SUPPRESSOR GENE ACTION IN THE TRYPTOPHAN SYNTHETASE SYSTEM OF NEUROSPORA CRASSA. II. BIOCHEMICAL STUDIES[1]

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SUPPRESSOR gene action in some cases may be explained by an effect on the primary structure of a mutant protein. This may entail the insertion of the wild type or a different but functional amino acid at the primary mutant site, or another amino acid substitution at a second site, thereby producing a functional double mutant enzyme. The existence of this type of suppressor mechanism was suggested from experiments with one suppressed tryptophan synthetase mutant of *Neurospora crassa* (YANOFSKY 1952). The formation of enzyme which is normal in catalytic, immunological, and physical properties has been shown for a group of suppressed CRM-negative alkaline phosphatase mutants of *Escherichia coli* (GAREN and SIDDIQI 1962). (CRM: crossreacting material.) A small amount of wild-type-like enzyme in addition to the mutant species was found in a suppressed CRM-positive tryptophan synthetase A protein mutant of *E. coli* (YANOFSKY and CRAWFORD 1959), and recently it was demonstrated that the wild-type-like A protein contained the original wild-type amino acid reinserted in its correct position in the polypeptide chain (BRODY and YANOFSKY 1963).

This paper is concerned with the nature of the tryptophan synthetase protein possessing wild-type activity which has been restored in suppressed td_{201} mutants as well as with the negligible effect of the td_{201} suppressors on the wild-type phenotype. The results are considered in the context of the existing hypotheses of suppressor-gene action. A preliminary report of part of this material has been presented (YOURNO and SUSKIND 1963).

MATERIALS AND METHODS

Growth analyses: Quantitative experiments were performed according to the method of RYAN (1950). For a comparison of the relative growth rates of each unsuppressed wild type ($td+$ $su+_{201}$) or suppressed wild-type ($td+su_{201}$) strain on various media, inocula were taken from an identical conidial suspension and still cultures were run concurrently. In direct comparisons of relative growth rates of $td+su+_{201}$ and $td+su_{201}$ strains on minimal medium, conidial suspensions were filtered once through cheesecloth and counted with a Petroff-Hauser counter. Suspensions then were adjusted to equal conidial concentration. Viable conidial counts were made by plating aliquots of the suspension on sorbose agar. These comparisons were made between

$td^+su^+{}_{201}$ and td^+su_{201} strains originating in the same tetrad to decrease the possibility of growth rate differences due to diverse genetic backgrounds. Inocula for all td^+su_{201} cultures were taken directly from ascospore-initiated cultures to insure the presence of the suppressor gene.

Preparation of extracts: Small quantities of crude extract for comparative assays were prepared as described by YOURNO and SUSKIND (1964). Large quantities of mycelia necessary for enzyme purification were grown in 10 or 20 liter carboy culture at 30°C as described (MOHLER and SUSKIND 1960). Wild types were grown on unsupplemented minimal medium. Suppressed and unsuppressed td_{201} mutants were grown on minimal medium supplemented with indole or tryptophan. All cultures were harvested after 72 to 96 hr incubation. The td^+su_{201} carboys were inoculated with ascospore cultures to insure the presence of the suppressor. Harvesting and lyophilization of mycelia, preparation of crude extracts, and purification of normal tryptophan synthetase (TSase) and mutationally altered tryptophan synthetase (CRM) to the $R_{25\text{-}31}$ stage (about 15–20 percent pure) were performed as previously described (MOHLER and SUSKIND 1960).

Artificial $R_{25\text{-}31}$ mixtures of wild-type and td_{201} TSase, in proportions designed to simulate TSase activity ratios in suppressed td_{201} mutants, were prepared either by mixing $R_{25\text{-}31}$ preparations of the two enzymes (Reconstitute $_a$) or by fractionation of mixed mycelia to the $R_{25\text{-}31}$ stage (Reconstitute $_b$). About 3 ml of $R_{25\text{-}31}$ fractions containing approximately 50 mg protein per ml were further purified on a 2×170 cm G-100 Sephadex column prepared as described (Pharmacia Brochure 1961) at room temperature with a solution A eluant (see *Heat inactivation*). Fractions of 3 ml were collected in a refrigerated fraction collector. G-100 fractions of maximum specific activity were used for acrylamide gel electrophoresis, Ouchterlony agar diffusion, and sucrose density gradient centrifugation.

Assay procedures: TSase reactions 1, 2, and 3f were assayed as described by YOURNO and SUSKIND (1964). Bovine serum albumin (0.8 mg/ml) was added to $R_{25\text{-}31}$ or G-100 preparation assay mixtures to stabilize the enzyme. Protein in crude extracts and $R_{25\text{-}31}$ fractions was determined by the microbiuret assay (YOURNO and SUSKIND 1964), and in the G-100 samples by the optical density at 280 mμ.

Physical, biochemical, and immunological characterization of TSase and CRM prepared from $su^+{}_{201}$ and su_{201} genotypes: Ratio of Reaction 2 enzyme to antigen in crude extracts of $td^+su^+{}_{201}$ and td^+su_{201} strains: The ratio of enzyme/antigen in crude extracts was determined according to published methods (SUSKIND 1957). Fresh crude extracts were prepared from young mycelia (grown in 200 ml aerated cultures at 30°C) to minimize loss of enzymatic relative to antigenic activity. Inocula for td^+su_{201} cultures were taken directly from ascospore cultures.

Heats of activation of reaction 1: $R_{25\text{-}31}$ preparations were diluted to 5–7 units of reaction 1 per ml in solution A containing 1 mg BSA per ml, and reaction 1 activity was assayed at 17°, 28° and 37°C. The initial velocity is expressed as μmoles indoleglycerolphosphate (InGP) disappearing per hr per ml extract. The activation energy for each preparation was calculated from the slope of the Arrhenius plot.

K_m *for indoleglycerolphosphate in Reaction 3f:* K_m for InGP in reaction 3f was determined with $R_{25\text{-}31}$ TSase diluted in pH 6.2 buffer to approximately 1 unit reaction 3f per ml. The reaction mixture contained 0.1 ml enzyme, 260 μmoles pH 6.2 phosphate buffer, 0.2 μmoles pyridoxal phosphate, 1 mg BSA, 2×10^{-3} M EDTA and 0.04 to 0.80 μmoles InGP in a final volume of 1.0 ml. In order to insure a linear rate of indole formation the reaction was stopped before reaching 10 percent completion.

pH optimum of reaction 3f: $R_{25\text{-}31}$ samples were diluted in pH 6.2 buffer to approximately 1 unit of reaction 3f activity per ml. The reaction mixture consisted of 0.1 ml enzyme in solution A, 250 μmoles phosphate buffer at the pH indicated, 1 mg BSA, 0.2 μmoles InGP, 0.2 μmoles pyridoxal-P and 6×10^{-3} M EDTA in a final volume of 0.9 ml.

Heat inactivation of TS ase and td_{201} CRM: $R_{25\text{-}31}$ preparations were diluted to 2 mg protein per ml in solution A, X or Z. Solution A contained 10^{-3} M EDTA, 0.12 μmoles pyridoxal-P and 100 μmoles phosphate buffer, pH 7.8, per ml. Solutions Z and X were identical to solution A except that solution Z contained only 0.014 μmoles pyridoxal-P per ml while solution X contained 0.5 μmoles pyridoxal-P and 10 μmoles DL-serine per ml. 0.5 ml aliquots were preheated

at 37°C for 3 to 5 min, then placed in a water bath at the appropriate temperature and swirled for the first 30 seconds. At specified times the tubes were plunged in ice water, swirled for 30 sec, and stored in an icebath until assayed.

Gel filtration: Sephadex gels were prepared as described (Pharmacia brochures 1961, 1962) and 1×20 cm columns were used. $td_{201}su_{201-8}$ or 1:1 mixtures of wild type and td_{201} R_{25-31} preparations (8 to 16 mg protein) were eluted at room temperature with either 0.1 M phosphate buffer pH 6.2 containing 10^{-3} M EDTA; a stepwise gradient consisting of 0.1 M phosphate buffer pH 6.2 containing 10^{-3} M EDTA and 0.5 M phosphate buffer pH 6.2 containing 5×10^{-3} M EDTA; or a linear gradient of 0.1 M to 0.9 M phosphate buffer pH 7.8 containing 10^{-3} M EDTA. In all experiments fractions of approximately 1.5 ml were collected.

Ouchterlony agar double diffusion: As a criterion of enzyme purity agar diffusion experiments were performed as described (OUDIN 1952). The center well in each experiment contained approximately 100 units of rabbit anti-TSase prepared against partially purified TSase (strain C-84). TSase preparations from G-100 columns were added to the outer wells in successive dilutions.

Acrylamide gel electrophoresis of TSase and CRM: Preparation of acrylamide gels and electrophoresis buffer, pH 9.6, application of samples on filter paper strips, and staining for protein were performed as described (SMALL, HARRINGTON and KEILLEY 1961). Samples from G-100 columns each containing 100–150 μg protein, were run for $3\frac{1}{2}$ hours at 6 v/cm.

Sucrose density gradient centrifugation: The procedure for measuring the molecular weight of an enzyme by determining the ratio of the sedimentation velocity of an unknown to a standard enzyme was followed (MARTIN and AMES 1961). The 4.55 ml linear gradients of 5 to 20 percent sucrose in 0.1 M phosphate buffer, pH 7.8, containing 10^{-3} M EDTA were prepared in 1.3×5.1 cm cellulose nitrate tubes. A 0.075 ml aliquot of a Sephadex G-100 fraction of TSase containing 0.11 to 0.15 mg protein and 0.025 ml of the catalase standard (mol wt 250,000) containing 0.03 mg protein were carefully layered on the sucrose gradient and centrifuged 13 hr at 38,000 rev/min in a refrigerated model L Spinco ultracentrifuge equipped with an SW-39 swinging bucket rotor. The bottom of each tube was punctured with a #26 needle, the needle withdrawn, and 31 ± 5 fractions of two drops each were collected and assayed. Drop size was found to increase somewhat with fraction number because of the decrease in hydrostatic pressure during emptying of the tube. Correction factors for increase in drop size were determined for each tube after collection of the enzyme fractions by refilling the tube with an identical sucrose gradient containing Bromphenol blue and plotting increase in absorbancy at 600 mμ with fraction number after collection of two drop fractions in a standard volume of distilled water. Molecular weight values calculated with the correction factor, however, did not change by more than ± 5 percent.

RESULTS

Suppressed td_{201} mutants were found to retain the high level of reaction 2 activity of TSase characteristic of the unsuppressed mutant. In addition they possess about 10 to 20 percent of the wild-type level of TSase reaction 1 activity. While reaction 3f activity cannot be detected in crude extracts of suppressed mutants, it is measurable in fractionated preparations.

Biochemical and physical studies of mutant, suppressed mutant, and wild-type TSase: Mixing experiments with crude extracts and deproteinized supernatants of crude extracts of td_{201} and td_{201} su_{201-8} strains showed that inhibitor or activator type relationships cannot be the basis of recovered reaction 1 activity in td_{201} su_{201-8}. No inhibition of td_{201} su_{201-8} reaction 1 activity by td_{201} extracts, nor evocation of this activity in td_{201} CRM by td_{201} su_{201-8} extracts was observed. Furthermore td_{201} su^+_{201-8} CRM does not acquire reactions 1 or 3f activity, nor do td_{201} su_{201-8} or td_{201} su_{201-2} extracts lose reaction 1 activity on fractionation.

R_{25-31} preparation of $td_{201}\ su_{201-8}$ and $td_{201}\ su_{201-2}$ and Sephadex G-100 preparations of $td_{201}\ su_{201-2}$ contain reaction 3f activity in a ratio to reaction 1 activity approximating that of wild-type strains, i.e., 1 to 50.

Characterization of recovered activities of suppressed mutants: Various physical and enzymatic characteristics of the TSase activities in R_{25-31} preparations of $td_{201}\ su_{201-8}$ and $td_{201}\ su_{201-2}$ were compared to those of wild-type enzyme as well as to those of artificial mixtures of td_{201} and wild-type enzyme mixed in proportions of TSase activities simulating those exhibited by the suppressed mutant enzyme.

Energy of activation of reaction 1: TSase reaction 1 activity of suppressed mutants possesses an energy of activation similar to that of wild type and of artificial mixtures of td_{201} and wild-type TSase, viz., 13,000 to 14,000 cal/mole InGp in the temperature range 17° to 37°C (Figure 1).

K_m for InGP and pH optimum of Reaction 3f: Reaction 3f activity of suppressed mutant TSase, like that of artificial mixtures, has a wild-type K_m value for InGP of 2.5 to 3.0 × 10^{-4}M (Figure 2) and exhibits a wild-type pH optimum of 6.2 to 6.4 (Figure 3).

Heat stability: Reaction 2 activity of suppressed mutant TSase was found to be far more heat stable than reactions 1 or 3f in phosphate buffer containing pyridoxal-P. The kinetics of loss of all three TSase activities are slightly greater than first order, but while reaction 1 and 3f exhibit a half-life of about 100 seconds at 60°C, reaction 2 has a half-life of approximately 25 minutes (Table 1). Under
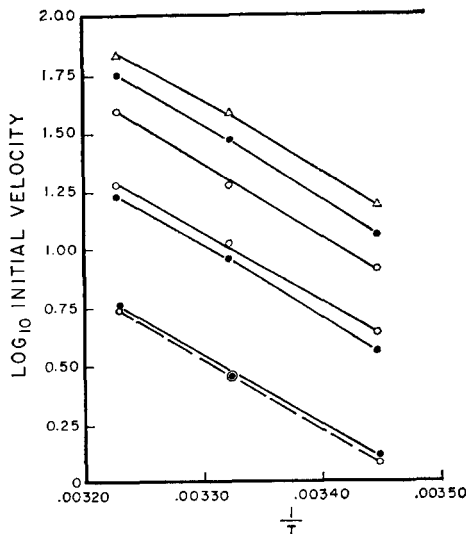

FIGURE 1.—Arrhenius plot of reaction 1 activity of R_{25-31} TSase from su^+_{201} and su_{201} genotypes. The activation energies, calculated from the slopes and expressed as cal/mole InGP are, from top to bottom: td^+_{201-2}, 13,300; $td^+su^+_{201-8}$, 14,200; td^+su_{201-8}, 13,900; Reconstitute $_a$ ($td_{201}su^+_{201-8}$ + $td^+su^+_{201-8}$), 13,100; $td_{201}su_{201-2}$, 13,800; $td_{201}su_{201-8}$, 13,400; Reconstitute $_b$ ($td_{201}su^+_{201-8}$ + $td^+su^+_{201-8}$), 13,600.
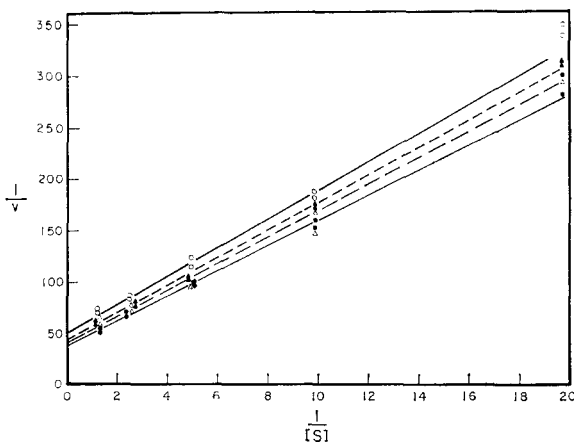
FIGURE 2.—K_m for InGP, reaction 3f, of R_{25-31} TSase from su^+_{201} and su_{201} genotypes. ○ $td_{201}su_{201-8}$, 2.8×10^{-4} M; ▲ Reconstitute $_b$ $(td_{201}su^+_{201-8} + td^+su^+_{201-8}$, 3.0×10^{-4} M; △ $td^+su^+_{201-8}$, 3.1×10^{-4} M; ● td^+su_{201-8}, 3.1×10^{-4} M. Preparations of $td_{201}su_{201-2}$ and td^+su_{201-2} TSase exhibited similar values, viz., 3.1×10^{-4} and 2.7×10^{-4} M respectively. S represents the molar concentration of InGP and v the initial reaction velocity.

the same conditions td_{201} CRM reaction 2 activity declines in a manner similar to that of suppressed mutant TSase reaction 2, both with respect to half-life and reaction order. In contrast, all three wild-type Tsase activities are inactivated initially with first-order kinetics and have a half-life of approximately 60 seconds at 60°C. Reconstituted mixtures of td_{201} and wild-type TSase behave as would be predicted from the properties of the individual components.

Wild-type TSase is protected from heat inactivation in the presence of both

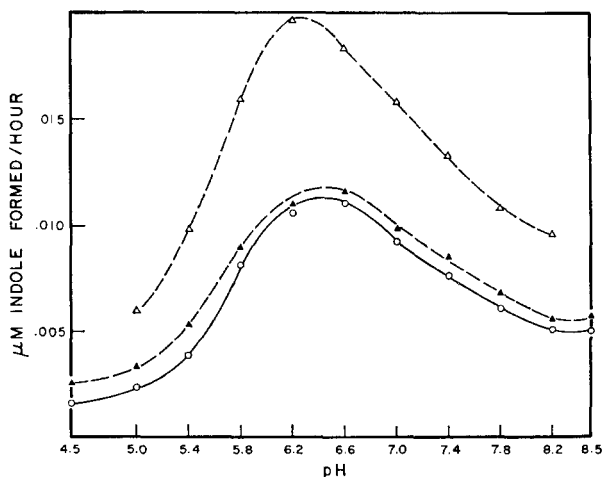FIGURE 3.—pH optimum, reaction 3f, of R_{25-31} TSase from su^+_{201} and su_{201} genotypes. △ $td^+su^+_{201-8}$; ▲ Reconstitute $_b$ $(td_{201}su^+_{201-8} + td^+su^+_{201-8})$; ○ $td_{201}su_{201-8}$. $td_{201}su_{201-2}$ TSase gave similar results.

TABLE 1

Heat inactivation of R_{25-31} preparations of TSase or CRM from $su^+{}_{201}$ and su_{201} genotypes

| | Inactivation at 60°C in phosphate-pyridoxal-P buffer | | | | | | Increase in $T_{1/2}$ at 58°C in presence of pyridoxal-P and serine | |
| | $T_{1/2}$ in seconds | | | Reaction order | | | | |
Enzyme	1	Reaction 2	3f	1	Reaction 2	3f	Reaction 1	2
$td^+su^+{}_{201\text{-}8}$	60	52	63	1°	1°	1°	4.6	4.6
$td^+su_{201\text{-}8}$	64	52	63	1°	1°	1°	..	..
$td_{201}su^+{}_{201\text{-}8}$	..	1200	..	.	>1°	.	..	1.1
$td_{201}su_{201\text{-}8}$	120	1400	90	>1°	>1°	>1°	5.6	1.2
Reconstitute $_b$ $(td^+su^+{}_{201\text{-}8} + td_{201}su^+{}_{201\text{-}8})$	56	1200	56	1°	>1°	1°	4.6	1.2

Reaction order and half-life of activity loss of R_{25-31} TSase or CRM was determined from semilog plots of heat inactivation data. $td_{201}su_{201\text{-}2}$ and $td^+su_{201\text{-}2}$ enzyme preparations were inactivated like those of $td_{201}su_{201\text{-}8}$ and $td^+su_{201\text{-}8}$ respectively. Inactivation of Reconstitute $_a$ was similar to that of Reconstitute $_b$.

DL-serine and pyridoxal-P (MOHLER and SUSKIND 1960). At 58°C the half-life of both reactions 1 and 2 of wild-type TSase is increased approximately fivefold by addition of these compounds while reaction 2 of td_{201} CRM is not significantly protected. Under the same conditions the half-life of reaction 1 of suppressed mutant TSase is increased approximately fivefold while the half-life of reaction 2 is increased no more than 10 to 20 percent. Reconstituted mixtures of td_{201} CRM and wild-type enzyme are indistinguishable from suppressed mutant TSase in this respect (Table 1).

Sephadex gel filtration: Attempts were made to separate td_{201} CRM and wild-type enzyme in artificial mixtures (1:1 by activity) and in suppressed mutant extracts by column chromatography of R_{25-31} fractions through a variety of ion-exchange and neutral Sephadex gels. Passage of a td_{201} $su_{201\text{-}8}$ preparation through a short DEAE-Sephadex (A-50) column failed to separate reactions 1 and 2. Neither did the artificial mixtures show detectable skewing of reactions 2 and 3f on short DEAE-Sephadex (A-50 or A-25), Sulfoether-Sephadex (SE-50), or neutral Sephadex (G-200) columns. Furthermore, large scale preparative fractionation of mutant, suppressed mutant, and wild-type R_{25-31} TSase preparations on Sephadex G-100 columns indicated similar behavior for all the samples. Specific activity peaks for reactions 1 and 2 of suppressed mutant TSase also coincided by this fractionation method.

Sucrose density gradient centrifugation: Physical characterization of the enzymes and further attempts at separation of mutant and wild-type species were performed with highly purified G-100 preparations. On Ouchterlony agar double diffusion the mutant and suppressed mutant enzyme preparations gave a single precipitin band when tested with anti-wild-type TSase while the wild-type enzyme preparation showed a weak second band as well.

Mutant, suppressed mutant, and wild-type enzyme were found to have similar

molecular weights as determined by sucrose density gradient centrifugation. The approximate value of 110,000 to 120,000 mol wt obtained with sucrose gradient centrifugation is in reasonable agreement with the value previously obtained by sedimentation and diffusion measurements (MOHLER and SUSKIND 1960; CARSIOTIS, APPELLA, and SUSKIND 1964; ENSIGN, KAPLAN, and BONNER 1964). Reaction 1 activity of suppressed mutant TSase sedimented coincidentally with reaction 2. As was expected by the similar fractionation behavior of mutant and wild-type enzyme, the reaction 1 activity of a small amount of wild-type TSase added to the td_{201} CRM preparation did not separate from td_{201} reaction 2 activity. This was confirmed by the uniform sedimentation of a 1:1:1:1 mixture of $td^+\ su^+_{201}$, $td^+\ su_{201}$, $td_{201}\ su_{201}$, and $td_{201}\ su^+_{201}$ preparations. Single and coincidental reaction 1 and reaction 2 peaks were obtained which showed no spread relative to the unmixed control enzyme profiles.

Acrylamide gel electrophoresis: Aliquots of these G-100 enzyme preparations were compared by acrylamide gel electrophoresis. Again no evidence of a physical difference between mutant, suppressed mutant, and wild-type enzymes was obtained. No additional component, representing a putative second enzyme species in addition to td_{201} CRM, was detected by electrophoresis of suppressed mutant samples.

Effect of td_{201} *suppressors on the wild-type phenotype:* To assess the phenotypic effect of td_{201} suppressor genes on wild-type strains a number of enzymic, immunological and nutritional properties of td^+su_{201} and $td^+su^+_{201}$ strains were compared. The presence of the td_{201} suppressor in the culture from which $td^+su_{201\text{-}2}$ TSase was prepared was confirmed by appropriate crosses of the harvested mycelia.

Biochemical and physical characterization of td^+su_{201} *TSase:* $R_{25\text{-}31}$ preparations of $td^+su_{201\text{-}8}$ and $td^+su_{201\text{-}2}$ TSase were similar to the TSase of the $td^+su^+_{201\text{-}8}$ control when judged by the Km for InGP in reaction 3f, energy of activation of reaction 1, heat stability of all three TSase activities in solution A, and ratios of TSase activities (Figures 1, 2; Table 1).

Several additional properties of suppressed and unsuppressed wild-type enzymes were examined. The purification patterns of enzymes from two $td^+su^+_{201}$–td^+su_{201} pairs were similar at the $R_{25\text{-}31}$ stage both with respect to degree of purification and recovery of enzyme activity. The $td^+su^+_{201\text{-}2}$ and $td^+su_{201\text{-}2}$ $R_{25\text{-}31}$ TSase preparations also fractionate similarly on Sephadex G-100 columns. Suppressed wild-type enzyme, like the unsuppressed, has a molecular weight of 110,000 to 120,000 as determined by sucrose density gradient centrifugation and is indistinguishable from the wild type on acrylamide gel electrophoresis. Like the $td^+su^+_{201\text{-}2}$ enzyme, the $td^+su_{201\text{-}2}$ G-100 TSase preparation used for these studies showed a very weak second precipitin band against anti-wild-type TSase in Ouchterlony agar double diffusion.

Immunological characterization of td^+su_{201} *TSase:* The ratio of reaction 2 enzyme activity to antigen in crude extracts of young mycelia is close to unity for two td^+su_{201}–$td^+su^+_{201}$ pairs originating in the same tetrad. No antigenic

TABLE 2

Comparison of ratios of enzyme/antigen in crude extracts of young mycelia of su_{201} and su^+_{201} genotypes

Strain		enzyme/antigen
$td^+su^+_{201\text{-}8}$	(2 experiments)	1.0 ± 0.1
$td^+su_{201\text{-}8}$		1.0 ± 0.1
$td^+su^+_{201\text{-}2}$	(2 experiments)	1.0 ± 0.1
$td^+su_{201\text{-}2}$		1.0 ± 0.1
$td_{201}su^+_{201\text{-}8}$	(2 experiments)	0.7 ± 0.05
$td_{201}su_{201\text{-}8}$		0.7 ± 0.05

The ratio of enzyme/antigen with respect to reaction 2 was determined for each crude extract as described in METHODS. Each experiment was performed with extracts prepared from different cultures.

difference was observed between crude extract TSase of $td_{201}su^+_{201\text{-}8}$ and td_{201} $su_{201\text{-}8}$ strains, both types exhibiting a ratio of reaction 2 enzyme activity to antigen of approximately 0.7 (Table 2).

Nutritional characteristics of td$^+$su$_{201}$ *strains:* The only consistent difference between td^+su_{201} and $td^+su^+_{201}$ strains from the same tetrad involved a slight decrease in the initial growth rate of td^+su_{201} on minimal medium (Figure 4). Nevertheless each td^+su_{201}–$td^+su^+_{201}$ pair attained comparable final growth levels and in all cases proved equally inhibitable by DL-tryptophan, although growth of the td^+su_{201} strains was less stimulated by complete medium (Table 3).
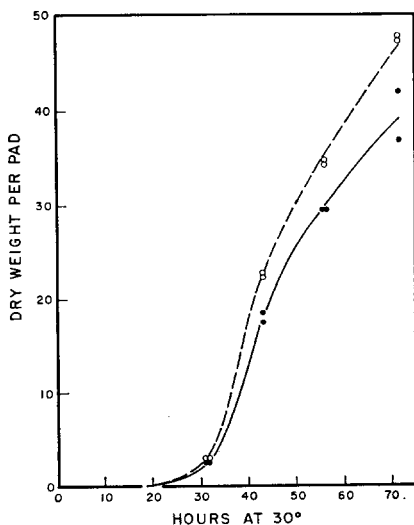

FIGURE 4.—Comparison of minimal medium growth rates of $td^+su^+_{201}$ and td^+su_{201} strains. O $td^+su^+_{201\text{-}2}$; ● $td^+su_{201\text{-}2}$. td$^+$ strains carrying either $su_{201\text{-}4}$, $su_{201\text{-}6}$, $su_{201\text{-}7}$, or $su_{201\text{-}8}$ exhibited a similar decrease in growth rate relative to $td^+su^+_{201}$ controls originating in the same tetrad. For each $td^+su^+_{201}$ — td^+su_{201} pair examined, inocula were adjusted to equal concentrations of viable conidia. Dry weight per pad is expressed in mg.

TABLE 3

Comparison of the growth of td$^+$su$^+_{201}$ *and* td$^+$su$_{201}$ *genotypes originating in the same tetrad*

Strain	Dry weight (mg) of terminal culture in minimal medium	Effect of 150 μg DL-tryptophan per ml on log phase growth rate*	Effect of complete medium on log phase growth rate*
td$^+$su$^+_{201\text{-}2}$	159	83	160
td$^+$su$_{201\text{-}2}$	150	63	108
td$^+$su$^+_{201\text{-}6}$	139	66	181
td$^+$su$_{201\text{-}6}$	150	70	149
td$^+$su$^+_{201\text{-}7}$	146	60	160
td$^+$su$_{201\text{-}7}$	146	55	113
td$^+$su$^+_{201\text{-}4}$	147	64	195
td$^+$su$_{201\text{-}4}$	141	57	70
td$^+$su$^+_{201\text{-}8}$	96	. .	. .
td$^+$su$_{201\text{-}8}$	104	. .	. .

* Expressed as percent rate in minimal medium.

DISCUSSION

It was shown by mixing experiments and by the fractionation behavior of mutant and suppressed mutant enzymes that the low level of activities of reactions 1 and 3f in suppressed td_{201} mutants does not appear to reflect activation or inhibition phenomena. Rather, the suppressor mutation seems to be responsible for restoring normal structure to a fraction of the enzyme molecules. The implication, by analogy to the studies of suppressor action in the *E. coli* TSase system (BRODY and YANOFSKY 1963), is that the original wild-type amino acid has been reinserted, although alternatively, a second residue change in each CRM molecule might have occurred (YANOFSKY, HENNING, HELINSKI and CARLTON 1963), resulting in a partially regained capacity of the CRM to catalyze reactions 1 and 3f. Unfortunately, attempts at physical separation of mixtures of td_{201} and wild-type enzyme or of the putative two enzyme species in suppressed mutant extracts were unsuccessful, so that we are unable to distinguish between these possibilities.

Reversion studies with the *E. coli* alkaline phosphatase (GAREN 1960) and Neurospora TSase (ESSER, DEMOSS, and BONNER 1960) systems suggest that all revertants with normal enzymatic characteristics are primary site revertants. In contrast, revertants with aberrant enzymatic characteristics test either as primary or as second site revertants. Fingerprint studies of revertant enzymes in the *E. coli* TSase A protein system (ALLEN and YANOFSKY 1963; HENNING and YANOFSKY 1962; YANOFSKY, HENNING, HELINSKI and CARLTON 1963) indicate that revertants with enzymatically normal A protein contain the wild-type or an equally functional amino acid reinserted at the primary mutant site in the polypeptide chain. Enzymatically abnormal revertant A proteins contain either a second mutant amino acid at a site distinct from that of the primary mutant site,

or a different amino acid than the wild-type residue at the primary mutant site.

In this light, it seems most reasonable to suggest that the low levels of reactions 1 and 3f activities in suppressed td_{201} mutants indicate the formation of a small amount of wild-type or wild-type-like TSase together with characteristic levels of CRM. The enzymological properties of the suppressed enzyme, i.e., InGP utilizing activities, are so similar to those of the wild-type protein that second-site reversion or the insertion of an amino acid less functional than the wild-type at the primary site seems unlikely. The slightly greater than wild-type half-life of heat inactivation observed for suppressed mutant TSase reactions 1 and 3f could reflect the presence of a functional amino acid different from the wild-type residue or to *in vivo* association of mutant and wild-type subunits (CARSIOTIS, APPELLA and SUSKIND 1964).

The properties of a suppressed td_{201} TSase have also been examined by RACH-MELER and ST. LAWRENCE (1964) who conclude the active enzyme is physically more similar to the mutant CRM. It appears that the site of this td_{201} suppressor mutation is not identical with ours (ST. LAWRENCE, personal communication).

In considering the mode of action of td_{201} suppressors an unrestricted "mistake" mechanism, whether at the level of the adaptor system (YANOFSKY and ST. LAWRENCE 1960; YANOFSKY, HELINSKI and MALING 1961) or the ribosome (DAVIES, GILBERT and GORINI 1964; GORINI and KATAJA 1964), might be expected to substitute one specific amino acid for another in all cell proteins, thereby producing considerable quantities of altered proteins.

Thus the lack of gross changes in the enzymatic and nutritional characteristics of suppressed strains found in this study seems more compatible with a "low-level" mistake mechanism of suppressor action, or one involving a switch of similar amino acid residues.

A "nonsense to sense" mechanism of td_{201} suppressor action does not appear as plausible. It appears (CRICK, BARNETT, BRENNER and WATTS-TOBIN 1961; SARA-BHAI, STRETTON, BRENNER and BOLLE 1964) that the sequential synthesis of the polypeptide chain may stop at the nonsense triplet. Therefore, mutants of this type very likely would be CRM-negative (SUSKIND, WICKHAM and CARSIOTIS 1963; KAPLAN, ENSIGN, BONNER and MILLS 1964). The td_{201} mutant site is known to be near the middle of the td locus (KAPLAN, SUYAMA and BONNER 1964). If this site corresponds to an approximate midpoint in the reading frame of the gene and the td_{201} suppressors act by a nonsense-to-sense mechanism, then some mechanism for reading past the nonsense triplet in td_{201} must exist, since td_{201} forms CRM of normal molecular weight.

To recapitulate, td_{201} suppressors were found to have little if any effect on td^+ genotypes (or on td_{201} genotypes aside from restoring some wild-type TSase activity). In consonance with these findings, RACHMELER and ST. LAWRENCE (1964) could detect no changes in gross amino acid composition of suppressed td_{201} or suppressed wild-type strains. A slight inhibition of growth was observed when the suppressor was present in a wild-type strain, and this was not relieved by any components of the complete medium. Similar results have been reported previously for a suppressed TSase mutant (YANOFSKY 1952). A site-specific sup-

pressor of an inositolless mutant of Neurospora also appears to exert an inhibitory effect on the wild-type (GILES and PARTRIDGE 1953). On the other hand, site-specific suppressors of *E. coli* alkaline phosphatase (GAREN and SIDDIQI 1962) and *Salmonella typhimurium* purine-requiring mutants (GOTS 1956) do not detectably alter the growth of wild types. Indeed, a site-specific suppressor of isoleucineless mutants of *Saccharomyces cerevisiae* has been reported to stimulate the growth of the wild type on minimal medium, or of the mutant grown in the presence of isoleucine (KAKAR 1963). One limitation of most of these growth tests is the unavailability of isogenic stocks for comparison. Although growth comparisons were made here only between $td^+su^+{}_{201}$–td^+su_{201} pairs originating in the same tetrad, no claim for isogenicity can be made. Hence the growth effect may be due to genes other than su_{201}.

SUMMARY

Enzymological and physical studies of TSase from suppressed td_{201} mutants suggest the presence of wild-type enzyme as well as CRM. When the td_{201} suppressors are crossed into wild-type strains there is no observable change in the enzymic, antigenic, or physical properties of wild-type TSase. Nevertheless a slight decrease in growth rate does occur on either minimal or complete medium. These data are considered in the context of current theories of suppressor gene action in protein synthesis.

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