A BIOCHEMICAL AND GENETIC STUDY OF REVERSION
WITH THE A-GENE A-PROTEIN SYSTEM
OF *ESCHERICHIA COLI* TRYPTOPHAN SYNTHETASE¹

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STRAINS which approach wild type in behavior can arise from mutant strains as a result of either reversion (mutation of the gene altered by the original mutation), or suppression (mutation of some other gene). Recently it has become evident that reversion is an exceedingly complex phenomenon, whether analyzed at the level of genetic fine structure or in terms of modifications of specific proteins. Reversion may not only occur at the same site as the original mutational alteration but at other sites within the same gene (JINKS 1961; CRICK, BARNETT, BRENNER and WATTS-TOBIN 1961; YANOFSKY, HELINSKI and MALING 1961; HELINSKI and YANOFSKY 1963). The specific enzyme activities that are restored by reversion may be associated with proteins resembling the wild-type protein, or with proteins that are clearly different (MAAS and DAVIS 1952; HOGNESS and MITCHELL 1954; FINCHAM 1957; GILES 1959; GILES, PARTIDGE and NELSON 1957; STADLER and YANOFSKY 1959; YANOFSKY and CRAWFORD 1959; GAREN 1960; and ESSER, DEMOSS and BONNER 1960). It is becoming clear, therefore, that when a mutant reverts it probably can undergo all possible single mutational changes that will permit the synthesis of a functional enzyme.

Reversion due to a genetic change at a second site within a gene, in addition to its intrinsic interest, could yield valuable information on one of the puzzling problems of gene action. This problem concerns the nature of CRMless³ mutants (SUSKIND, YANOFSKY and BONNER 1955), mutants which appear to lack any protein resembling a specific wild-type protein as a result of a mutational change.

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³ The following abbreviations are used throughout: T⁺, tryptophan independent; T⁻, tryptophan requiring; his⁻, histidine requiring; cys⁻, cysteine requiring; FR, full revertant; PR, partial revertant; SU, suppressor; 5BU, 5-bromouracil; 5BDU, 5-bromodeoxyuridine; 2AP, 2-aminopurine; 5MT, 5-methyl tryptophan; TSase, tryptophan synthetase; In, indole; T, tryptophan; InGP, indoleglycerol phosphate; CRM, an altered protein (cross reacting material) structurally related to the wild-type protein.

One possible explanation of some mutants of this type is that mutational alteration has led to a nonsense nucleotide sequence, i.e., a nucleotide sequence that does not specify an amino acid (CRICK, GRIFFITH and ORGEL 1957). A single nonsense sequence in a gene could conceivably prevent the formation of an intact polypeptide chain. If this explanation is correct, reversion of CRMless mutants would not be expected to result from mutations in a second amino acid coding unit in the gene altered by the original mutation. The finding of a second-site reversion of this type in a CRMless mutant would establish that at least some CRMless mutants do not arise by mutations leading to nonsense sequences.

The present study is concerned with a genetic and biochemical examination of reversion with a well defined gene-protein system. It was hoped that by examining many characteristics of revertants it would be possible to determine the number and types of different revertants that are obtainable from each mutant. From this information and from gene and protein structural studies, it eventually should be possible to describe each reversion event in terms of a nucleotide change and a corresponding amino acid change.

The system selected for study is the *A*-gene – *A*-protein system of the tryptophan synthetase of *Escherichia coli*. The *A* gene controls the formation of the *A* protein, one of the two protein subunits which comprise the tryptophan synthetase system of this organism. The other subunit is designated the *B* protein (CRAWFORD and YANOFSKY 1958). Together these proteins catalyze the following three reactions: (1) indole + L-serine $\rightarrow$ L-tryptophan; (2) indoleglycerol phosphate $\rightleftharpoons$ indole + 3-phosphoglyceraldehyde; and (3) indoleglycerol phosphate + L-serine $\rightarrow$ L-tryptophan + 3-phosphoglyceraldehyde (CRAWFORD and YANOFSKY 1958; CRAWFORD 1960). Reaction (3) is believed to be the physiologically important reaction and only those strains capable of carrying out this reaction can grow in the absence of tryptophan (YANOFSKY and RACHMELER 1958; YANOFSKY and CRAWFORD 1959). A large number of well characterized ultraviolet-induced mutants which were incapable of forming a functional *A* protein were available for this study (YANOFSKY, HELINSKI and MALING 1961). Some of these mutants produced an altered *A* protein (*A*-CRM) that could combine with the *B* protein and carry out reaction (1), while the others did not. Revertants of both types of mutants were examined.

Terminology: The term “phenotypic revertant” (STADLER and YANOFSKY 1959) is used to describe any tryptophan-independent (T^+) strain arising from a tryptophan auxotroph, regardless of genetic type. The term “reversion” or “revertant” applies only to strains in which the mutational event restoring prototrophy appears to have occurred in the gene that was altered by the original mutation, e.g., the *A* gene for *A* mutants. Strains in which the wild-type enzymatic activity appears to be fully restored are called “full revertants” (FR). Those strains in which reversion has restored only partial activity in the physiologically important reaction are termed “partial revertants” (PR). The term “suppressor” (SU) describes a phenotypic revertant in which a mutation outside the *A* gene restored the ability to grow without exogenous tryptophan.

“Primary-site reversion” refers to a presumed change at the nucleotide posi-

tion of the original mutation, while "second-site reversion" describes an event occurring at a nucleotide position different from the one altered by the original mutation. This could be in the same amino acid coding unit or in a different one. Mutants with alterations in the same small region of the *A* gene will be called members of the same cluster. Within each cluster, mutants will be said to map at the same site if they give less than 0.001 percent recombination with one another in appropriate transduction tests (MALING and YANOFSKY 1961).

MATERIALS AND METHODS

Escherichia coli strains: The mutant strains used in this study were isolated by penicillin selection following ultraviolet irradiation of the K-12 strain of *E. coli* (ADELBERG and MYERS 1953) and have been described previously (YANOFSKY, HELINSKI and MALING 1961). A-protein mutants of both types (CRMless and CRM-formers) can grow on indole since they form large amounts of the B protein which can convert indole to tryptophan slowly in the absence of A. The A mutants accumulate indoleglycerol and cannot grow on minimal medium or medium supplemented with anthranilic acid.

Media: L-broth was used for the growth of mutants (LENNOX 1955). Where L-broth lacking cysteine was required, the medium was depleted of cysteine by growing a cysteine auxotroph in the L-broth. The minimal salts medium of VOGEL and BONNER (1956) with 0.2 percent glucose was used and was supplemented with amino acids or indole when required. Histidine (30 $\mu\text{g/ml}$), indole (10 $\mu\text{g/ml}$) and L-tryptophan (20 $\mu\text{g/ml}$) were used as growth supplements.

Analogs and other chemicals: Aminopterin (California Corporation for Biochemical Research) was usually autoclaved with the plating medium. For use as a supplement in liquid media, a stock solution was prepared containing 2 mg/ml in 0.01N sodium hydroxide, and stored in the dark at 5°C. Solutions containing 5-bromouracil (5BU) (8 mg/ml), 5-bromodeoxyuridine (5BDU) (8 mg/ml) (California Corporation) and 2-aminopurine (2AP) (40 mg/ml) (California Corporation) were autoclaved immediately before use.

Spontaneous reversion experiments: Each A mutant examined was grown overnight in L-broth from a small inoculum to a final population of approximately 5×10^9 cells per ml. The cells were centrifuged and washed twice with 5 ml of sterile 0.85 percent saline. The cells were resuspended and diluted with saline. Aliquots of the dilutions were plated on a medium containing minimal agar with a low tryptophan supplement (0.01 $\mu\text{g/ml}$). The plates were incubated for five days at 37°C. The number of bacteria plated was determined by dilution and plating on minimal agar containing 20 $\mu\text{g/ml}$ of L-tryptophan. After a preliminary experiment to estimate the reversion frequency, sufficient cells of each mutant type were plated to give 100 to 200 revertant colonies per plate. The T⁺ colonies appearing on this supplemented medium probably represent independent mutational events since it was shown by resspreading experiments that most of the reversion events occurred after plating.

Reversion experiments with base analogs: Each A mutant was grown to a

population of 5×10^8 cells/ml in L-broth. The bacterial suspensions were centrifuged, washed, resuspended and diluted if necessary and aliquots added to tubes containing 2.5 ml of melted soft agar (0.65 percent Difco agar, 0.5 percent NaCl and 0.8 percent Difco Bacto-nutrient broth powder) at 47°C. Immediately after addition of the cells the mixture was poured onto the agar surface of a previously poured plate. The bottom layer consisted of minimal agar with 0.1 gm Difco Bacto-nutrient broth powder per liter. When 5BU or 5BDU was to be used, 50 µg/ml of aminopterin was added to the medium. After the agar had hardened a sterile filter paper disc, one-half inch in diameter, was placed on the agar surface in the center of the plate. A small volume (0.05 ml) of the 2AP, 5BU, or 5BDU solution was pipetted onto each disc. An equal volume of sterile distilled water was added to discs on control plates. The plates were incubated for eight days at 37°C.

Routine handling of tryptophan-independent (T^+) colonies: Colonies of widely varying sizes appeared after incubation of the experimental plates. These colonies were picked and streaked on minimal agar to obtain single colony isolates before further tests were performed. Following the initial purification, T^+ strains were examined using the first three tests listed in Table 1. The full and partial revertants were detected and distinguished by these tests and were then examined in the other tests in the table.

Accumulation studies: An aliquot of 1 ml of a liquid minimal culture of each revertant was removed and mixed with 1.5 ml of FeCl_3 reagent (YANOFSKY 1956). Within a few minutes after mixing this reagent gives a red-purple color with indoleglycerol.

5-methyl tryptophan sensitivity: Two tests of sensitivity to growth inhibition by 5-methyl tryptophan (5MT) were employed. The first test was qualitative and involved streaking dilutions of cultures on minimal plates containing 0.01 µg/ml 5MT and on control plates without 5MT. The plates were inspected for growth inhibition after 24 hours. In the second test, 10^6 cells were added to a tube containing 2.5 ml of melted minimal soft agar (minimal medium plus 0.65 percent agar) and the mixture poured onto a minimal agar plate. When the agar hardened a sterile filter paper disc was placed in the middle of the plate. A sterile 5MT solution (0.1 µg or 1.0 µg) was pipetted onto the disc. After incubation for 24 hours at 37°C the radius of the zone of inhibition around the disc was measured in millimeters. The variation between duplicate plates did not exceed ten percent and rarely reached that level.

Colony size studies: A culture of each revertant was diluted and plated to obtain 20 to 30 colonies per plate. The plates were incubated for five days at 37°C and the diameters of 5 to 10 colonies measured. The experimental variation observed between colonies on the same plate or on duplicate plates in the same experiment did not exceed 10 per cent.

Whole-cell assay of tryptophan synthetase activity: The ability of cell suspensions to catalyze the conversion of indole and serine to tryptophan was determined, as described by EISENSTEIN and YANOFSKY (1962). The experimental

TABLE 1

Tests employed in the examination of full and partial revertants

Test	Observation	Interpretation
indoleglycerol accumulation	a. accumulation b. no accumulation	defective tryptophan production tryptophan synthesis not limiting
colony size determination	a. smaller than wild type b. same as wild type	defective tryptophan production tryptophan synthesis not limiting
genetic test of linkage of reversion site to mutant site	a. no mutants recovered b. a few mutants recovered	reversion at or near site of original alteration second-site reversion
5-methyl tryptophan sensitivity	a. more sensitive than wild type b. same sensitivity as wild type	defective tryptophan production tryptophan formed at the same rate as in wild type
whole cell TSase activity (reaction 1)	a. higher than wild type b. same as wild type c. lower than wild type	derepression due to defective protein the revertant protein may be as active as the wild-type protein labile or less active protein or less protein formed
A specific-activity (reaction 1)	a. higher than wild type b. same as wild type c. less than wild type	depression due to defective protein a. fully functional A protein if $A/B = 1$ to 2. b. defective protein if $A/B = < 0.5$ labile or less active protein, or less protein formed
A/B ratio (reaction 1)	a. < 1 to 2 b. $= 1$ to 2	restored protein is labile or produced in smaller amounts than B equal production of both proteins
activity ratio: $\frac{\text{In} \rightarrow \text{T (reaction 1)}}{\text{InGP} \rightarrow \text{T (reaction 3)}}$	a. > 2.5 b. $= 2.5$	restored protein is less active in the physiologically essential reaction as active as the wild-type protein in both reactions
heat stability	a. $>$ wild type b. $=$ wild type c. $<$ wild type	not wild-type protein protein <i>may</i> be wild-type not wild-type protein
acid precipitability	a. $>$ wild type b. $=$ wild type	not wild-type protein protein <i>may</i> be wild-type
relative neutralization by antibody	a. $=$ wild type b. $<$ wild type	normal activity/antigen ratio protein less active enzymatically or a mixture of enzymatically active and inactive proteins

variation obtained with duplicate tubes within the same experiment or from different experiments frequently reached 30 percent.

Preparation and examination of extracts: Extracts were prepared by treatment of bacterial suspensions in 0.1M Tris buffer at pH 7.8 for 10 minutes in a 10Kc Raytheon sonic oscillator. Glucose-minimal medium was employed for the growth of revertant cultures for enzyme studies.

To obtain sufficient amounts of the A proteins for purification from the revertants, double mutants were prepared with strain T-3, a mutant which is blocked in the synthesis of anthranilic acid. When strains with the T-3 block are grown on low levels of indole, they form large amounts of the two protein components of TSase (CRAWFORD and YANOFSKY 1958). The techniques used to determine enzymatic activity, protein concentration, the neutralization of A activity by antiserum, and the heat and acid stability of proteins have been described previously (CRAWFORD and YANOFSKY 1958; MALING and YANOFSKY 1961). One unit of TSase activity in any of the reactions corresponds to the conversion of 0.1 μ mole of substrate to product in 20 minutes at 37°C.

Peptide pattern studies: The A proteins were purified by the procedure of HENNING, HELINSKI, CHAO, and YANOFSKY (1962) and peptide patterns prepared as described by HELINSKI and YANOFSKY (1962a).

Genetic procedures: Suppressed mutants and true reversions (within the A gene) were distinguished by genetic tests (STADLER and YANOFSKY 1959) using transduction with phage P1kc (LENNOX 1955). An adaptation of the penicillin selection technique was utilized in the test for reversion at a second site. A standard transduction mixture was incubated for 20 minutes to permit adsorption of the phage, and then centrifuged. The sedimented cells were washed with 5 ml sterile saline and resuspended in L-broth lacking cysteine. These cells were grown overnight with shaking at 37°C, 0.05 ml was transferred to 10 ml of the same medium and the culture grown to log phase. The cells were centrifuged, washed twice with sterile minimal medium, and diluted to 10^6 /ml in minimal medium plus histidine and glucose. The culture was incubated for six hours and then penicillin (300 units/ml) was added. The culture was incubated overnight with shaking at 37°C, centrifuged, and the cells resuspended in 0.4 ml minimal medium. Aliquots of 0.1 ml were removed and plated in duplicate on plates containing L-histidine and L-tryptophan. After incubation for two days at 37°C the colonies were replicated to medium containing L-histidine as sole supplement to detect tryptophan-requiring colonies.

RESULTS

Spontaneous reversion studies: The initial experiments in the study of reversion in the A gene were concerned with the isolation and preliminary characterization of spontaneous revertants obtained from a selected group of A mutants. The procedure employed for the isolation of revertants was designed to insure that most of the revertants obtained from each strain arose by independent mutational events. An attempt was made in these studies to isolate representa-

tives of all of the phenotypically different revertant types that were derived from each mutant. Accumulation tests, colony size measurements and genetic tests (Table 1) were performed with each purified revertant to provide a basis for their initial assignment to different categories. The results of these tests are presented in Table 2. It is obvious that many of the mutants differed in their reversion patterns and the frequency with which they gave T⁺ colonies. Some strains gave only full revertants while others gave several distinguishable revertant types as well as suppressed mutants.

Of the mutants listed in Table 2, strains A-1, A-3, A-11, A-26 and A-33 have

TABLE 2

Frequency and types of colonies found among spontaneous phenotypic revertants

Mutant	T ⁺ colonies per 10 ⁹ cells plated	Number of colonies picked	T ⁺ type*	Relative colony size†	Mutant	T ⁺ colonies per 10 ⁹ cells plated	Number of colonies picked	T ⁺ type*	Relative colony size†
A-1	1	6	FR	100	A-15	2 × 10 ³	2	FR	100
A-2	6	10	FR	100			5	PR	96
		22	SU	18			1	PR	43
A-3	4	3	FR	100	A-16	1 × 10 ²	5	PR	100
		4	SU	74			2	SU	15
		1	SU	21	A-17	2 × 10 ³	4	FR	100
A-4	4 × 10 ⁵	6	FR	100			4	SU	13
		2	SU	16	A-23‡	7	4	FR	100
A-7	2 × 10 ³	2	FR	100			2	PR	28
		6	SU	15			3	PR	78
A-8	1 × 10 ⁵	4	FR	100			3	PR	88
		1	PR	51	A-26	10	5	FR	100
		3	SU	16			1	PR	41
A-9	2 × 10 ⁵	2	FR	100			3	SU	15
		6	PR	100	A-28‡	50	5	FR	100
A-10	7 × 10 ²	3	FR	100			1	PR	23
		3	SU	12			2	PR	81
A-11	4 × 10 ²	3	FR	100			4	PR	90
		1	PR	55	A-33	0			
		2	SU	16	A-34	1	4	FR	100
A-12	20	3	FR	100			2	PR	57
		1	PR	100			2	PR	100
		1	PR	70			4	SU	13
		3	SU	19	A-36‡	6	4	FR	100
A-13	2 × 10 ³	2	FR	100			2	PR	29
		1	PR	64			2	PR	80
		1	SU	81			4	PR	88
		3	SU	19	A-46	6	3	FR	100
A-14	1 × 10 ³	1	FR	100			6	PR	44
		2	PR	71			1	PR	100
		1	PR	50					
		4	SU	13					

* Of the strains tested only the FR type did not accumulate indoleglycerol.

† Expressed as percent of the colony size of wild-type colonies.

T⁺=colonies not requiring tryptophan, FR=full revertant, PR=partial revertant, SU=suppressor.

‡ In other experiments suppressed mutants have been isolated from these strains.

been shown to be altered at the same genetic site (MALING and YANOFSKY 1961). Enzymological investigations and suppressor specificity data obtained with these mutants indicated that only A-11 and A-26 could be identical (MALING and YANOFSKY 1961). The reversion studies summarized in Table 2 are consistent with this conclusion. Of the five mutants altered at the same site, only A-11 and A-26 appear to have similar reversion patterns. Recent peptide pattern studies with the A proteins of these mutants also suggest that they are identical (HELINSKI and YANOFSKY 1962b). Strains A-23, A-28 and A-36 are also altered at the same site and have identical reversion patterns. Peptide pattern studies and primary structure analyses with the A proteins of these strains have shown that the same amino acid replacement occurred at the same position in these proteins (HELINSKI and YANOFSKY 1962b). The A-10, A-15, A-16 and A-17 alterations are located in the same cluster in another region of the A gene, but only A-10 and A-17 have the same reversion pattern.

Full and partial revertants were selected from the strains isolated in these experiments and subjected to a series of *in vivo* and *in vitro* tests (Table 1) to obtain additional information on the number and types of different revertants that can be isolated from each mutant. The data summarized in Tables 3, 4 and 5 describe each different type of revertant recovered from each mutant. If only one full revertant or partial revertant type is listed, it usually indicates that only this type was observed.

The characteristics of five full revertants from CRM-forming mutants were examined (Table 3) and on the basis of the tests performed all appear to be in-

TABLE 3
Characteristics of revertants of CRM-forming mutants

Strain	5MT sensi- tivity*	InG accumu- lation	Colony size†	Cell TSase	A-protein specific activity‡	A/B§	$\frac{\text{In} \rightarrow \text{T} }{\text{InGP} \rightarrow \text{T}}$	Heat inactivation¶	Acid loss**	Antibody inhibition††
Wild type	+	—	...	0.6	2.10	1.2	2.5	15.0	62.0	1.00
A-1 FR(1)	+	—	100	0.5						
A-11 FR(1)	+	—	100	0.8	1.55	0.95	4.41	17.2	59.3	...
A-11 PR(1)	—	+	55	12.0						
A-23 FR(1)	+	—	100	0.6	2.1	...	...	13.4	58.4	...
A-23 PR(1)	—	+	28	6.0	0.86	0.04	5.31	2.5	0	0.49
A-23 PR(2)	—	+	78	3.5	14.3	1.13	236.	3.5	7.89	0.60
A-23 PR(3)	—	+	88	3.6	19.2	1.81	715.	1.4	9.95	...
A-26 FR(1)	+	—	100	1.2						
A-26 PR(1)	—	+	41	11.0						
A-46 FR(1)	+	—	100	0.9	2.2	...	...	21.0	46.7	0.98
A-46 PR(1)	—	+	44	8.9	56.5	0.78	1580.	11.4	21.3	0.97
A-46 PR(2)	—	+	100	4.6	19.7	1.38	1390.	2.5	35.9	0.46

* + = resistant, — = sensitive to 5-methyl tryptophan at the concentration employed.

† Expressed as percent of the colony size of wild-type colonies.

‡ A enzyme units in reaction (1) per mg protein.

§ Ratio of A and B activities in reaction (1).

|| Ratio of A protein activity in reactions (1) and (3).

¶ Time for 50 percent inactivation of A protein activity in reaction (1) at 52°C.

** Percent of A protein activity remaining in acid supernatant after treatment at pH 4.0.

†† Inhibition relative to the inhibition of normal A. Normal A value set at 1.00 and all other values related to this. Inhibition measured in reaction (1).

TABLE 4

Characteristics of revertants of CRM-forming mutant A-34

Strain	5MT sensi- tivity*	InG accumu- lation	Colony size†	Cell TSase	A-protein specific activity‡	A/B§	In→T InGP→T	Heat inactivation¶	Acid loss**	Antibody inhibition††
Wild type	13	—	...	0.6	2.10	1.2	2.5	15.0	62.0	1.00
A34 FR(1)	13	—	100	0.9						
A34 PR(1)	46	+	67	15.9	41.1	0.372	380.	10.5	42.9	1.01
A34 PR(2)	52	+	52	14.3	18.1	1.63	55.4	11.3	60.7	...
A34 PR(3)	32	+	100	4.5	17.5	1.65	855.	13.4	...	0.43
A34 PR(4)	36	+	100	13.7	13.8	0.719	196.	5.0	24.6	...
A34 PR(5)	51	+	52	7.2	13.1	0.436	82.	6.9	25.6	...
A34 PR(6)	19	+	100	1.5	3.08	0.832	120.	13.9	52.1	...

* Zone of inhibition in millimeters.

† Expressed as percent of the colony size of wild-type colonies.

‡ A enzyme units in reaction (1) per mg protein.

§ Ratio of A and B activities in reaction (1).

|| Ratio of A protein activity in reactions (1) and (3).

¶ Time for 50 percent inactivation of A protein activity in reaction (1) at 52°C.

** Percent of A protein activity remaining in acid supernatant after treatment at pH 4.0.

†† Inhibition relative to the inhibition of normal A. Normal A value set at 1.00 and all other values related to this. Inhibition measured in reaction (1).

distinguishable from the wild-type strain. The slightly elevated value for the ratio of activities in the two reactions with A-11 FR(1) A protein is probably not significant. Of the seven partial revertants that were studied all were sensitive to 5MT and accumulated indoleglycerol, but one, A-46 PR(2), grew at the wild-type rate. Elevated whole-cell TSase activity was detected with all the partial revertants, indicating that a defective A protein was formed and, as a result, TSase formation was derepressed. The A-protein specific activity values support this conclusion with the exception of the value obtained with strain A-23 PR(1).

TABLE 5

Revertants of CRMless mutants

Strain	5MT sensi- tivity*	InG accumu- lation	Colony size†	Cell TSase	A-protein specific activity‡	A/B§	In→T InGP→T	Heat inactivation¶	Acid loss**	Antibody inhibition††
Wild type	+	—	100	0.6	2.10	1.2	2.5	15.0	62.0	1.00
A-2 FR(1)	+	—	100	1.1	3.60	1.38	6.38	12.1	55.4	1.17
A-7 FR(1)	+	—	100	2.9	2.54	1.32	4.39	15.2	63.3	1.06
A-8 FR(1)	+	—	100	2.2	1.78	1.59	3.67	14.4	59.6	1.04
A-8 PR(1)	—	+	48	8.6	14.5	0.294	55.	1.9	4.4	0.30
A-9 FR(1)	+	—	100	1.3	1.02	0.81	2.55	3.0	17.8	...
A-9 PR(1)	—	+	100	0.2	0.156	0.016	7.26	...	...	...
A-12 PR(1)	—	+	67	6.7	5.27	0.237	162.	2.1	3.0	0.45

* + = resistant, — = sensitive to 5-methyl tryptophan at the concentration employed.

† Expressed as percent of the colony size of wild-type colonies.

‡ A enzyme units in reaction (1) per mg protein.

§ Ratio of A and B activities in reaction (1).

|| Ratio of A protein activity in reactions (1) and (3).

¶ Time for 50 percent inactivation of A protein activity in reaction (1) at 52°C.

** Percent of A protein activity remaining in acid supernatant after treatment at pH 4.0.

†† Inhibition relative to the inhibition of normal A. Normal A value set at 1.00 and all other values related to this. Inhibition measured in reaction (1).

This low value can be accounted for by the lability of the A protein it forms (see heat and acid stability). In view of the low specific activity value compared to the whole-cell TSase value and the abnormally low A/B ratio, it seems likely that only a fraction of the activity present in the cells of this partial revertant survives the extraction procedure. The antibody inhibition data suggests that inactive A protein is present or the A protein that is formed is relatively inactive catalytically. The high ratios of the activities in the two reactions (Table 3, column 8) obtained with the partial revertant proteins are a clear indication that the restored A proteins are relatively inactive in the physiologically essential reaction, Reaction (3).

The revertants obtained from mutant A-34 are listed separately because a quantitative test of 5MT sensitivity was performed to distinguish different revertant types derived from this mutant. The results obtained with the revertants illustrate the advantage of employing many tests. Revertants could be distinguished that otherwise would have been considered identical on the basis of many of the tests performed. For example, A-34 PR(1) and A-34 PR(2) appear very similar in some tests but clearly, A-34 PR(1) produces a protein that is less active in Reaction (3). A-34 PR(3) and A-34 PR(4) also have many characteristics in common, but the PR(4) A protein is less active in Reaction (3) and is more heat labile. It is apparent that the more tests that can be employed for examining different properties of revertants, the more likely that distinguishable types will be detected. The fact that six distinct partial revertant types were derived from the same mutant indicates that reversion cannot always take place at the nucleotide position at which the original mutational alteration occurred. The same conclusion has been reached on the basis of similar observations with other systems (ESSER, DeMOSS and BONNER 1961; STADLER and YANOFSKY 1959).

The results of tests with the revertants of the CRMless mutants (Table 5) indicate that they have many characteristics in common with the revertants of CRM-formers. The most notable difference in these and other experiments is that partial revertants of CRMless mutants generally form labile A proteins. This can be seen from the data presented in Table 5 and is immediately apparent from the A/B ratios. For example, tests performed with extracts of A-9 PR(1) failed to detect significant levels of A protein activity. Nevertheless, since this revertant grows as well as wild type on minimal medium it is likely that a functional A protein can be formed. One full revertant was isolated, strain A-9 FR(1), which forms a heat- and acid-labile A protein. It is obvious in this case that reversion did not restore the wild-type A gene and A protein.

Base analog studies: It has been postulated by BAUTZ and FREESE (1960) that the mutagenic base analogs, 2AP and 5BU, cause nucleotide substitutions of preferred types in phage DNA. Induction and reversion of mutants of *Salmonella typhimurium* by base analogs has also been studied in an attempt to establish the nucleotide substitutions associated with mutational events (RUDNER 1961a, b; KIRCHNER 1960). If there is a preferred change with one of these analogs it would presumably be possible to obtain some information on the nucleotide sub-

stitutions associated with forward and reverse mutations in the A gene by examining the response to the base analogs. Furthermore, since many of the A mutants have complex reversion patterns it should be possible to determine which reversion classes are increased by one or both of the analogs.

Base analog reversion studies were carried out in liquid medium (FREESE 1959) and on solid medium. Reversion studies with liquid medium were not as reliable and suffered from the inherent disadvantage that many of the revertants which were detected were formed by division of other revertants. The latter difficulty can be minimized, if not eliminated, by plating the mutants on solid medium and permitting reversion to occur on the plates. Presumably, under these conditions, each reversion event can only lead to a single colony. For these reasons, solid medium was generally used in testing for an increase in reversion. In the examination of reversion of the A mutants, each strain was plated in duplicate at three different cell concentrations. The increase in reversion due to the base analogs was estimated by comparing the number of colonies obtained on plates with and without the base analogs. This estimate is not a valid indication of reversion frequency since the concentration of analog and number of revertants decreased with increasing distance from the disc. Furthermore, the bacterial population was not constant since the responding bacteria could divide several times on the plating medium employed. In the experiments with liquid media, cultures grown both in the presence and absence of analog were plated on solid medium and the number of revertant colonies counted. Table 6 presents the results of these experiments. It can be seen that both analogs increase the reversion of some, but not all of the A mutants. Comparison of the results obtained with 2AP in liquid and solid medium indicates that qualitatively similar results are obtained with both treatments. The exceptions to this conclusion are mutants A-3, A-11, A-26 and A-34 which do not appear to have an increased reversion rate on solid medium but do in liquid medium. However, in these cases the results from different experiments utilizing liquid medium were frequently contradictory. The experiments with solid medium were repeated at least three times using 12 plates per mutant and gave consistent results. Consequently, they are considered more reliable than the experiments with liquid medium.

When the results of the reversion studies with 2AP and 5BU are compared it is evident that some strains respond only to 2AP (A-1), some only to 5BU (A-9), some to both (A-2, A-10), and some to neither (A-14, A-15). BALBINDER (1962), in an investigation of reversion of 23 *Salmonella typhimurium* tryptophan-requiring mutants, found that 22 of the mutants behaved identically when treated with 2AP and 5BU. Clearly, the mutants studied by BALBINDER exhibit a greater degree of homogeneity when treated with base analogs than do the mutants of *Escherichia coli* investigated here.

Since many of the mutants studied gave several types of revertants, it was possible to determine whether specific revertant types are produced more frequently as a result of treatment with base analogs. T⁺ colonies were picked from plates containing 2AP and from control plates and were purified by streaking.

TABLE 6

Approximate increase in numbers of revertants induced by base analogs

Strain	5BU	2AP	
	Solid	Solid	Liquid
A-2	+ over 100×	+ 2-12×	+ 15×
A-4		—†	—‡
A-7	+ over 100×	+ 2×	—‡
A-8		—‡	—‡
A-9	+ over 50×	—	—
A-10	+ over 100×	+ 2×	+ 2-17× (only large colonies increased)
A-12		—	—†
A-13	+ 2×	+ 2×	+ 25× (large colonies); 2--× (small colonies)
A-14	—	—	—
A-15	—	—	—
A-16		—†	—
A-17	+ over 100×	+ 5-15×	+ 3-10× (only large colonies increased)
A-44	+ 60×	+ 2-3×	
A-87	+ 60×	+‡	
A-97	+ 50×	+ over 100×	
A-104	+ 30×	+ over 100×	
A-1§	—	+ 5-13×	+ 20×*
A-3	—	—	± 5-15×
A-11	—	—	+ 100× (large colonies); 34× (small colonies)
A-26	—	—	± 27-160×
A-33	—	—	—
A-23§	—#	+ 2×	+ 10-36×
A-28	—	+ 3-8×	+ 20-28×
A-36	—	+ 2-19×	+ 90×
A-34§	—	—	+ 15×
A-75	—	—	
A-85	—	—	
A-89	—	—	
A-46§	—#	+ 4×	+ 5-100×
A-61	—	+ 5-10×	
A-76	—	+ 2-9×	
A-86	—	+ 2-12×	
A-95	—	+ 3-8×	
A-58§	—	+ 30-100×	
A-78	—	+ 2-3×	
A-90	—	+ over 100×	

* + = an increase in reversion rate; — = no increase, ± = conflicting results in different experiments. The number indicates the approximate increase in T⁺ colonies after treatment. When two values are given they are the low and high values of several experiments.

† Indicates that the spontaneous reversion was high, and therefore it was difficult to determine whether there was an increase.

‡ Indicates scoring difficult due to small colonies which were not T⁺ which clustered around the large ones.

§ Mutants grouped together map at the same site.

|| Experiment not performed.

More recent studies have detected a slight increase in reversion with 5BDU.

The T⁺ isolates were then tested for several properties, such as sensitivity to 5MT and indoleglycerol accumulation. Revertants of mutants A-1, A-2, A-7, A-10, and A-23 were classified as to type. A-1 normally yields only full revertants while A-2, A-7 and A-10 give both full revertants and suppressors and A-23 produces full revertants, a suppressor and at least two distinctly different partial revertants. In each case treatment with 2AP increased reversion to the full revertant type. In only one case (A-7) was another class, the suppressor class, increased.

Second-site reversion: Revertants of both CRM-forming and CRMless mutants were examined genetically to determine if they arose by a second-site change in the A gene. Transducing phage prepared on revertants were adsorbed to his⁻ cys⁻ cells, and the cells were plated on a medium supplemented with tryptophan and histidine. The cys⁺ recombinants were then examined for T⁻ recombinants by replication to a medium lacking tryptophan. The system used is represented in Diagrams 1 and 2. Crossing over between the reversion site and the mutant site could lead to the recovery of the original A mutant among the cysteine-independent progeny.

To increase the sensitivity of this test the transduction mixture was subjected to penicillin selection to enrich for the T⁻ mutants produced by recombination. The results obtained are given in Table 7. It can be seen that mutants were recovered from A-9 PR(1), A-23 PR(1) and A-23 PR(2). The mutants recovered were found to be indistinguishable from A-9 and A-23 in transduction tests. To estimate the recovery of mutants during this treatment a mixture of A-9 cells and

TABLE 7

Tests for second-site reversion
Transduction: Revertant → cys⁻ his⁻

Revertant	T ⁻ colonies/survivors after penicillin	Revertant	T ⁻ colonies/survivors after penicillin
A-2 FR(1)	0/910	A-11 FR(1)	0/1800
A-7 FR(1)	0/36	A-11 FR(2)	0/1500
A-8 FR(1)	0/11	A-12 PR(1)	0/48
A-9 PR(1)	21/1350	A-15 PR(1)	0/48
A-9 PR(2)	0/16	A-23 PR(1)	5/600
A-9 PR(3)	0/4	A-23 PR(2)	5/1600
A-9 PR(4)	0/22	A-23 FR(1)	0/1350
A-9 FR(1)	0/460	A-46 FR(1)	0/2600
		A-9 PR(1) + A-9 (104:1)	138/656

T⁻ = Colonies requiring tryptophan.

A-9 PR(1) cells was subjected to the same conditions. It can be seen in Table 7 (last line) that out of 656 colonies recovered, 138 were mutants, indicating that the ratio of mutants to wild-type was increased 2000-fold by the penicillin treatment. In no case were mutants recovered if the transduction mixture was not treated with penicillin.

The frequency of transduction for the A gene with the P1kc-*Escherichia coli* K-12 system is about one transduction per 5×10^5 lytic phage under standard conditions (YANOFSKY, unpublished). In the procedure employed in these tests one half of the mutants would be lost because there is about 50 percent recombination between the cysteine marker and the tryptophan region. One would thus expect to recover only one mutant from an experiment utilizing 10^{11} phage in a standard transduction procedure, if the second-site reversion were 0.001 map units from the mutant site.

In the experiments summarized in Table 8, a total of 21 T⁻ colonies were recovered from A-9 PR(1). This is the yield from four experiments, each utilizing one ml of lysate with a titer of 10^{10} phage/ml. Using these values the second-site reversion could be placed approximately 0.05 map units from the A-9 site. A distance of 0.05 map units would correspond to approximately 18 nucleotides if one assumes that there are three nucleotide pairs in the A gene for every amino acid residue in the protein (a total of 840 nucleotide pairs for the entire protein). These considerations suggest that the reversion in A-9 occurred at a second-site in the A gene, presumably in a coding unit other than the one altered by the primary mutation.

Examination of peptide patterns of revertant proteins: The physical and enzymatic characteristics of the proteins restored by reversion were described in a preceding section of this paper. Changes in these properties imply changes in the structure of the protein. To determine whether structural alterations had occurred, peptide patterns of some selected proteins were examined. Those studied to date include the A proteins of full revertants of mutants A-2, A-11, A-23 and A-46. The revertants of A-23 and A-46 were of particular interest because the amino acid changes characteristic of the A-23 and A-46 mutant proteins were known (HENNING and YANOFSKY 1962a; HELINSKI and YANOFSKY 1962b).

The peptide patterns obtained with the A proteins of A-2 FR(1), A-11 FR(1), A-11 FR(2) and A-23 FR(1) were identical with the wild-type peptide patterns (Figure 1). The pattern obtained with the A protein of A-46 FR(1) showed a difference in the position of a single peptide (Figure 1). This peptide is the same one that is altered in the A-46 protein. This finding indicates that reversion led to an amino acid substitution in the same peptide that is altered in mutant A-46. It has been shown recently that a glycine residue in the wild-type peptide is replaced by a glutamic acid residue in the peptide from mutant A-46. This glutamic acid residue of the A-46 peptide is replaced by an alanine residue in the peptide from the A-46 FR(1) protein (HENNING and YANOFSKY 1962b).

DISCUSSION

Tryptophan-independent phenotypic revertants obtained from the A mutants

FIGURE 1.—Peptide patterns of A proteins of full revertants. Upper left—tracing of wild-type pattern with the position of mutant peptides indicated. Peptide A is found on patterns of mutant A-11, peptide B on patterns of mutant A-23, and peptide C is the wild-type peptide that is replaced by peptide B on patterns of mutant A-23. Upper right—A-23 FR(1); middle left—A-2 FR(1); middle right—A-46 FR(1); bottom left—A-11 FR(1); bottom right—A-11 FR(2). The altered peptide on the A-46 FR(1) pattern is marked by the arrow.

were found to arise by either suppressor mutation or reversion. Among those that resulted from changes in the A gene, many different types were recovered. Some of the revertants were indistinguishable from the wild-type while others

were clearly separable on the basis of phenotypic characteristics. By employing many different tests, particularly those that examined different properties of the A proteins of revertants, it was possible to detect significant differences between strains that were indistinguishable in phenotypic tests. Thus it became apparent that many mutants have complex reversion patterns and that the number of distinguishable types detected depends in part on the tests employed. Even in those cases in which complex reversion patterns were not evident, it is possible that revertants that appear to be identical arose by different amino acid replacements in the defective protein. The findings with revertant A-46 FR(1) support this conclusion. The complexity of reversion when dealing with single gene-single protein systems has also been noted by others (GILES 1959; ESSER, DEMOSS and BONNER 1960; STADLER and YANOFSKY 1959).

The A proteins of partial revertants were all found to be qualitatively different from the wild-type protein. The partial revertants of CRMless mutants, with very few exceptions, had one distinguishing characteristic—a low A/B ratio. The explanation for this is not known; however, it almost certainly indicates that many of the mutations leading to CRMless mutants have a different effect on A protein formation or structure than those that give CRM-forming mutants.

The question of whether CRMless mutations are nonsense mutations was examined by genetic analysis of revertants derived from a number of CRMless strains. In one of the partial revertants studied, strain A-9 PR(1), the site of the reverse mutation was found to be separable from the site of the original mutational alteration and probably in a different amino acid coding unit. In view of this finding, it seems unlikely that CRMless mutant A-9 arose by a nonsense mutation in the A gene. If nonsense mutations do lead to an interruption in the synthesis of a polypeptide chain, it would be difficult to see how an amino acid change in another region of the protein could compensate for this defect. An alternative explanation for CRMless mutations of the A-9 type is that the reversion and the original mutation involved nucleotide additions or deletions. In this case, this is unlikely since A-9 is reverted by 5BU. It would appear, therefore, that an inactive but intact A protein is formed by mutant A-9, but it cannot be recognized by the detection methods employed. The fact that A-9 PR(1) grows well without tryptophan, although A protein activity could not be conclusively demonstrated in its extracts, may also indicate that the protein is present, but in an unrecognizable form. Instances of second-site reversion were also detected with CRM-forming mutants.

According to current interpretations of the action of the mutagens 2AP and 5BU (FREESE 1959), mutants which respond to the analogs probably arose by transitions of one purine base to another purine, or of one pyrimidine base to a different pyrimidine. Those which do not respond could have arisen by transversions of a pyrimidine to a purine, a purine to a pyrimidine, or other changes. It has also been concluded (BAUTZ and FREESE 1960) that these base analogs have a preferred mutagenic direction, with 2AP generally causing a transition of an adenine:thymine base pair to a guanine:cytosine pair, while 5BU favors the reverse transition. Treatment of the various A mutants with the base analogs 2AP

and 5BU gave many different responses; some of the mutants were reverted by one or the other of these analogs, some by both, and some by neither. In addition, in those cases in which the classes of revertants increased by 2AP were determined, it was found in general that only one class was increased. This could be interpreted as indicating that only one type of reversion event is favored by treatment with the mutagen. However, in view of the findings with strain A-46 FR(1), it would appear that it is unwise to rely on phenotype alone in interpreting the specificity of a chemical mutagen.

SUMMARY

Tryptophan-independent (T^+) phenotypic revertants were obtained from a large number of tryptophan-requiring A mutants of *E. coli* strain K-12. The reversion patterns of the CRM-forming and CRMless A mutants were examined. Genetic studies showed that T^+ phenotypic revertants arise both by suppressor mutations and by reversions. Reversions of some CRMless and CRM-forming mutants were found to be due to changes at second-sites with the A gene. *In vivo* and *in vitro* studies of the proteins restored by reversion showed that the restored proteins frequently differed quantitatively and qualitatively from the normal enzyme. Partial revertants of CRMless mutants produced A proteins which proved to be especially labile. The effect of the base analogs 2AP and 5BU on the reversion of a series of A mutants was examined. The mutants reacted specifically to the base analogs, some responding while others did not. Only certain known classes of the revertants produced by these mutants were found to be increased by action of the analogs. Examination of the peptide patterns of several revertant proteins which appeared identical to the wild-type A protein on the basis of all other tests revealed that the primary structure of the A protein of one of the revertants differed from that of the wild-type A protein.

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