3×10^{-2}	1.5×10^{-1}
30	1.7×10^8	8.2×10^{-5}	3.5×10^{-2}
40	3.8×10^8	5.0×10^{-5}	5.0×10^{-4}
50	2.7×10^8	1.8×10^{-5}	2.7×10^{-5}

TABLE 8

Proportion of color variants among survivors of ultraviolet-irradiated suspensions of Serratia marcescens, HY strain, with and without photoreactivation

ULTRAVIOLET DOSE	DARK SURVIVORS		LIGHT SURVIVORS	
	No. colonies counted	Color variants*	No. colonies counted	Color variants*
sec		%		%
10	555	4.1	1,310	1.3
20	1,310	12.4	2,872	4.4
30	1,142	19.9	592	19.8
40	1,876	12.0	1,717	18.8
50	446	6.1	7,480	19.1

* Total per cent pink and white variants.

In these experiments no spontaneous pink or white variants were detected among 1,385 colonies counted on the nonirradiated control plates.

The survival data from a typical experiment are summarized in table 7. When the fractions of cells surviving in the dark and in the light were plotted against the ultraviolet dose, survival curves similar to those described by Kelner (1949) for *Escherichia coli*, strain B/r, were obtained. From such curves the dose-reduction ratio for the HY strain *S. marcescens* was calculated and found to be 1.6, indicating that visible light reduced the lethal effects of ultraviolet irradiation by 37 per cent. There was a decrease in the rate of inactivation for populations treated with ultraviolet doses higher than 30 seconds due, presumably, to the clumping of the bacteria.

The proportions of conspicuous pink and white color-variants among both the dark and light survivors are presented in table 8. Irradiation produced the usual increase in color-variants among the dark survivors with a decrease in the apparent numbers of variants after 30 seconds. Photoreactivation reduced the number of color-variants among the light survivors up to the critical 30 second dose associated with cell clumping. Subsequently, the proportions were increased by reactivation, but the increase never exceeded the maximum frequency detected among unphotoreactivated survivors. Experiments in which suspensions of the pink and white variants types were irradiated and exposed to visible light demonstrated that they were capable of being photoreactivated to the same extent as the red parental type.

Considering only the data obtained with the lower doses of ultraviolet light it was evident that reactivating light reduced both the lethal and genetic effects of the irradiation. The dose-reduction ratios calculated for the mutagenic effects of ultraviolet irradiation at different doses were not constant, however, as they were for lethality, but rather they decreased with increasing ultraviolet dose. A difference between dose-reduction ratios for lethality and mutagenesis has been reported also by Newcombe and Whitehead (1951) in their studies of ultraviolet induced color-response mutants in *E. coli*, strain B/r, on mannitol-tetrazolium agar.

All of the experimental results obtained with ultraviolet light support the hypothesis that the stable variants are caused by gene mutations similar to those found in higher forms. The uniformity of pattern of induced variation following treatment with ultraviolet light is illustrated by the collection of data presented in table 9 showing the results of 30 seconds' exposure in 6 different experiments performed on different days. No spontaneous pink or white variants were seen in over 12,000 colonies examined on the control plates whereas in each assay 20 to 30 per cent of the colonies from the irradiated suspensions were conspicuously pink or white. In no experiment was there any significant shift in the proportion of cells giving speckled colonies. Thus, the pattern of variation displayed by red population of the HY strain of *S. marcescens* following treatment with ultraviolet light was

strikingly reproducible. Irradiation was capable of inducing a variety of color-mutants including the conspicuous pink and white types which were especially valuable in following the process quantitatively. There was no evidence that it had any effects on the production of variants of the unstable colorless type.

The effect of ultraviolet light also was tested on a few other strains of *Serratia*. These preliminary observations will not be reported in detail, but enough was done to show that different responses may be expected from different strains. For example, very few stable white or pink types were obtained from strain 274 following irradiation. The pattern of induced variation produced with a given strain seemed just as characteristic as its pattern of spontaneous variation.

Experiments with chemical mutagens. The striking effectiveness with which ultraviolet light increased the proportions of dark red, pink, and white color-variants in treated suspensions of red cells of the HY strain of *S. marcescens* encouraged us to hope that it might be relatively simple to demonstrate the mutagenic action of chemical agents with this biological system. Such, however, did not prove to be the case.

The first attempts at inducing color-variation with chemical agents were made with the surface-active compounds sodium desoxycholate and sodium lauryl sulfate. Witkin (1947) and Latarjet (1948) had reported that sodium desoxycholate induced phage-resistant mutants in *E. coli*. Low concentrations of these agents had been found to modify the proportions of color-variants in aging cultures of strain 274 of *S. marcescens* (Bunting, 1942), but this phenomenon had been shown to be due to selection rather than any mutagenic action of the compounds (Bunting, 1950). In later experiments with the HY strain the surface-active agents were used in the same concentrations as those employed by Witkin and Latarjet, but although every effort was made to follow the procedures they had used no increase was noted in the relative numbers of color-variants following treatment with the mutagens.

The only chemical agent which gave any suggestion of mutagenic activity when tested for its ability to induce color-variation in the HY strain of *S. marcescens* was nitrogen mustard. The methyl bis (8-chloroethyl) amino hydrochloride (Merck) was obtained in vials containing 10 mg of salt. Ten ml of sterile distilled water were added to a vial giving an acidic aqueous solution which was relatively stable. Suitable dilutions were made in buffer at pH 6.9, and the agent was allowed to stand only two minutes before it was added to the bacterial suspensions which were incubated for one hour in a water bath at 37 C together with a control tube containing only bacterial suspension and buffer. Aliquots from the tubes were diluted and plated on synthetic agar in the usual manner.

Preliminary trials established the killing curve and showed that 0.25 mg of nitrogen mustard per ml killed 99.96 per cent of the bacteria and gave a population containing about 2.5 per cent of stable pink and white variants. Multiple runs with 0.15 mg HN-2 per ml gave from 1.2 to 2.8 per cent stable variants where the untreated controls showed none. There was no evidence of any shift in the number of cells giving speckled colonies following treatment with the nitrogen mustard. However, the evidence for mutagenic activity of the nitrogen mustard was not conclusive since later experiments in which mixtures of red and variant types were treated showed that in this case the red cells were appreciably more sensitive to the lethal action of the agents which could have accounted for the observed increase in the per cent of variants.

TABLE 9

Induced color-variation in suspensions of red HY cells treated with ultraviolet light

ULTRA-VIOLET DOSE	FRACTION SURVIVING	PER CENT OF COLONIES		
		Pink	White	Speckled
sec				
30	4.0×10^{-4}	17.8	13.9	1.5
30	2.3×10^{-3}	15.2	12.2	0.8
30	3.5×10^{-3}	17.0	6.5	1.5
30	1.1×10^{-4}	14.5	11.3	1.2
30	2.1×10^{-4}	17.0	7.5	0.6
30	8.6×10^{-3}	13.1	7.0	0.2
0	6 control platings	0	0	1.1 ± 0.6

Many other chemical agents were employed with negative results. Three basic dyes (acriflavine, methyl violet, and methylene blue) were tested for their possible effects on color-variation. Suspensions of *Serratia* were treated both in the dark and in the light. The dyes exerted a much greater killing effect in the light (exposed to 500 watt tungsten filament projection lantern

placed 2 inches from glass-fronted water bath) than in the dark but had no detectable effect on the proportions of color-variants.

Following Demerec's report (Demerec *et al.*, 1950) of the highly mutagenic properties of manganous chloride when used to produce reversions from streptomycin-dependence to non-dependence in *E. coli*, this compound was tested also for its ability to cause color-variation in *Serratia*. However, even though parallel experiments with *E. coli*, strain B/r/Sd-4, were performed to make sure that the highly specific conditions essential for mutagenic activity with *coli* cultures were fulfilled, and even though a high yield of mutants was obtained with *coli*, no increase in color variants was noted with *Serratia*.

Negative results were obtained also with urethane (ethyl carbamate) and pyrogallic acid which had been reported by Auerbach (1949) to have mutagenic activity. Concentrations from 1 mg per ml to 0.1 mg per ml were used, and the cells were exposed for 1 hour at 37 C in a water bath. A few pink variants were noted in platings from two of the urethane tubes, but these were found to revert to the red parental type when streaked on synthetic agar.

Although disappointing, it was perhaps not altogether surprising that the chemicals tested here failed to induce color-variation in *Serratia*. As Witkin (1950) demonstrated in the case of acriflavine and *E. coli*, a rather delicate relationship may exist between survival and the rate of induced mutation. Demerec *et al.* (1950) have shown also with *E. coli* that highly specific conditions unrelated to survival are essential for the demonstration of the mutagenic activity of manganous chloride. It appears that the conditions required for the demonstration of mutagenic activity for any one chemical and any one organism must be determined empirically and that a different set of conditions may be required when either the chemical or the test organism is varied. Possibly better yields of variants would have been obtained if resistant strains had been developed as shown by Bryson (1948).

In conclusion it may be stated that although it was relatively easy to induce color-variation by treatment with ultraviolet light this has not been the case with chemical agents.

DISCUSSION AND CONCLUSIONS

The pattern of distribution of spontaneous color-variants in the HY strain of *S. marcescens* was found to be characteristic of the strain and strikingly different from that previously found with strain 274 (Bunting, 1940). Strain 274 rarely produced stable variants of any color and was never observed to give rise to an unstable variant of the colorless type found so abundantly in HY cultures. It produced unstable bright pink variants which readily reverted to the dark red type and occasionally gave rise to paler types which were even more unstable. It might be difficult to identify red cultures of the two strains from their general appearance, but they could be distinguished easily by the variants which they produced.

The most conspicuous color-variants of the HY strain were stable pink and white types which arose infrequently and irregularly as spontaneous mutants but were found in relatively large numbers following exposure of red cells to ultraviolet irradiation. Photoreactivation with white light reversed the mutagenic action of the ultraviolet. The data on incidence, stability, and behavior of these variants suggested that they arose as gene mutations and that they offered promising material for the experimental study of induced mutations. However, all attempts to induce color-variation with chemical mutagens were unsuccessful.

Color-variants also were observed which showed biochemical deficiencies. Presumably these types as well as the more stable prototrophic forms mentioned above were caused by gene mutations. It would be of interest to test the effectiveness of chemical mutagens in inducing auxotrophs from the HY strain in view of our inability to induce color variation with these agents.

The commonest color-variant found in HY cultures was an unstable, colorless form which reverted readily to the pigmented type. All pigmented cultures were found to contain an appreciable number of these variants; the proportion varied with cultural conditions. Young cultures in fresh synthetic medium contained large numbers of unstable, colorless cells whereas older synthetic cultures or cultures grown in the presence of peptone or yeast extract contained very few. Colonies which developed from color-

less variants inoculated on synthetic agar plates were white with pigmented specks whereas those developing from similar cells on agar supplemented with peptone or yeast extract soon became uniformly colored and were indistinguishable from colonies arising from the pigmented form. Ultraviolet irradiation had no detectable effect on the production of the colorless variants.

Every red HY colony analyzed contained colorless variants, and all of these variants gave colonies with demonstrable areas where reversion to the pigmented form had taken place. It was evident, therefore, that both forms were highly unstable. The data were not sufficient to determine whether selection or some other mechanism was responsible for the reproducible shifts in the proportions of the two types found under different cultural conditions. In many respects the system resembled that described by Spiegelman *et al.* (1950, 1951) for long-term adaptation in yeasts. Pigmented cells of *Serratia* in the absence of supplement rapidly lost the ability to make pigment just as adapted yeast cells in the absence of galactose lost the ability to ferment that sugar. Under appropriate conditions some of the progeny from each colorless variant regained the ability to make pigment in much the same manner that the yeasts readapted in the presence of galactose.

Whether the reversible instability of pigmented HY cells was due to a high rate of gene mutation or to cytoplasmic alterations of a mechanism resembling that responsible for changes in antigenic type in paramecia (Sonneborn, 1948; Beale, 1952) was difficult to determine in the absence of a demonstrable sexual cycle. How many other examples of reversible instability affecting colonial and other characteristics of bacterial populations are of a similar nature remains to be investigated.

The general conclusion from this study of *Serratia marcescens* is that several distinct kinds of color-variants are produced, possibly as a result of different kinds of cellular events. Knowledge of the characteristics and behavior of specific variant types is essential to a correct interpretation of the significance of their appearance in experiments with suspected mutagenic and other agents. The data indicated that quantitative determinations of the numbers of stable pink and white color-variants could be used to evaluate the action of mutagenic agents but that the number of unstable, colorless variants was influenced greatly by cultural conditions. The mechanism responsible for the production of these unstable variants is being investigated.

SUMMARY

Red cultures of the HY strain of *Serratia marcescens* produced spontaneous color-variants of several different kinds. Irradiation with ultraviolet light increased the proportion of stable variant types; photoreactivation reversed the mutational as well as lethal effects of the ultraviolet light. No success was attained in using HY cultures to demonstrate mutagenic activity of chemical agents.

An unstable colorless variant which gave speckled colonies on synthetic media was found in all cultures. Ultraviolet irradiation had no apparent effect on the production of these variants but cultural conditions greatly influenced their numbers.

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