* For example, variations of ±0.3° in the water temperature or ±2.5° in the desiccant temperature will each cause an error of ±2 per cent in r, or a maximum combined error of ±4 per cent.

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*THE FORMATION OF A NEW ENZYMATICALLY ACTIVE PROTEIN AS A RESULT OF SUPPRESSION**

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Suppressor mutations are changes which, though occurring at a genetic locus distinct from the site of a primary mutation, reverse the effect of the primary mutation. Depending on the nature of the primary mutation[1] there may be several means by which suppressor genes act. In certain instances the suppressor mutation may allow the synthesis of a required substance by an alternate pathway. A second possible mechanism involves relief of inhibition; if the primary mutation has resulted in the formation of an enzyme susceptible to inhibition by some normal cellular component, any mutation reducing the level of the inhibitor within the cell will, in effect, be a suppressor mutation. Examples of suppression by both the above mechanisms are known.[2-4] In addition it has been suggested that certain suppressor genes might act by permitting the formation of new, effective enzymes to function for the absent or defective ones. In *Neurospora crassa* several cases of suppression are known in mutants which form an altered protein immunologically related to the enzyme tryptophan synthetase;[5] in the presence of the suppressor gene normal enzymatic activity appears. Moreover, two instances are known in *Escherichia coli* where mutants lacking a specific enzyme or any protein immunologically related to it regain a protein with wild-type activity upon suppression.[6] In these instances, however, it has not yet been shown that a *second* protein (one the unsuppressed mutant is incapable of forming) is responsible for the activity observed, since an inhibition relief mechanism or a quantitative effect on enzyme formation might be proposed to account for the experimental findings.

In the present study examination of a suppressed mutant derived from a tryptophan-requiring strain of *E. coli* which forms a mutationally altered protein has revealed the presence of a new, enzymatically effective protein *in addition to* the altered one. This second protein is responsible for the suppressed mutant's ability to grow on unsupplemented media.

The enzyme system employed in this study, tryptophan synthetase, has been shown to catalyze the following three reactions.[7, 8]

(1) Indole + L-Serine → L-Tryptophan

(2) Indoleglycerol phosphate ⇌ Indole + Triose phosphate

(3) Indoleglycerol phosphate + L-Serine → L-Tryptophan + Triose phosphate

Of these the third is the one which appears to be responsible normally for growth in the absence of added tryptophan. The enzyme system consists of two separable protein components, termed A and B, both of which must be present for the appearance of appreciable activity in any of the three reactions.[8] The synthesis of these two proteins is controlled by separate though neighboring genes.[9] Mutationally altered forms of both components are known which, when present with the normal second component, can catalyze but one of the three reactions.[10] The mutant used in these experiments forms an altered component A which, when combined with component B, is fully active in reaction 1 but inactive in reactions 2 and 3.[10]

Since enzyme effective in permitting growth in the absence of supplement must be able to convert indoleglycerol phosphate (InGP) to tryptophan, estimates of the levels of effective and altered enzymes in an extract can be made by comparing the activities evidenced in reactions 3 and 1. Any significant deviation from a constant ratio of these two activities at different stages of purification would constitute evidence of the presence of two different enzymatically active proteins.

Materials and Methods.—The origin and certain characteristics of *E. coli* strain T–3, the mutant strain A–3 and the suppressed mutant, strain A–3 su–3, have been described previously.[10] Strain T-3 is blocked at an early step in tryptophan formation; when grown on a limiting amount of supplement it forms large amounts of the normal components A and B of tryptophan synthetase. All strains were grown on a glucose-minimal medium for extract preparation; a growth-limiting supplement of indole (2.5 μg/ml) or anthranilic acid (2 μg/ml) was added for strains A–3 and T–3 respectively. The cells were harvested at maximal growth, washed, resuspended in 0.1 *M* Tris buffer at pH 7.8, and disrupted in a 10 Kc sonic oscillator. Reversion controls were performed on aliquots of all cultures at the time of harvesting; wild-type colonies could easily be distinguished from A–3 su–3 colonies on unsupplemented minimal agar on the basis of their colony size, for after 48 hours A–3 su–3 colonies were barely visible while wild-type colonies were 2 mm in diameter or larger.

Purification and chromatography: Two procedures were used to prepare crude extracts for chromatography on DEAE cellulose. For both the first step was the precipitation of nucleic acids by the addition of a twentieth volume of 1 *M* $MnCl_2$. In method I component B was then precipitated by the slow addition of acetic acid to pH 4.0. After centrifugation the component A-containing supernatant was returned to pH 8.0 by the addition of 10 *N* NaOH, centrifuged again and brought to 60 per cent saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in a small volume of 0.01 *M* KPO_4 buffer, pH 7.2, and dialyzed against this buffer for 4–8 hours. Method 2 was used when partial purification of both component A and component B was desired. In this case the $MnCl_2$ supernatant was adjusted to pH 6.2 with acetic acid, and $(NH_4)_2SO_4$ was slowly added to 40 per cent saturation. The resulting precipitate was collected by centrifugation, dissolved in a small amount of 0.01 *M* KPO_4 buffer at pH 7.2 (containing 2 μg of pyridoxal phosphate/ml), and dialyzed 4–8 hours against this same mixture.

Following either purification method the chromatographic procedure was essentially as previously described.[8] A KCl gradient was employed for the elution of

the protein components from DEAE cellulose. When component B was present all eluting solutions contained 2 μg of pyridoxal phosphate/ml.

Assays and transduction methods: The assays for the conversion of InGP and indole to tryptophan by measuring tryptophan appearance and indole disappearance respectively have been described.[10] A unit of activity in either reaction corresponds to the conversion of 0.1 μM of reactant to product in 20 minutes at 37°C.

The transduction techniques employed, using phage P1kc, have been described elsewhere.[9]

Results.—Characterization of the suppressor mutation: Proof that strain A–3 su–3 is in fact a suppressed mutant rests on the results of transduction experiments in which strains resembling the original mutant, A–3, were recovered from it. The experiments were performed by selecting for a marker (*cys-4*) closely linked to the A–3 marker.[9] Cys$^-$ cells were infected with phage grown on strain A–3 su–3 and plated on a medium containing tryptophan but lacking cysteine. A similar experiment with phage grown on strain A–3 served as a control. The cysteine independent colonies which appeared in both transductions were picked and restreaked on test media to score the frequency of tryptophan dependence (Table 1). Approximately the same ratio of tryptophan dependent to independent colonies was

TABLE 1

TRANSDUCTION EXPERIMENTS WITH MUTANT AND SUPPRESSED MUTANT STRAINS

Donor	Recipient	Recombinants		tryp$^-$ mutants per cent
		cys$^+$tryp$^-$	cys$^+$tryp$^+$	
A–3	*cys4*$^-$	54	83	39
A–3 su–3	*cys4*$^-$	48	59	45

obtained whether strain A–3 or strain A–3 su–3 had served as the donor in this experiment. Several of the tryptophan-requiring strains recovered in this fashion from strain A–3 su–3 were tested in growth and accumulation tests and were indistinguishable from strain A–3.

Having shown that a second gene is responsible for the tryptophan independence of strain A–3 su–3, the allele specificity of this gene was examined by transduction experiments of a second type. Here strain A–3 and its allelic strains A–1 (which also forms an altered component A) and A–2 (which does not)[10] served as recipients for phage grown on strain A–3 su–3. Following adsorption, cells were plated on minimal medium. Only with A–3 cells as recipients were slow-growing, A–3 su–3-like colonies obtained in these experiments, showing that the suppressor gene under investigation is allele specific.

Comparison of normal and altered components A: In previous studies it was shown that the elution pattern of normal component A during chromatography on DEAE cellulose varies depending on the presence or absence of component B.[8] Without component B a single peak of A protein appears in the early fractions; in the presence of B, however, a biphasic distribution ensues since a portion of the A protein remains combined with B protein and is eluted in later fractions. Before examining the suppressed mutant A–3 su–3 the chromatographic behavior and properties of the abnormal component A from strain A-3 were examined. It was found that the A protein from this mutant behaves exactly like normal component A during purification. Following pH 4.0 treatment (purification method 1), and the consequent elimination of component B, this altered component is inseparable

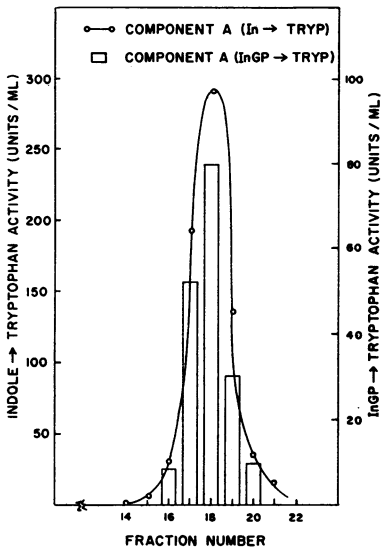

FIG. 1.—Chromatography of a mixture of T-3 and A-3 components A. Both extracts were prepared by method 1 (see Methods). Histograms represent InGP→tryptophan activity due to normal component A. The smooth curve represents indole→tryptophan activity, the sum of the activities of normal and abnormal components A.

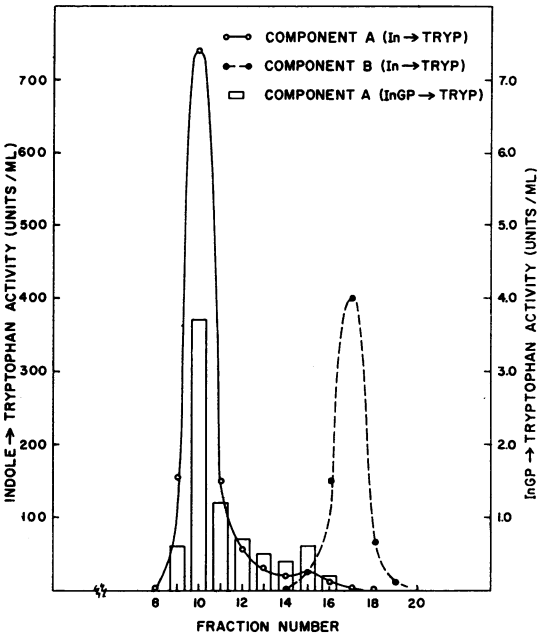

FIG. 2.—Chromatography of an extract of strain A-3 to which a small amount of T-3 component A was added. The A-3 extract was purified by method 2 (see Methods). Histograms represent InGP→tryptophan activity due to the added normal component A. The smooth curve represents indole→tryptophan activity, the sum of the activities of normal and abnormal components A.

from normal A by chromatography on DEAE cellulose. Figure 1 shows the result of co-chromatography of the two A components purified separately and combined just prior to dialysis. Normal component A is assayed in reaction 3, while values obtained in reaction 1 represent the sum of the activities of both components. It can be seen that the normal and abnormal components behave alike under these chromatographic conditions.

Standing in contrast, however, is the behavior of altered component A when chromatographed in the *presence* of component B following purification by method 2. The biphasic distribution of A activity expected from previous experiments with normal A protein is scarcely detectable, for very little of the altered component A appears in the area of the expected "combined A-B" peak. This difference is illustrated in Figure 2, the chromatogram of an A-3 extract to which a small amount of normal component A was added just prior to chromatography. Clearly a larger proportion of the normal than the abnormal component remains associated with component B. The distribution of the normal component in this chromatogram does not seem to differ significantly from that observable in the absence of altered A protein.

On the premise that the chromatographic behavior just illustrated might result from a small difference in the affinities of normal and abnormal components A for the B protein, an investigation of the saturation of component B with each of the A proteins was undertaken. The results are shown in Figure 3; altered component A

shows a somewhat poorer affinity for component B than does its normal counterpart. The units of A required for one-half the maximal reaction rate using 2.9 and 2.8 units of B were 1.3 and 1.7 respectively for the normal and altered components. The difference, though small, was found consistently in several experiments.

Chromatography of A–3 su-3 tryptophan synthetase: As reported earlier,[10] there is a small amount of InGP → tryptophan activity present in extracts of strain A–3 su–3. As with wild-type extracts, pH 4.0 treatment inactivates the preparation, but activity can be restored by the subsequent addition of component B. This suggests that an A–like protein forms part of the effective enzyme system. An A–3 su–3 extract was purified by method 2, chromatographed in the presence of component B, and the fractions obtained assayed for both InGP → tryptophan and indole → tryptophan activity. The results of this experiment are shown in Figure 4. Though the method of representation is the same as for Figure 2, here the InGP → tryptophan activity found originated in the suppressed mutant extract itself, not in added normal component A. A definite enrichment for InGP utilizing activity in the A component of the second or "combined A–B" peak of the A–3 su-3 chromatogram is apparent. This contrasts with the results of similar experiments previously performed with *normal* tryptophan synthetase from strain T–3 where the ratio of InGP to indole utilizing activities held constant (at about 40 per cent) throughout the entire chromatogram.[8] It appears that the suppressed mutant extract contains two types of A protein. Neither in this nor in several similar experiments with A–3 su–3 extracts did more component A InGP → tryptophan activity appear in the chromatographic fractions than had been placed on the column. When assayed with normal component A, the InGP → tryptophan activity of component B in the suppressed mutant extract (not shown in Figure 4) was in constant and normal ratio to the indole → tryptophan activity throughout the B-containing area of the chromatogram, supporting the earlier finding that this component is not affected by either the original (A–3) or the suppressor (su-3) mutation.[10] The recovery of all activities in this chromatogram was in the neighborhood of 90 per cent.

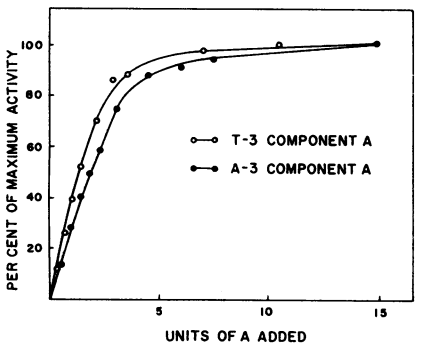

FIG. 3.—Saturation of component B with T–3 and A–3 components A. Both A components prepared by purification method 1 (see Methods). The amount of normal component B present was 2.9 units for the experiment with T–3 A and 2.8 units for the A–3 A experiment. The extent of reaction was measured in the indole→tryptophan assay.

The numerical ratio (in per cent) of the InGP → tryptophan to indole → tryptophan activity of the A–3 su–3 extract at various stages of purification is shown in Table 2, along with similar ratios from the artificial mixture experiment of Figure 2 for comparison. In each case the ratios are reasonably constant during the early stages of purification, but an enrichment of InGP utilizing activity of about 5-fold appears in the second chromatographic peak, with an inverse change in the ratios of the early fractions.

TABLE 2

RATIO OF INGP TO INDOLE UTILIZATION IN AN EXTRACT OF A-3 SU-3 AND A MIXTURE OF A-3 AND T-3 EXTRACTS AT VARIOUS PURIFICATION STAGES

Purification Stage	A-3 su-3 Extract $\frac{\text{InGP} \rightarrow \text{tryp} \times 100}{\text{indole} \rightarrow \text{tryp}}$	A-3 + T-3 Extracts $\frac{\text{InGP} \rightarrow \text{tryp} \times 100}{\text{indole} \rightarrow \text{tryp}}$
Crude extract	0.32	7.1
$MnCl_2$ supernatant	0.24	7.4
$(NH_4)_2SO_4$ supernatant	0.27	...
Dialysate	0.19	5.9
Chromatogram fractions		
1st peak (A alone)	0.09	3.5
2nd peak (A + B)	1.7	28

In one additional experiment the fractions in the "combined A–B" area of the chromatogram of an A–3 su–3 extract were concentrated by ammonium sulfate precipitation and rechromatographed under similar conditions. The pattern of Figure 4 reappeared, and a further enrichment of InGP → tryptophan activity (about 4-fold) was achieved in the new "combined A–B" area. The ratio of InGP to indole utilization in the best fraction still did not approach that of normal component A, however.

Examination of A–3 extracts for an inhibitor: If the A–3 mutation had resulted in the production of an A component sensitive to inhibition by some cellular constituent, the suppressor gene might act by reducing the level of the inhibitor in the cell. Under this hypothesis, the effective A component in strain A–3 su–3 would be susceptible enzyme freed of the inhibitor. Since chromatography of A–3 su–3 extracts yielded fractions with reasonable levels of InGP → tryptophan activity, it was possible to determine the susceptibility of this activity to inhibition by substances in an A–3 extract. When crude extracts of the mutant were added to suitable A–3 su–3 fractions, however, no inhibition was demonstrable (Table 3). Care was taken to maintain the usual 3 to 1 ratio of component B to component A in these assays.

TABLE 3

THE EFFECT OF CRUDE A–3 EXTRACT ON THE CONVERSION OF INGP TO TRYPTOPHAN BY A–3 SU–3 COMPONENT A

Column purified A–3 su-3 component A *ml*	Crude A-3 extract *A units*†	Partially purified component B* *units*†	InGP → tryptophan activity *units*
0.1	..	30	0.32
0.1	23	100	0.32
0.1	46	175	0.32
...	46	150	0

* This preparation of component B was free of component A.
† Determined in the indole → tryptophan reaction.

Discussion.—The results obtained in this study of suppressed mutant A–3 su–3 of *E. coli* show that in this instance suppression permits the formation of an active protein which the unsuppressed mutant cannot produce. The mechanisms of suppressor gene action established in previous studies, alternate pathway utilization and relief of inhibition, appear unlikely in this case. The suppressed strain A–3 su–3 can form tryptophan in the usual way, by the conversion of InGP to tryptophan. The inhibition release explanation also seems ruled out not only by the fact that crude mutant extract does not impair the InGP → tryptophan activity of

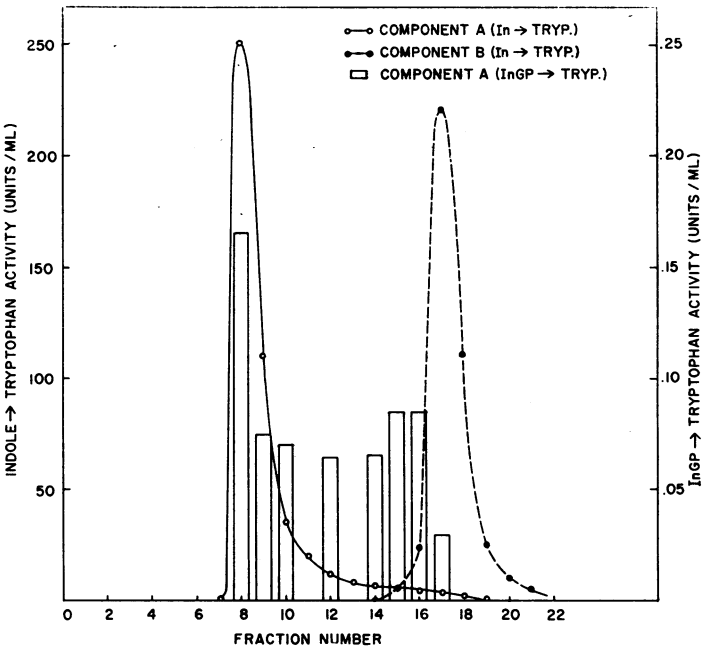

FIG. 4.—Chromatography of an extract of strain A–3 su–3 following purification by method 2 (see Methods). The method of representation is the same as in Figure 2. Fractions 7–17 were concentrated 20-fold by $(NH_4)_2SO_4$ precipitation prior to assay.

enzyme from the suppressed mutant, but by the fact that a high degree of purification, such as is attained by the procedures used,[8] does not result in either the appearance of InGP utilization in the mutant extract or the liberation of *additional* InGP utilizing ability in the suppressed mutant extract. On other grounds the likelihood of an inhibitor being closely bound to the "altered" A protein seems remote since this protein (in the presence of component B) has the same activity to antigen ratio in the indole → tryptophan reaction as does normal component A.[10] Thus the hypothetical inhibitor would have to eliminate completely one reaction catalyzed by the protein without having any effect on its participation in a second reaction.

The possibility that the gene responsible for suppression in strain A–3 su–3 can cause the *de novo* synthesis of a protein which can serve as an effective component A was ruled out in this case by the finding that the suppressor gene in question is specific for the A–3 allele and does not alleviate the tryptophan requirement of other A alleles.

It remains possible that the original mutant, A–3, is potentially capable of producing effective component A in addition to the altered protein, and that the effect of the suppressor gene is to relieve an inhibition of this synthetic ability. At the present time, however, it seems more likely that the effect of the suppressor gene is to modify somehow a small portion of the product of the mutant gene. Such an interpretation is not only in accord with the experimental data but forms a close analogy with results recently obtained in allele heterocaryon complementation studies in *Neurospora crassa*.[11] Assuming, as seems likely from their similar proper-

ties, that the normal and mutationally altered component A proteins differ but slightly, it is possible that in the presence of the mutant form of the suppressor gene a short amino acid sequence similar to that of the affected area of component A might be exchanged with the altered region. Any mechanism (analogous to transpeptidation if operative at the protein level but possibly occurring at an earlier stage of protein synthesis) permitting exchange in this manner might allow the elaboration of a small amount of effective enzyme. A somewhat similar mechanism has been postulated to explain the ability of certain pairs of alleles in *N. crassa* to form "normal" enzyme in a heterocaryon.[11]

Whatever the mechanism by which the effective component A in strain A–3 su–3 is formed, it is clear that the distribution of this protein in chromatograms where component B is present is very similar to that of normal, mutationally unaltered component A under similar conditions. The present experiments do *not*, however, permit the conclusion that the effective protein *is* normal component A, in the sense of being identical with the protein present in wild type cells. Analogous phenomena may account for several unexplained instances of suppression at other loci and in other organisms.[12–15] A determination of the prevalence of the mechanism of suppression uncovered in these experiments with *E. coli* strain A–3 su–3 must await the successful application to other systems of more refined protein separation and identification techniques, however.

Summary.—Certain tryptophan auxotrophs of *E. coli* form a defective A protein, one of the components of the tryptophan synthetase enzyme system. Examination of a suppressed mutant of one such strain has revealed that, in addition to the altered protein characteristic of the unsuppressed mutant, an A protein with normal catalytic activity is present. Hypotheses for the formation of the second A protein are discussed.

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† Postdoctoral fellow of the National Foundation.

[1] In this paper we confine discussion to primary mutations which exert a direct effect on an enzymatically active protein. If instead, for instance, the primary mutation were to cause the synthesis of an inhibitory substance, other forms of suppression would be possible.

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