EFFECTS OF AZATRYPTOPHAN ON BACTERIAL ENZYMES AND BACTERIOPHAGE*

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Recent discoveries have shown that certain amino acid analogs are readily incorporated into bacterial proteins[1,2,3,4]. It is the purpose of this paper to learn whether such analogs prevent the appearance of active agents such as inducible enzymes and bacteriophages. The proteins of these materials are advantageous for study because although initially absent they may be formed rapidly in response to outside stimuli.

Experiments were performed to determine whether the tryptophan analog DL-7-azatryptophan[5] interfered with the appearance of activities of several enzymes in *Escherichia coli*, or with the production of bacteriophage T2. Studies of the isolated phage permits determination of whether inactivation is a consequence of incorporation of the analog into the phage structure, or occurs as a result of effects on material formed prior to synthesis of actual phage components. Formation of different proteins is of importance at different times during phage development[6-10], and azatryptophan was therefore added at various times in the hope that such experiments would lead to insights into the roles of proteins during various stages of phage synthesis.

MATERIALS AND METHODS

E. coli, strain B or ML was grown on either a salts–glycerol medium[11] or a low phosphate medium[12]. The latter in some experiments was supplemented with 0.5 mg/ml of an acid hydrolysate of casein (Difco Casamino Acids). The medium for the tryptophan requiring mutant 19-2 was supplemented with 10 μg/ml of L-tryptophan, while that of the pyrimidineless mutant 6386 contained 20 μg/ml of uracil. These mutants were isolated by Dr. B. D. DAVIS. The tryptophan-requiring mutant B/1,t[6] was isolated by Dr. A. NOVICK. Bacteria were grown at 37° C with aeration by shaking, and their concentration was measured by turbidity determined in the Klett-Summerson colorimeter using green light. Viable counts were made by spreading suitably diluted aliquots on broth-agar plates.

For phage infection, bacteria were removed from the growth medium by centrifugation, and resuspended in a fresh medium lacking both a carbon and a nitrogen source. Phages were added at a multiplicity of about 3 for metabolic experiments or 0.1 otherwise; after a 5 minute adsorption period the bacteria were sedimented to remove free phage and resuspended in the original growth medium to allow phage development[13]. The phages T1 and T2r were used. Standard phage techniques were used for determination of phage titer[14]. Purification of phage was accomplished by centrifugation at 10,000 × *g* for 5 minutes to remove bacterial debris, centrifugation for 90 minutes at 15,000 × *g* to sediment the phage, and a final low speed centrifugation.

Proteins, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) were determined on samples that had been precipitated with 10% trichloroacetic acid (TCA), by the Folin[15], orcinol[16], and indole[17] methods, respectively. Radioactive phosphorus, provided as phosphate, or carbon, provided as 2-^{14}C leucine, was determined with the Geiger-Müller counter, usually after TCA

* Aided by a grant from the University of California Cancer Research Funds and by a grant from the Rockefeller Foundation.

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precipitation of the bacteria, followed by washing the precipitate to remove the TCA, and drying. Starch electrophoresis and sonic oscillation were performed as described previously[18].

The enzymes β-galactosidase[19], D-serine deaminase[11], and aspartate carbamyl transferase[18] were determined as described previously.

Hydrolysis and chromatography of DNA was accomplished by the method of WYATT AND COHEN[20].

Chemicals were obtained from commercial sources except for DL-7-azatryptophan and 7-azaindole[5] which were gifts from Dr. M. M. ROBISON, tryptazan[4] from Dr. H. R. SNYDER, DL-6-F-tryptophan[21] from Dr. F. LIPPMANN, other tryptophan analogs from Dr. K. PFISTER, 6-azauracil[22] from Dr. A. D. WELCH, and ε-methyl-lysine[23] from Dr. A. D. MCLAREN. Chloramphenicol (Chloromycetin) was a gift from Parke, Davis, and Co. Methyl-β-D-galactoside was synthesized by Mr. GEORGE RUSHIZKY, and homoarginine by Mr. GEORGE HUBERT.

RESULTS

Substitutions by analogs of amino acids

A variety of compounds were tested in order to determine whether they were able to substitute for an amino acid. The criterion used was to determine whether the analog would permit turbidity increase and protein synthesis by a mutant which normally required the amino acid for growth. Of ten tryptophan analogs tested, seven did not permit much "growth" of mutant 19-2 (Table I). Three compounds [DL-7-azatryptophan (azatryptophan), DL-tryptazan, and DL-6-F-tryptophan] did support

TABLE I

EFFECTS OF TRYPTOPHAN ANALOGS ON GROWTH OF *E. coli* MUTANT 19-2

Compound	*μg/ml*	*% Increase*	
		Protein	*Turbidity*
None	—	6	6
L-Tryptophan	1	140	134
DL-7-Azatryptophan	2	75	90
DL-Tryptazan	2	71	87
DL-6-F-tryptophan	2	55*	65*
DL-5-Methyl-tryptophan	2	19	41
7-azaindole	1	12	27
Indole	1	10	18
DL-6-Methyl tryptophan	2	3	21
DL-α-Methyl-tryptophan	2	1	21
DL-α-Methyl-homotryptophan	2	9	16

E. coli mutant 19-2 in exponential growth was washed to remove tryptophan, and resuspended in salts–glycerol medium. Compounds as given above were added to aliquots and the cultures were incubated with aeration at 37° C. Samples were taken for protein determinations at 98 minutes and for turbidity readings at 110 minutes. Very similar results were obtained with 5 times the above concentrations of analogs.

* By interpolation of times.

growth, protein synthesis, and RNA formation for about one generation. Of the analogs of other amino acids tested, ethionine was able to substitute for methionine, as in animals[24], and permitted doubling of protein in 105 minutes. The following compounds were not effective in substituting for the amino acid listed after them in parentheses: ε-methyl-lysine (lysine), ornithine (lysine), homoarginine (arginine), canavanine (arginine), homoleucine (leucine).

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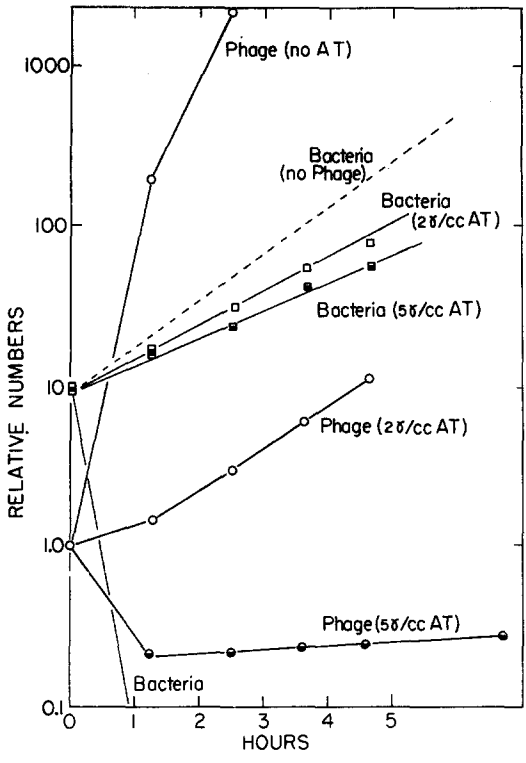

Fig. 1. Effect of azatryptophan on bacterial growth and reproduction of T2 phage. *E. coli* B grown on low phosphate medium were infected with T2 phage (multiplicity 0.1) and then aliquots were diluted into media containing 0, 2, or 5 μg/ml azatryptophan. Samples taken at intervals were diluted into media containing 10 μg/ml tryptophan and were plated at once for infective centers (plotted as infective centers × 10^{-7}), and after 60 minutes to obtain the burst size, which was obtained by dividing the number of plaques found after 60 minutes by the number found at the beginning of the 60 minute period.

Effects of azatryptophan on bacteria

Growth of *E. coli*, strain B exposed to 30 μg/ml azatryptophan was not inhibited for an hour. Five μg/ml of the analog permitted growth for at least 5 hours at about 60% the rate of the control. The viable count was affected similarly to turbidity: 20 μg/ml of the analog permitted a 3.5-fold increase in colony formers in 3 hours, and 5 μg/ml allowed a gradual exponential increase in viable counts for several hours (Fig. 1). The analog (20 μg/ml) allowed *E. coli*, strain B to form RNA and protein at nearly the normal rate and 80% as much DNA in 1 hour as in the control culture.

Azatryptophan permitted a rapid doubling of protein and RNA in the mutant 19-2[1]; it also permitted DNA synthesis. No loss of viability was observed in several generation times. In contrast to 19-2, another tryptophan-requiring mutant, B/1,t in F medium[6] did not form protein or nucleic acids when supplied with the similar analog, tryptazan. Presumably not all strains of *E. coli* will show the same response to the analogs.

No mutagenic effects of azatryptophan were noted. Exposure of *E. coli* strain B to the analog for an hour or more resulted in less than 1 in 10^7 bacteria converted to streptomycin resistance; nor did tryptophan-independent mutants of 19-2 occur in observable numbers.

Azatryptophan was found to block the tryptophan biosynthetic pathway at a step prior to the formation of anthranilic acid. It was noted that anthranilic acid (measured by its fluorescence) accumulated in the salts–glycerol medium in which mutant 19-2 was suspended, but if tryptophan or azatryptophan was present,

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accumulation of anthranilic acid did not occur. This result is similar to that obtained with 4-methyl-tryptophan[25].

Effects on enzyme formation

Azatryptophan inhibited the appearance of a number of enzyme activities in *E. coli*, strain B or 19-2, grown on either minimal or low phosphate medium, in spite of the fact that protein synthesis was not immediately inhibited. No increased ability to oxidize glucose, lactate, glycerol, or melibiose occurred when mutant 19-2 previously adapted to these carbon sources was subsequently exposed to them in the presence of 10 μg/ml azatryptophan without added tryptophan. Also, with bacteria grown on glucose no adaptation to glycerol, melibiose, or galactose occurred during several hours in the presence of azatryptophan. These measurements were made by

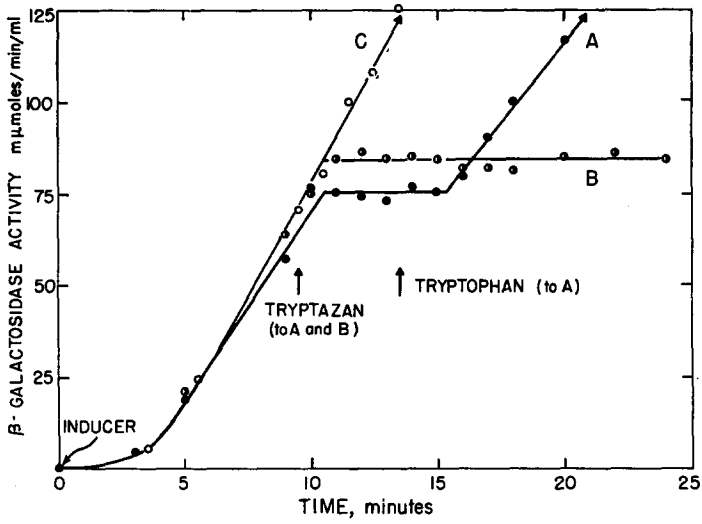

Fig. 2. Kinetics of β-galactosidase inhibition. *E. coli* strain ML was grown on glycerol-salts medium to a density of $5 \cdot 10^8$ bacteria per ml. The experiment was started by addition of 200 μg/ml methyl-β-D-galactoside to each of three cultures. Thirty μg/ml tryptazan was added to cultures A and B at 9.5 minutes, and 100 μg/ml of tryptophan was added to A and C at 13.5 minutes. At intervals 0.4 ml aliquots were removed from each culture into tubes on ice containing 0.6 ml 20 μg/ml chloramphenicol and 0.06 ml toluene, and the contents of the tubes were assayed for β-galactosidase. The initial optical density at 660 mμ of the culture was 0.118 and the optical densities at 29 minutes were A = 0.156, B = 0.166, C = 0.171.

determination of oxygen uptake with the Warburg respirometer. Induced formation of β-galactosidase under the influence of lactose, melibiose, or methyl-β-D-galactoside was completely blocked by 10 μg/ml of azatryptophan.

Kinetics of inhibition and reversal by the analog tryptazan were determined. Even when the inhibitor was added to the culture after induction was well under way, formation of additional β-galactosidase ceased within 1.0 minute after the analog was added (Fig. 2). There was less than 1.8 minutes lag in synthesis of the enzyme upon reversal with tryptophan.

Syntheses of two other enzymes were only partly blocked by the analog. In determinations of aspartate carbamyl transferase advantage was taken of the fact that this enzyme is present at a low level when the bacteria are grown with excess

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TABLE II

EFFECT OF AZATRYPTOPHAN ON ASPARTATE CARBAMYL TRANSFERASE FORMATION AND ON OTHER SYNTHESES

E. coli	*Additions*	*μg/ml*	*Time (min)*	*ACTase*	*Protein*	*Turbidity*
6386	—	—	0	0	92	40
	—	—	60	187	158	67
	Azatryptophan	20	60	85	150	64
	5-Me-tryptophan	40	60	1	92	49
19-2	—	—	0	0	80	34
	Tryptophan	10	60	78	105	39
	Azatryptophan	20	60	68	104	39
	none	—	60	2	79	32

E. coli mutant 6386 (uracil-less) was grown on salts-glycerol medium plus 20 μg/ml uracil to $5 \cdot 10^8$ bacteria/ml. The bacteria were removed from the medium and resuspended in fresh medium with 20 μg/ml orotate substituted for uracil, and containing the additions listed, and also 1 mg/ml lactose. Samples for assay were taken at the start and after 60 minutes aeration. The second experiment was performed similarly but with mutant 19-2 (tryptophanless). The mutant was grown in medium containing 10 μg/ml tryptophan, and was resuspended in minimal medium plus 20 μg/ml 6-azauracil, lactose, and the additions listed. Aspartate carbamyl transferase (ACTase) is given as mμ moles product formed per minute per ml culture, protein as μg/ml, and turbidity in arbitrary units.

TABLE III

EFFECT OF AZATRYPTOPHAN ON β-GALACTOSIDASE AND D-SERINE DEAMINASE FORMATION

Addition	*μg/ml*	*β-galactosidase (Time in minutes)*				*D-serine deaminase (Time in minutes)*			
		3	*30*	*45*	*60*	*3*	*30*	*45*	*60*
—	—	1 3	6.6	14.7	20 1	0	7.4	13.2	18.6
Tryptophan	10	1.2	6.1	12.7	19.8	0	6.8	11.2	17.1
Azatryptophan	10	1.2	2.1	2.6	3.0	0	4.4	7.2	8.1

To exponentially growing *E. coli* strain B ($4 \cdot 10^8$ bacteria/ml) were added the compounds listed. Two minutes later 1 mg/ml lactose and 0 15 mg/ml D-serine were added to each culture as inducers. At intervals, shown in the Table, aliquots were removed and were assayed for the enzymes. Activities are given as mμ moles of substrate reacted per minute per ml of culture.

uracil whereas much larger amounts are present in bacteria grown in a medium in which the pyrimidine supply is limited[18]. Hence a large amount of enzyme formation takes place when the bacteria are transferred from the former to the latter medium. Formation of aspartate carbamyl transferase was far less strongly inhibited by azatryptophan (Table II) than was β-galactosidase. In contrast to 93% inhibition of β-galactosidase, D-serine deaminase formation was only 56% inhibited by 10 μg/ml of azatryptophan (Table III), and increasing the concentration to 40 μg/ml did not increase the inhibition of D-serine deaminase. Complete inhibition of formation of both D-serine deaminase and aspartate carbamyl transferase by 5-methyl-tryptophan, as well as partial inhibition by azatryptophan, and absence of formation by a tryptophan-deficient mutant deprived of the amino acid, shows that these enzymes have a tryptophan requirement for formation.

Assuming that azatryptophan substitutes completely for tryptophan, one explanation for these observations is that for those enzymes which show activity, the

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tryptophan analog is built into positions of small importance for catalytic activity, while those that do not increase in activity incorporate tryptophan analogs in the active part of the enzyme. Direct evidence for formation of such "enzymes" is not yet available. However, the hypothesis would appear more likely if it could be shown that the kinds of proteins made in the presence of the analog are like those normally

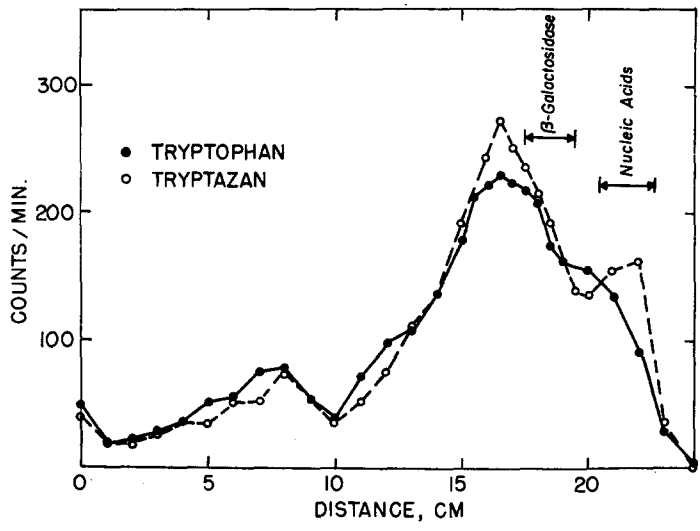

Fig. 3. Starch electrophoresis of proteins made in the presence of tryptophan on tryptazan. Two cultures of *E. coli* strain ML were grown in 30 ml glycerol-salts medium each, to a density of $5 \cdot 10^8$ bacteria per ml. To one was added 15 μg/ml of tryptophan and to the other 30 μg/ml tryptazan. One minute later 1 μg/ml L-leucine ^{14}C ($3 \cdot 10^5$ counts) was added to each. Fifteen minutes later the cultures were harvested by centrifugation, 10^{11} non-radioactive *E. coli* (grown in the presence of 1 mg/ml lactose) were added as carrier, the bacteria were disrupted by sonic oscillation, and the proteins were fractionated by precipitation twice with 50% $(NH_4)_2SO_4$. The precipitate (60% of the protein) was taken up in water, dialyzed to remove salt and fractionated on the starch electrophoresis apparatus. The starch was then cut into 0.5 cm blocks and every other sample was counted for ^{14}C.

made. Evidence of this sort is provided by ammonium sulfate fractionation and starch electrophoresis of cultures that took up ^{14}C-leucine in the presence and absence of tryptazan (Fig. 3). The experiment shows that the electrophoretic properties of proteins made in the presence of tryptazan and in its absence are very similar. The formation of enzymically active β-galactosidase was completely inhibited by the analog.

Effects on phage production

Azatryptophan inhibited the multiplication of the phage T2r in *E. coli*, strain B. The analog had a strong inhibitory effect at 2 μg/ml, and at 5 μg/ml less than 20% of the infective centers were maintained (Fig. 1). These results are in strong contrast to the relatively negligible effects on the bacteria. Thus a sort of chemotherapy was achieved—cultures of bacteria infected with low multiplicities of phage could continue to grow for many hours at about half the normal rate if azatryptophan was present. However, it was not possible to abolish the infection completely. Even high concentrations of the analog did not bring about disappearance of all the

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infective centers, and these went on to lyse the culture upon reversal of inhibition by addition of tryptophan.

The effect of a higher concentration (10 μg/ml) of azatryptophan on the number of infective centers and on the burst size is shown in Fig. 4. After 15 minutes the number of bacteria capable of producing at least one phage began to decrease, as

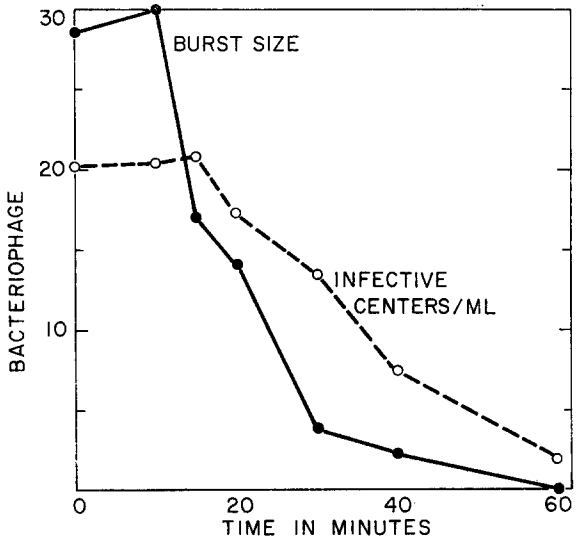

Fig. 4. Reversal of azatryptophan inhibition at various times. *E. coli* B were infected (multiplicity 1.5) with T2r in buffer and were suspended in medium containing 10 μg/ml azatryptophan. Aliquots were diluted into media containing 10 μg/ml tryptophan and were plated at once for infective centers (plotted as infective centers × 10^{-7}), and after 60 minutes to obtain the burst size, which was obtained by dividing the number of plaques found after 60 minutes by the number found at the beginning of the 60 minute period.

did the average number of phages released upon 1 hour further incubation in the absence of the analog. Some irreversible reactions, damaging to the infection process, took place, as with 5-methyl-tryptophan[26], but more rapidly.

Measurement of the number of intracellular phages ruled out the possibility that these effects were mainly the result of an inhibition of lysis; (although it was observed that lysis was affected because in the presence of the analog neither a turbidity decrement nor a solubilization of bacterial contents occurred). Bacteria infected at a multiplicity of 1.5 were incubated in the presence of azatryptophan (20 μg/ml), and intracellular phages were liberated by addition of $CHCl_3$[27]. Their number commenced to rise at about 30 minutes, from a background level of less than 0.01 per original infected bacterium, to 0.1 at 1 hour. The number of infectious centers dropped at 1 hour to 0.2 per original bacterium. Thus there was a definite production of new phage under these conditions. Similar results were obtained when cyanide was used to measure the number of intracellular phage.

No mutagenic effect of azatryptophan on T2-phages was found; *i.e.*, no appreciable number of new types of plaques was observed.

Azatryptophan led to abortive T1 infection: 20 μg/ml of the analog added at the time of infection (multiplicity 0.02) reduced the infective centers to half by 20 minutes. The control gave an average burst of 10 in two experiments.

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Early effects in phage-infected bacteria

Azatryptophan presumably inhibits phage production by preventing the formation of one or more biologically active proteins. At present, these proteins may be divided into three groups: those necessary for development of phage[6,7,8,26]; those, formed later, which are built into the phage structure[9,10,28]; and a lytic enzyme which

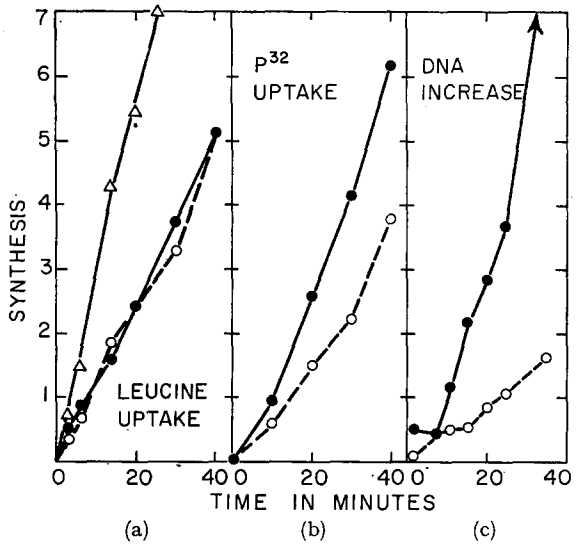

Fig. 5. Effects of azatryptophan on syntheses by infected bacteria. Three experiments were performed separately, but in a similar manner. *E. coli* ($2 \cdot 10^8$/ml) were infected with T2r phage (multiplicity 2 to 4), centrifuged, and resuspended in their growth medium, and at subsequent times aliquots were removed into 10% TCA, washed, and the precipitates were analyzed. The first graph shows uptake of 2-^{14}C-leucine by bacteria in low phosphate medium in the presence and absence of 20 μg/ml azatryptophan. The leucine was added with 1.2 μg/ml of non-radioactive carrier and was all taken up by the uninfected bacteria in about 25 minutes. The second graph shows results of an identical experiment except that $^{32}PO_4^{-3}$ was added instead of leucine. In the third experiment DNA was measured; it was performed in a medium containing amino acids, and the concentration of azatryptophan was 60 μg/ml. –●– Phage-infected, no azatryptophan; –○– Phage-infected, plus azatryptophan; –△– Uninfected, no azatryptophan.

appears to be formed[29]. To determine whether azatryptophan inhibits the action of the first group of proteins, one must learn whether certain events which normally occur in the first 10 minutes after infection, and before the formation of phage protein, can take place in the presence of the inhibitor.

In order to determine whether azatryptophan is inhibitory in the initial minutes of this period, one can see whether removal of inhibition at 10 minutes permits appearance of intracellular phages in an additional time which is less than the normal eclipse period of about 12 minutes. Such an experiment revealed that intracellular phages were not formed until 15 minutes after reversal of inhibition. Therefore, one or more processes vital to virus reproduction at a very early stage must not have occurred. There was a more gradual intracellular phage production after reversal of inhibition, so some processes, presumably defective, must have occurred. It was found that development of infection proceeds to the extent of about 3 minutes during the process of adsorption in buffer, before the inhibitor is added, so the process blocked occurred some time shortly after 3 minutes.

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The effects of azatryptophan on synthetic abilities of the infected bacteria was studied next. Little influence on over-all protein synthesis, as measured by the incorporation of 2-^{14}C-leucine into TCA-insoluble material, was observed (Fig. 5a). The infected bacteria took up radioactivity 65% as rapidly as the uninfected control culture, irrespective of the presence of the analog.

In contrast to uninfected bacteria, $^{32}PO_4^{-3}$ incorporation into the TCA-insoluble parts of infected *E. coli* was inhibited approximately 40% by the analog (Fig. 5b) in low phosphate medium. When DNA was measured colorimetrically, its synthesis in infected bacteria was inhibited 75% by azatryptophan, both in low phosphate

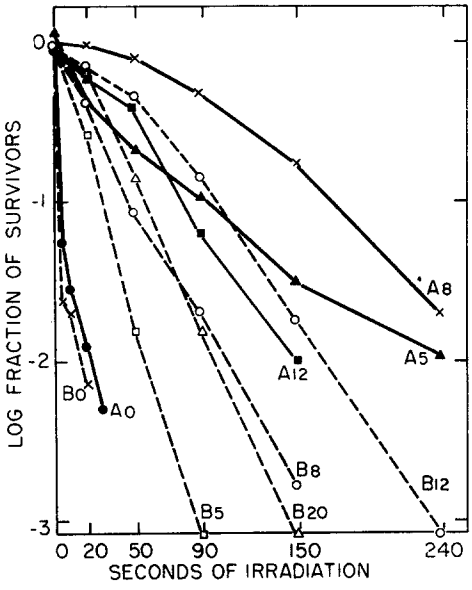

Fig. 6. Ultraviolet resistance of infective centers. *E. coli* strain B grown in low phosphate medium plus casein hydrolysate were infected in salts solution (multiplicity 0.02) and resuspended in used medium with and without 60 μg/ml azatryptophan. Aliquots were diluted into iced tubes of the medium salts solution at various times, and resistance to various periods of irradiation was determined. Platings and subsequent incubations were done in dim light to avoid photoreactivation. A, without azatryptophan; B, with azatryptophan.

medium and in this medium supplemented with amino acids (Fig. 5c). Determination of the base composition of the DNA present at 16 minutes in infected bacteria (grown with amino acids in the medium) revealed at least a 70% inhibition of 5-hydroxymethyl-cytosine synthesis when 60 μg/ml azatryptophan was added 5 minutes before infection. (Sixty μg/ml of azatryptophan is required in this supplemented medium for the same inhibition of phage production given by 10 μg/ml in low P medium.) This result indicates a strong inhibition of phage DNA synthesis. A requirement for protein synthesis for the subsequent synthesis of DNA has been shown previously, by a variety of techniques[26,6,7,8]; the present result suggests that the protein made in the presence of azatryptophan does not fulfill this function satisfactorily.

The greatly increased resistance, by about 12 minutes, of the infected bacteria to inactivation by ultraviolet light (UV)[13] has been used as a criterion for early progress of phage infection[7]. Experiments to determine UV resistance in the presence of the analog were performed both in the low phosphate medium and in the presence of casein hydrolysate—the latter being added to approach more closely the rich media used by other workers. When the bacteria were infected in the presence of 0.002*M* KCN (to prevent phage development before resuspension in the growth medium) there was only a small increase in UV resistance by 12 minutes if 20 μg/ml

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azatryptophan was present. Chloramphenicol, and tryptophan deficiency (in B/1,t) also were found to block the increase in UV resistance, in confirmation of the earlier reports[6,7,8]. But when a slight development occurred before addition of azatryptphan, the analog only moderately decreased the rate at which a high UV resistance was attained (Fig. 6). Apparently azatryptophan strongly inhibits only the very first minutes of development of UV resistance.

Effects on phage-structural proteins

It may now be asked whether the analog has any effects on the proteins which are an integral part of the phage structure. When bacteria were exposed to azatryptophan 15 minutes after infection (one intracellular phage per bacterium), the number of intracellular phages increased only slightly subsequently, so that by 40 minutes there were only 10 phages per bacterium as compared to a burst of 50 in the control culture. Therefore, the analog interfered strongly with some process required for completion of the phage, and also with other processes required at an earlier stage (as shown previously).

Bacteria infected with T2 in the presence of azatryptophan eventually lysed slowly, but produced few viable phages. Instead they released material with properties to be expected of incomplete or imperfect phages (Table IV). The separation method used to obtain this material was the same as that used for the purification of T2, and therefore the material must have been similar in size to the phage, assuming a similar density. The yield, as measured by optical density at 260 mμ, was as much as half of that of the control in some cases but was quite variable. Viability measurements revealed very few infective phages. Azatryptophan thus appeared to permit formation in infected bacteria of material similar in size to phage, and in considerable amount, but non-infective. This result recalls the effect of proflavin in permitting the formation of incomplete or unstable phage precursors[28], although the mechanisms must be quite different.

Properties of these "imperfect phages" were next investigated with the aim of

TABLE IV

PHAGE PRODUCTION IN THE PRESENCE OF AZATRYPTOPHAN

Azatryptophan			*Phage* $\times 10^{-7}$ *per ml original culture*			
μg/ml	*Added at* min	*Collected at* min	*Total phage* +AT	*Total phage* −AT	*Viable phage* +AT	*Viable phage* −AT
20	−5	90	53	250	0.5	370
10	+1	50	100	270	1.0	290
20	1	50	340	490	0.7	360
20	5	50	320	490	17.0	360
10	5	100	145	700	2.5	280
20	5	90	100	640	0.6	660
40	15	160	270	530	5.0	120

Each row represents a different experiment. In each, *E. coli* strain B in the exponential phase of growth (about $3 \cdot 10^8$ bacteria/ml) in low phosphate medium were infected at a multiplicity of 1 to 2, in the presence or absence of azatryptophan, added at the times and concentrations given above. The phage were collected by differential centrifugation at the times shown, and the resuspended samples were assayed for "Total Phage" by optical density at 260 mμ (10^{11} phage/ml = optical density of unity) and for "Viable Phage" by plating.

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discovering the type of lesion which made them non-infective. Electron microscopy of metal-shadowed specimens was used to obtain information regarding their structure. No intact T2 phages could be seen. Pictures were also made of bacteria infected 5 minutes after addition of azatryptophan to the culture, or 10 minutes before addition of tryptazan, and harvested shortly before the expected release of phage. Those bacteria when dried released their contents on the specimen film but no phages were seen. Control cultures liberated numerous phages under these conditions, so it is concluded that azatryptophan did not permit any nearly complete phages to be made.

The ultracentrifuge was used to gain an idea of the size of imperfect phages. No boundary which sedimented like T2 was observed with UV optics; instead slowly moving material (sedimentation coefficient = 39 S) was seen. These data show that the imperfect phages must be quite unstable, although they were originally readily sedimentable once during the process of purification.

The imperfect phages contained large amounts of nucleic acid, as shown by the high ratio, similar to T2, of optical densities at 260 and 280 mμ (1.30 to 1.40). This nucleic acid consisted largely of T2-type DNA, as shown by the base composition, and especially by the presence of 5-hydroxymethyl-cytosine (Table V).

TABLE V

BASE RATIOS OF "PHAGE" MADE IN THE PRESENCE AND ABSENCE OF AZATRYPTOPHAN

	Base ratios of preparations	
	+AT	*−AT*
Guanine	0.15	0.15
Adenine	0.32	0.30
OH-Me-Cytosine	0.14	0.17
Thymine	0.40	0.39

E. coli strain B was infected with T2 phage, in the absence of azatryptophan, and with the analog (10 μg/ml) added at 5 minutes. The purified "phage" were hydrolyzed and chromatographed to determine their base composition. The material formed in the presence of azatryptophan had a ratio of viable phage to "total phage" of 0.06.

Protein was also found in these preparations, as demonstrated by the Folin method (4.3 μg protein/μg DNA) and by the presence of incorporated radioactive leucine, which had been added to the medium at the time of addition of azatryptophan. These results show that considerable non-phage protein (or phage that had lost its DNA) was present as a contaminant, since the ratio of protein to DNA in phage is about 1; and it was not possible to determine how much phage-structural protein was present. However, the protein did seem able to hold the DNA together in a sedimentable form. Hydrolysis and chromatography revealed about 0.5% azatryptophan, approximately the anticipated amount if the analog replaced tryptophan in the phage, in the amino acids of the phage preparations made in the presence of azatryptophan. This poses the question of the nature of this protein.

The protein apparently does not completely surround the DNA, as is shown by the observation that the DNA is susceptible to the action of deoxyribonuclease (DNase). When a sample (optical density 0.2) was incubated for 20 minutes at 37° C with 40 μg/ml of pancreatic DNase in the presence of 0.02 *M* $MgSO_4$, 60% of the optically dense material became soluble in 7% $HClO_4$ (with 1% serum albumin added

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as a co-precipitant). For controls, it was found that 50 to 60% of a sample of thymus DNA became soluble, and only 10% or less of a T2 preparation went into solution under similar treatment. DNase did not destroy either the small infectious activity of the imperfect phage preparation, or that of the control T2 preparation. These results showed that the DNA was not protected by its protein coat, unlike the DNA of T2, and also confirmed the conclusion that much of the optically dense material was DNA.

The above results suggest that a major defect in phages produced in the presence of azatryptophan lies in the protein portion of the phage. Certain properties of phage proteins, other than ability to surround the DNA, are known[9, 10, 28]. These include ability to kill bacteria, to adsorb to bacteria, and to combine with anti-phage serum. These properties of imperfect phages were tested and all were possessed to only a small extent (less than 20%) by the imperfect phages, as compared with T2.

The inability of imperfect phages to kill *E. coli* is shown in Table VI. An 8-fold

TABLE VI

PURIFIED LYSATES: KILLING AND ^{32}P UPTAKE

	Total phage added/ml $\times 10^{-7}$	*Viable phage found/ml* $\times 10^{-7}$	*Bacteria found/ml* $\times 10^{-7}$	% ^{32}P *in supernatant*
Control	25	16	69	52
	50	20	51	48
	500	61	1.6	46
AT	25	0.2	88	84
	50	0.4	84	107
	500	3.7	76	98

The preparations were made by infection of *E. coli*, strain B with T2r at a multiplicity of 1.0 in buffer, adsorption for 5 minutes, and resuspension in fresh medium containing $^{32}PO_4^{-3}$. After 5 minutes 10 μg/ml of azatryptophan was added to half of the infected culture (AT), and not to the other half (control). At 135 minutes the bacterial debris was removed by centrifugation at low and then at high speed (see METHODS). The phage preparations were then dialyzed for 15 hours at 0° against adsorption buffer.

To test for killing and ^{32}P adsorption, various amounts of the preparations, measured by optical density at 260 mμ, were mixed with $66 \cdot 10^7$ *E. coli* per ml in broth and incubated at 37° for 10 minutes. The samples were plated for viable bacteria and for phage, and the fraction of ^{32}P not removed with the bacteria by sedimentation was determined.

multiplicity (measured by optical density) of the phage preparation made in the absence of azatryptophan killed 98% of the bacteria while the same amount of the preparation made in the presence of the analog killed a negligible number since growth permitted an increase in the number of bacteria by 14%. Similar results were obtained with a preparation in which the azatryptophan had been added 8 minutes after the phage.

Table VI also shows that the ^{32}P of the imperfect phages is not taken up by the bacteria. By contrast, half of the ^{32}P of the control was taken up in 10 minutes. Similar experiments were performed with preparations labeled with ^{14}C-leucine. In every case, two or three times as much radioactivity was absorbed by the bacteria from the control compared to the sample prepared in the presence of azatryptophan. However, the high background readings made the results of only qualitative significance (Table VII).

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TABLE VII

LYSATES: PROTEIN ADSORPTION AND ANTISERUM PRECIPITATION

	^{14}C-counts/min/ml			
Sample	*Total*	*TCA-precipitable*	*Adsorbed by E. coli*	*Precipitated by antiserum*
Control	766	308	174	113
AT	640	311	114	26
Initial	833	4	93	0

The preparations were made by adsorption of phage T2r (multiplicity of 2.0) to *E. coli*, centrifugation and resuspension of 1/3 into fresh medium containing 1.2 μg/ml of ^{14}C-leucine (3500 counts/μg) and harvested after 30 minutes (control); and 2/3 into fresh medium to which 20 μg/ml of azatryptophan had also been added. One half of this latter culture was harvested after 3 minutes (initial) and the other half after 50 minutes (AT). Counts were made for total ^{14}C, and ^{14}C precipitable by 10% TCA.

For determination of adsorption of ^{14}C to *E. coli*, portions of the preparations were briefly centrifuged to remove intact bacteria, and incubated at 45° for 10 minutes with a surplus of *E. coli* previously irradiated strongly with ultraviolet light (to reduce uptake of free leucine). The bacteria were centrifuged, washed once with cold 0.85% NaCl, and were counted for adsorbed ^{14}C.

For determination of precipitation by antiphage serum, the preparations were disrupted in the sonic oscillator and then any free bacteria were removed by centrifugation. An excess of anti-T2 serum and $3 \cdot 10^{12}$ carrier T2 phages were added. After 2.5 hours at 25° and 15 hours at 0°, the mixtures were centrifuged, the precipitates were washed, and were counted for ^{14}C.

The imperfect phages showed little tendency to be precipitated by anti-phage serum (Table VII). About 20% as much ^{14}C was brought down from crude preparations of infected bacteria exposed to azatryptophan as by the controls. Also, the former preparation did not possess the ability to neutralize anti-phage serum. To demonstrate this, a crude lysate of T2-infected *E. coli* was inactivated with UV and then was mixed with an equivalent amount of anti-T2 serum, and after 3 hours at 45° the absence of residual antibody was determined by addition of viable phages and plating after 4 more hours. By contrast, a lysate prepared in the same way, but with azatryptophan added 8 minutes after infection did not show appreciable combining ability. From these tests it is concluded that the protein associated with the imperfect phages has only the property of permitting its sedimentation with phage through one cycle of purification. Other characteristics of phage protein are absent.

DISCUSSION

The results presented are most readily interpreted to mean that incorporation of azatryptophan into an enzyme usually results in an inactive protein. That is, a tryptophan moiety somewhere in the protein is essential for activity and the analog is similar enough to substitute for it but not similar enough for catalytic function. However, other explanations are plausible. For example, in the absence of tryptophan the protein molecule analogous to the enzyme may not be formed because azatryptophan may not possess sufficient similarity to tryptophan to permit the combination of the amino acids into this particular protein (in contrast to the majority of proteins). Or possibly, azatryptophan may be joined by a peptide bond to one of its neighbors but not to the other with the result that breaks exist in the peptide bond sequence. To distinguish these possibilities, inactive proteins corresponding to the enzymes will have to be isolated, and studies of their composition, end groups, immunology and physical chemistry will be necessary.

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The observation that aspartate carbamyl transferase is synthesized readily in the presence of azatryptophan may mean that tryptophan is not in a position vital for catalytic action of this enzyme, although tryptophan (or a close analog) appeared to be essential, directly or indirectly, for formation of the enzyme. Further studies with a purified preparation of the enzyme will be required to determine whether or not the enzyme contains tryptophan.

The incomplete inhibition of the appearance of the above enzyme and of D-serine deaminase may indicate that altered enzymes can be created with limited catalytic activities; *i.e.*, enzyme activity may not be an all-or-none property of a protein but might vary in degree. Before such a conclusion can be made, the various considerations above must be investigated, and also the possibility that traces of tryptophan provide for the synthesis of some enzymes.

Phage formation showed a requirement for tryptophan not satisfied by azatryptophan, both in early and in late stages of development. The inhibition of phage development after the first few minutes was accompanied by a strong but not complete inhibition of DNA synthesis (presumably owing to inadequately active enzymes). By contrast, the appearance of UV-resistance was only slightly inhibited indicating that some of the processes of phage development were taking place. Production of a full complement of new DNA therefore is not required to bring about increased UV-resistance.

Inhibition of phage production by azatryptophan during the later stages of development is connected with formation of inadequate structural proteins of the phage. Isolation of material in some ways resembling phages permitted study of its properties. The protein of this material contained azatryptophan[1] and was sedimented with phage-like DNA through one cycle of centrifugation, but otherwise the protein had none of the properties of phage. It seems likely that the effect of azatryptophan is not simply to substitute for tryptophan and for this reason alone to inactive the phage, but to cause extensive disorganization in the entire process of phage protein synthesis.

A point of special interest is the much greater lethal effect of low concentrations of azatryptophan on replicating phage than on bacteria. This "chemotherapeutic effect" might be explained by postulating that since bacteria possess a large number of each kind of protein molecule, some of each sort may be active by consequence of using tryptophan rather than azatryptophan in their synthesis (at low concentrations of the latter), and these active enzymes may carry on the processes of growth in spite of the inactive molecules. By contrast, phages may have only one or a few of each sort of active molecules, so that the probability of achieving an inactive "phage" by elimination of all representatives of any one kind of molecule would be great. In agreement with this argument is the observation that a few active phages are always formed at any concentration of azatryptophan; these would be the rare ones that contain tryptophan at all essential positions.

ACKNOWLEDGEMENTS

We are indebted to Mr. MICHAEL ROSSMAN for preliminary testing of various analogs, to Miss ROSE LITMAN for chromatography of the DNA samples, to Mr. LEWIS DAVIS for taking the electron micrographs, to Dr. D. HOARD for a sample of DNA, to Miss

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E. McFALL for antiphage serum, to Drs. ROBISON, SNYDER, LIPMANN, PFISTER, WELCH AND McLAREN for gifts of analogs, and to Dr. DAVIS AND NOVICK for mutants.

SUMMARY

The influence of a number of amino acid analogs on the ability of *Escherichia coli* to form proteins was investigated. The analog of tryptophan, 7-azatryptophan, is incorporated into proteins and permits synthesis of proteins and nucleic acids, but the majority of enzymes, and also bacteriophages T1 and T2, did not appear in active forms. Two enzymes, aspartate carbamyl transferase and D-serine deaminase, were formed in about half the maximal amounts in the presence of azatryptophan, but not when the tryptophan supply was limited by various other means. Kinetics of inhibition showed that under the experimental conditions 1 and 1.8 minutes were required to establish and reverse the inhibition of β-galactosidase formation, respectively.

Phage formation was much more strongly inhibited by the analog than was bacterial growth, thereby providing a kind of chemotherapy. Inhibition of phage development was observed in both the early and late periods after infection. When added a few minutes after the time of infection, azatryptophan inhibited the formation of phage DNA much more strongly than the development of resistance to ultraviolet light; this suggests that the former is not necessary for the latter phenomenon. Protein made in the presence of azatryptophan and isolated in inactive, centrifugable particles had none of the properties of phage protein, but was associated with DNA of the phage type. The analog acts so as to cause major imperfections in the phage protein rather than by simply being incorporated in place of tryptophan.

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Received July 30th, 1957