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Protein Biosynthesis in *Escherichia coli*

PURIFICATION AND CHARACTERISTICS OF A MUTANT G FACTOR

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SUMMARY

Five mutant strains of *Escherichia coli* were selected for resistance to the steroid antibiotic fusidic acid. The protein synthetic activity of the cell-free extracts of only one of these was resistant to the inhibitory effect *in vitro* of the antibiotic. G factor, one of the soluble proteins required for peptide chain elongation, was purified to virtual homogeneity from both sensitive parent and resistant mutant strains. Aside from their differing sensitivity to fusidic acid inhibition, the two G factors were indistinguishable by conventional immunological, chromatographic, and electrophoretic analyses. The two factors differed, however, in two other respects. The specific activity of the sensitive strain was approximately twice that of the resistant strain throughout purification. In addition, the Michaelis constant for the ribosomal dependent hydrolysis of GTP calculated for the sensitive factor was half that for the resistant. The study, therefore, characterizes this fusidic acid-resistant mutation as one affecting G factor and establishes a biochemical basis for further genetic studies. It also suggests that the active enzymatic site for the ribosome-dependent hydrolysis of GTP is associated with the soluble G fraction.

G factor, one of the soluble *Escherichia coli* proteins involved in peptide chain elongation, is required for extension of peptides beyond their two initial amino acids (1-3). This process, thought to involve transfer of peptidyl-tRNA from a recognition to a holding site on the ribosome, is generally referred to as translocation. Using an antibody to homogeneous G factor, we have shown that G factor comprises over 2% of the soluble protein of rapidly growing *E. coli*. This corresponds to a ratio of about 2 to 3 G factor molecules per ribosome in such cells (4). G factor is, therefore, one of the most predominant of the soluble proteins of *E. coli*, yet only one of several soluble protein factors required for initiation, elongation, and termination of the polypeptide chain (*cf.* review in Reference 5).

In view of the frequently observed genetic relationship among enzymes involved in a given metabolic pathway (6), it seemed reasonable to suppose that there is a similar, coordinately regulated relationship among many of the elements involved in protein biosynthesis. In order eventually to test this possibility we have sought mutants which affect the soluble protein synthetic elements. Indeed, one such temperature-sensitive mutant affecting G factor has already been reported (7). The finding by Tanaka, Kinoshita, and Masukawa (8) that the steroid antibiotic, fusidic acid, specifically inhibited the action of G factor raised the possibility that a mutation resistant to this antibiotic might specifically affect G factor. Earlier reports by Kinoshita, Kawano, and Tanaka (9), Tocchini-Valentini, di Girolamo, and Felicetti (10), and ourselves (11) suggest, in fact, that certain resistant mutants contain resistant G factor.

In order to establish a biochemical basis for future genetic studies, we wish to describe the preparation and the characteristics of a highly purified mutant G factor which we have obtained from one of several fusidic acid-resistant strains of *E. coli*.

MATERIALS AND METHODS

Polyuridylic acid was obtained from Miles Chemical Company; ^{14}C-phenylalanine was from New England Nuclear; ^{32}P-GTP was from International Chemical and Nuclear; *E. coli* B tRNA was from General Biochemicals; acrylamide and *N*,*N'*-methylenebisacrylamide were from Eastman; agarose was from Bausch and Lomb; microgranular DEAE-cellulose was from Whatman; DEAE-Sephadex was from Pharmacia. Fusidic acid was the generous gift of Dr. W. O. Godtfredsen of Leo Pharmaceutical Company, Ballerup, Denmark.

METHODS

Preparation of Ribosomes and Transfer Factors

All components were prepared from early log phase harvested cells. Ribosomes were washed seven times in 1 M NH_4Cl_2 according to Erbe, Nau, and Leder (12). Transfer factors T, containing Tu and Ts, and G were purified by ammonium sulfate fractionation and DEAE-Sephadex chromatography by a procedure modified from that of Lucas-Lenard and Lipmann (13) by Erbe *et al.* (12). Fusidic acid-resistant G factor was further purified by acid DEAE-cellulose chromatography as described by Leder, Skogerson, and Nau (14). This step provided resistant G factor which was homogeneous by the criterion of disc electrophoresis, and the ammonium sulfate elution and crystallization steps were not required. One G factor unit corresponds to 1 $\mu\mu$mole of phenylalanine polymerized per standard reaction

TABLE I

Specific activity of sensitive and resistant G factor during purification

Components of reaction mixtures and conditions of incubation are as given under "Methods."

Purification step	G factor specific activity
	units[a]/μg protein
Crude $10^5 \times g$	
Resistant	4.4
Sensitive	10.1
DEAE-Sephadex	
Resistant	29
Sensitive	48.5
DEAE-cellulose	
Resistant	236
Sensitive	348

[a] One unit corresponds to 1 μμmole of phenylalanine polymerized per standard reaction per 10 min.

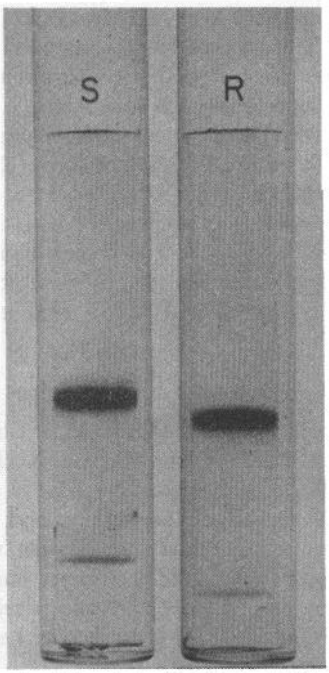

FIG. 1. Polyacrylamide gel electrophoresis of purified G factors obtained from the sensitive and resistant strains. In both cases 30 μg of protein were applied to the gel. The conditions of pH 8.3 electrophoresis are described in detail in Reference 8. The resistant factor is indicated by *R*; the sensitive enzyme is indicated by *S*. The *light line* close to the bottom of the gel is a dye marker.

per 10 min. ^{14}C-Phe-tRNA with 19 other ^{12}C-aminoacyl-tRNAs was prepared as described previously (12).

Assays

Polyphenylalanine Synthesis—Each 0.05-ml reaction mixture contained 0.05 M Tris-acetate (pH 7.2), 0.01 M $MgCl_2$, 0.05 M NH_4Cl_2, 0.25 A_{260} *E. coli* MRE 600 ribosomes, 8 μg of *E. coli* MRE 600 T factor, 0.029 μg of G factor (source as indicated), 6.3 μg of poly U, 11.5 μμmoles of ^{14}C-Phe-tRNA, and 0.01 M GTP. Incubation was at 37° for 10 min. Radioactivity incorporated into 10% $Cl_3CHCOOH$-precipitable material was measured on dried nitrocellulose filters in a scintillation counter.

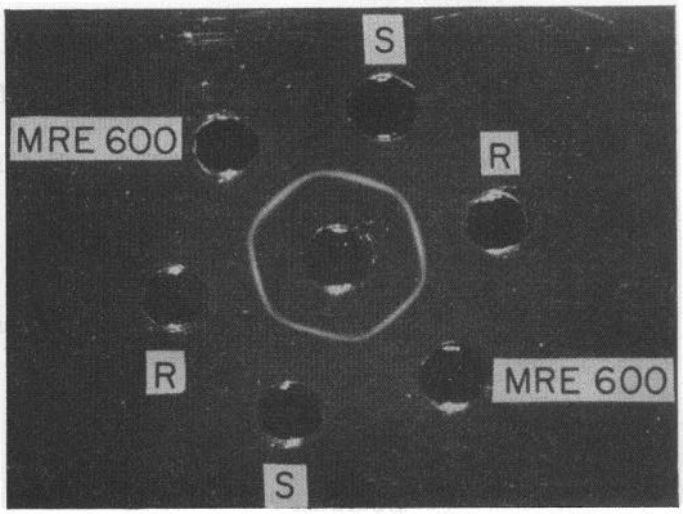

FIG. 2. Immunological cross-reactivity of sensitive and resistant G factor. Identification of the G factor by immunodiffusion technique according to Ouchterlony (17). The *central well* was filled with anti-G factor antiserum obtained against homogeneous G factor purified from *E. coli* MRE 600. The wells at the *top* and *bottom* were filled with G factor purified from the strain MRE 600 and the other wells as indicated by *S* and *R*, sensitive and resistant, respectively.

GTP Hydrolysis—Each 0.05-ml reaction mixture contained 0.05 M Tris-acetate (pH 7.2), 0.01 M $MgCl_2$, 0.05 M NH_4Cl_2, 1 A_{260} *E. coli* MRE-600 ribosomes, 0.5 μg of G factor (source as indicated), and 0.01 M γ-^{32}P-GTP (specific activity 4.9 μCi per μmole). Incubation was at 30° for 20 min. ^{32}P not absorbed by charcoal was measured as hydrolyzed GTP (12).

Protein Concentration—The method of Murphy and Kies (15) was used.

Analytical Procedures

Polyacrylamide Gel Electrophoresis—This was carried out at pH 8.3 as described by Davis (16). Thirty micrograms of sample were applied for each analysis.

Double Immunodiffusion—Antibody to homogeneous *E. coli* MRE-600 G factor was obtained as described by Leder, Skogerson, and Roufa (4). Double immunodiffusion was carried out as described by Ouchterlony (17).

Isolation and Preliminary Identification of Fusidic Acid-resistant Mutants—Fusidic acid-sensitive *E. coli* N156, an Hfr H strain, was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg, Mandel, and Chen (18). Colonies which grew rapidly on nutrient agar containing 0.2 mg per ml of fusidic acid were selected for further screening. The effect of fusidic acid on the incorporation of radioactive phenylalanine in crude cell-free extracts of each strain was measured according to Nirenberg (19). Only one of the five strains tested was resistant *in vitro*. This strain was the source of the resistant G factor used in these studies.

RESULTS

Purification and Characteristics of Factors—The activity of G factor, purified simultaneously from the sensitive parent and resistant mutant strains, was measured by assaying the factors' ability to support polyphenylalanine synthesis in otherwise saturated protein synthetic mixtures. The characteristically

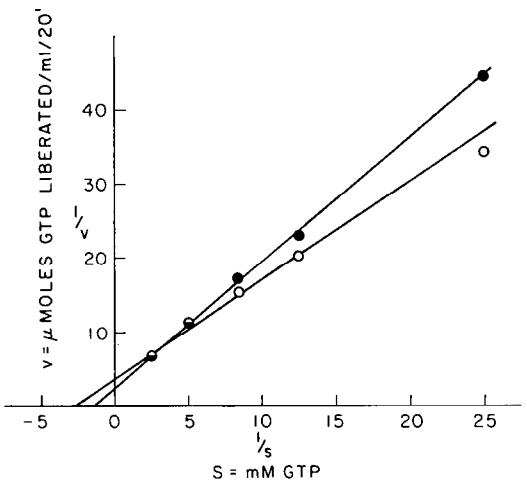

FIG. 3. Double reciprocal plot of the velocity of the ribosomal-dependent GTPase reaction as a function of GTP concentration in the presence of sensitive and resistant G factor. Experimental results are plotted according to Lineweaver and Burk (20). ○, sensitive G factor; ●, resistant G factor.

limited increase in specific activity of the sensitive and resistant factors during purification is shown in Table I. The purification resulted in 35- and 54-fold increases in the specific activity of the G factor isolated from the sensitive and resistant strains, respectively. This modest increase in specific activity, despite what is shown below to be a high degree of purification of both factors, reflects the large amount of each factor present in crude soluble extracts. Polyacrylamide disc electrophoretic analyses of the factors obtained after DEAE-cellulose chromatography are shown in Fig. 1. The factor derived from the resistant strain (Fig. 1, Sample *R*) ran as a single band having an R_F (calculated from the light green dye marker shown) identical with that of the parent sensitive factor (Fig. 1, Sample *S*). At this stage of purification, the sample derived from the parent strain was contaminated by very slight amounts of two slow moving components, barely visible in the reproduction shown in Fig. 1.

Although sensitive and resistant factors fractionated identically during purification, there was a consistent difference, shown in Table I, in their specific activities at each step of the purification. The factor derived from the resistant mutant was one-third to one-half less active than that derived from the sensitive strain. Nevertheless, both parent and mutant strains had approximately the same doubling time in enriched media.[1]

The factors were also detected during purification by their formation of a precipitin line on double immunodiffusion against an antibody to homogeneous *E. coli* MRE 600 G factor. A reaction of immunological identity between G factor isolated from *E. coli* MRE 600 and the parent and mutant strains used in these studies is shown in Fig. 2. The result suggests that, by this criterion, no immunological determinants have been lost by the mutant factor. The antibody assay, which is extremely convenient and independent of the factors' activity, indicated specific G factor antigen in chromatographic fractions which correspond to polyphenylalanine synthetic activity at each chromatographic step during purification.

Although immunologically, chromatographically, and electrophoretically indistinguishable, the consistently reduced specific

[1] Unpublished results.

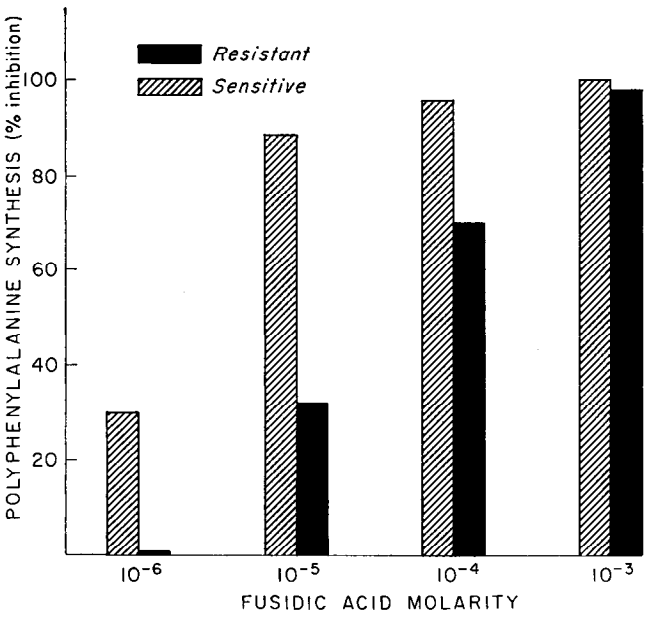

FIG. 4. Effect of fusidic acid on polyphenylalanine synthesis in the presence of sensitive and resistant G factor. Ribosomes from the *E. coli* MRE 600 were used. Components of reaction mixtures and conditions of incubation are described under "Methods." *Black bars*, percentage inhibition of resistant factor; *striped bars*, sensitive factor. Control values, in absence of fusidic acid, were approximately 4 μμmoles of phenylalanine polymerized per 10 min per standard reaction.

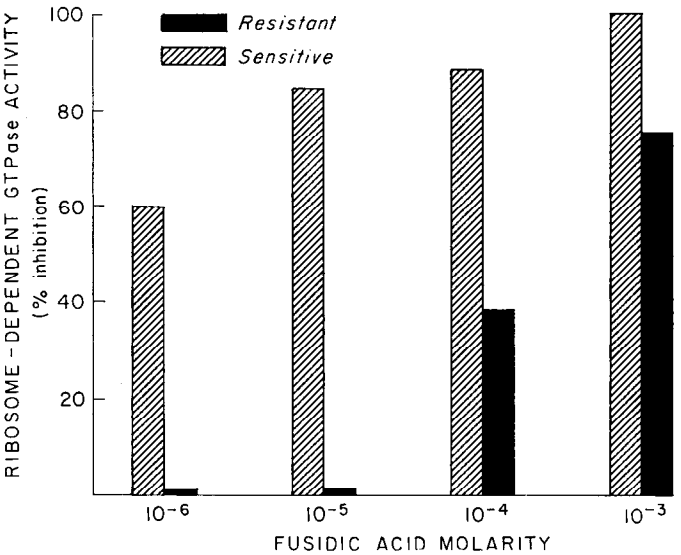

FIG. 5. Effect of fusidic acid on ribosome-dependent GTPase activity in the presence of sensitive and resistant G factor. Components of reaction mixtures and conditions of incubation are as described under "Methods." *Black bars*, percentage inhibition of resistant factor; *striped bars*, sensitive factor. Control values, in absence of fusidic acid, were between 40 and 80 μμmoles of GTP hydrolyzed per 20 min for each standard reaction indicated.

activity of the G factor isolated from resistant strain as compared to that of the parent strain suggests that the factors are not identical. This difference is further supported by kinetic studies of the ribosomal dependent GTPase activity associated with G factor. A double reciprocal plot of the velocity of the GTPase reaction as a function of GTP concentration in the presence of factors from both strains is shown in Fig. 3. The Michaelis con-

TABLE II

Additive effect of sensitive and resistant G factor on rate of polyphenylalanine synthesis in presence of fusidic acid

Components of reaction mixtures and conditions of incubation are as under "Methods." In addition, each reaction mixture contained 1×10^{-5} M fusidic acid and 2.6 units of sensitive and 1.7 units of resistant factor as indicated in the table.

Enzyme	^{14}C-Polyphenylalanine synthesis
	μμmoles/10 min
Sensitive	0.59
Resistant	1.46
Both	2.05

TABLE III

Fusidic acid inhibition of polyphenylalanine synthesis as function of G factor purity

Components of reaction mixtures and conditions of incubation are as given under "Methods." Fusidic acid concentration is indicated in the table. Percentage inhibition represents percentage of uninhibited control reaction (without fusidic acid) which was approximately 4 μμmoles of phenylalanine polymerized per 10 min per standard reaction mixture.

Purification step	Fusidic acid concentration	Factor	
		Resistant	Sensitive
	M	*% inhibition*	
Crude $10^5 \times g$	10^{-3}	50	86
DEAE-Sephadex	10^{-3}	80	100
DEAE-cellulose	10^{-3}	100	100
	10^{-4}	71	94
	10^{-5}	31	89

stant at 30° of the factor described from the resistant strain is twice that of the factor derived from the parent strain, 8×10^{-4} M and 4×10^{-4} M, respectively. Similarly, V_{max} for the GTPase reaction in the presence of the resistant factor is twice that of the sensitive factor, 4×10^{-4} and 2.5×10^{-4} M/20 min, respectively. These differences, although small, have been obtained several times, always in duplicate, and are quite reproducible.

Effect of Fusidic Acid on G Factor Derived from Sensitive and Resistant Strains—Preliminary studies on crude extracts derived from five separately isolated fusidic acid-resistant mutants yielded only one which retained the fusidic acid-resistant phenotype when tested in cell-free protein synthetic extracts. Obviously, mechanisms of drug resistance which do not directly involve the protein synthetic elements arise frequently. G factor isolated from the single strain which showed resistance *in vitro* retained this resistance throughout purification. The effects of fusidic acid on polyphenylalanine synthesis in the presence of equivalent amounts of highly purified G factor derived from parent and mutant strains are shown in Fig. 4. In this experiment, the other elements required for protein biosynthesis, ribosomes and T factor, were derived from *E. coli* MRE 600, a sensitive strain. The reactions containing G factor derived from the resistant strain were comparatively resistant to fusidic acid inhibition throughout a concentration range of 10^{-3} to 10^{-6} M fusidic acid. At a fusidic acid concentration of 10^{-4} M, for example, the resistant factor retained 30% of its activity, whereas the sensitive factor retained only 5% of its uninhibited activity.

TABLE IV

Effect of fusidic acid on ribosomal dependent GTPase and polyphenylalanine synthesis in presence of G factor or ribosomes from sensitive and resistant strains

Components of reaction mixtures and conditions of incubation are as given under "Methods." Concentration of fusidic acid was 1×10^{-5} M. Control values for polyphenylalanine synthesis were approximately 4 μμmoles of phenylalanine polymerized per 10 min per standard reaction; for ribosome-dependent GTPase, 40 to 80 μμmoles of GTP hydrolyzed per 20 min for each standard reaction indicated.

Source of added components	Activity	
	Ribosome-dependent GTPase	Polyphenylalanine synthesis
	% inhibition	
Experiment 1. Control ribosomes (MRE-600)		
Resistant G factor	2	32
Sensitive G factor	85	89
Experiment 2. Control G factor (MRE-600)		
Resistant ribosomes	59	41
Sensitive ribosomes	66	43

Similar results were obtained when the effect of fusidic acid upon the ribosomal dependent GTPase activity of G factor derived from sensitive and resistant strains was determined. In these experiments, shown in Fig. 5, ribosomes derived from *E. coli* MRE 600 (fusidic acid-sensitive) were used. Again, reaction mixtures containing G factor derived from the resistant strain were comparatively resistant to the inhibitory effect of fusidic acid concentrations varying from 10^{-3} to 10^{-6} M.

The possibility that the resistant factor, despite its apparent homogeneity, contained an activity which inactivated fusidic acid or that the sensitive factor contained a potentiating activity was tested in the experiment shown in Table II. Rate-limiting comparable amounts of G factor (factor units) were used separately and together to permit synthesis of polyphenylalanine. The concentration of fusidic acid used in this experiment was 10^{-4} M. This concentration of fusidic acid reduces the rate of polyphenylalanine synthesis to 60% of control values in the presence of the resistant G factor to 10% in the presence of the sensitive G factor. As can be seen in Table II, the effect of adding both resistant and sensitive G factors to a single reaction mixture is strictly (in this case, exactly) additive, indicating the absence of inactivating or potentiating activities in either G preparation.

The advantage of using highly purified G factor is shown by comparing the inhibitory effect of fusidic acid on sensitive and resistant G factors at several stages of purification. As can be seen in Table III, 10^{-3} M fusidic acid produces only a 50% inhibition in the rate of polyphenylalanine synthesis in the presence of crude supernatant. Inhibition is virtually complete, however, in the presence of an equivalent amount of highly purified sensitive or resistant G factor (DEAE-cellulose). The mechanism of this protection from inhibition is not yet known, but may account, at least in part, for the leaky or incompletely inhibitory effect that the antibiotic has on the growth of *E. coli*. It also indicates that small differences in sensitivity to fusidic acid may be difficult to detect in crude extracts.

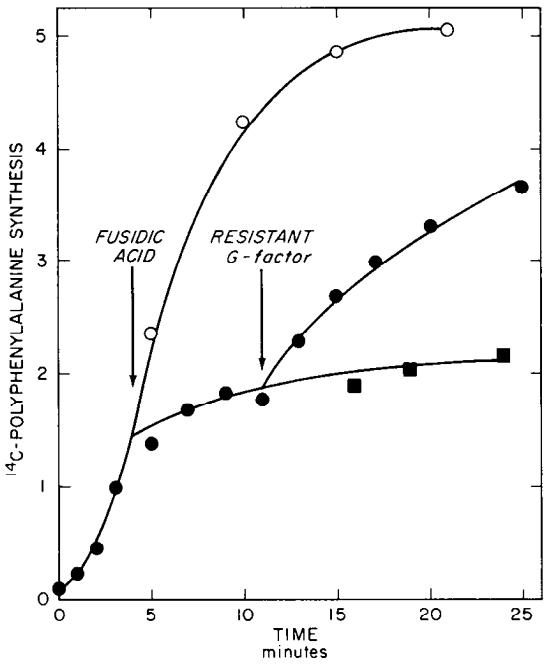

FIG. 6. Effect of resistant G factor on fusidic acid-inhibited polyphenylalanine synthesis. Components of reaction mixtures were as described under "Methods," except that a 2.0-ml reaction mixture contained: ribosomes, 24 A_{260}; poly U, 31.5 μg; T factor, 656 μg; ^{14}C-Phe-tRNA, 625 $\mu\mu$moles; and GTP, 2.5 μmoles. The reaction was initiated by the addition of 1.6 μg of sensitive G factor and a 0.5-ml aliquot was removed. To another 0.5-ml aliquot, fusidic acid, 10^{-4} M, was added at 5 min. To the remaining aliquot, fusidic acid, 10^{-4} M, was added at 5 min and resistant G factor, 0.46 μg, at 11 min. Incubation was at 30°. The reactions were followed by assaying 0.05-ml aliquots at the times indicated. ○——○, no addition; □——□, fusidic acid; ●——●, fusidic acid followed by resistant G factor. Experiments (not shown) were also carried out with additional sensitive G factor in place of the resistant factor.

In contrast to polypeptide chain elongation which involves a number of complex components, only ribosomes and G factor are required for the ribosome-dependent, uncoupled hydrolysis of GTP. As has been shown by Tanaka *et al.* (8) and confirmed above, this reaction is also inhibited by fusidic acid. That ribosomes derived from the resistant strain do not possess resistant phenotype is illustrated by the experiments shown in Table IV. Whereas in the presence of resistant G factor, GTP hydrolysis is virtually unaffected by 10^{-5} M fusidic acid, inhibition is 85% in the presence of sensitive G factor (Table IV, Experiment 1). In contrast, there is no significant difference in the effect of fusidic acid upon ribosomes derived from sensitive and resistant strains in the presence of control G factor derived from *E. coli* MRE-600 (Table IV, Experiment 2). Parallel results are shown in Table IV when polyphenylalanine synthesis is measured. With respect to both activities, the resistant phenotype depends upon the G factor used.

Reversibility of Fusidic Acid Inhibition—The inhibitory effect of fusidic acid on polyphenylalanine synthesis can be overcome by the addition of resistant G factor. This is illustrated by the experiment shown in Fig. 6. Addition of fusidic acid to the synthetic reaction mixture 4 min after its initiation results in an abrupt reduction in the rate of polyphenylalanine synthesis. Reversal of inhibition is brought about by the addition of highly purified resistant G factor at 11 min. The addition of an equivalent amount of sensitive G factor (not shown) failed to affect the rate of incorporation.

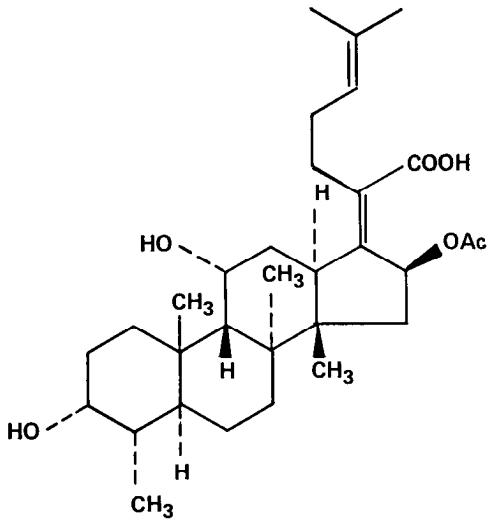

FIG. 7. Structural formula of fusidic acid (21)

DISCUSSION

Fusidic acid is a steroid antibiotic of known structure (21) (Fig. 7) which inhibits protein biosynthesis (22). Furthermore, it is able to inhibit, to a lesser extent, DNA and RNA synthesis as well (22). Its mode of action on protein biosynthesis has received far more attention and it is now fairly well understood. Tanaka *et al.* (8) have, in fact, shown that fusidic acid specifically inhibits the activity of the *E. coli* elongation factor G. Fusidic acid also inhibits the factor T-II (23), the mammalian analogue of factor G (24). Unfortunately, the chemical basis for the action of this steroid, as with so many antibiotics, remains unclear.

We have presented evidence indicating that homogeneous G factor obtained from a fusidic acid-resistant mutant of *E. coli* possesses the fusidic acid-resistant phenotype. Resistance is, therefore, due to a single mutational event affecting this protein. Although the resistant G factor could not be distinguished from the sensitive parent G factor by conventional immunogenetic, chromatography, or electrophoretic techniques, it differed from the wild type factor in two other important and probably related respects. The specific activity of the resistant factor was consistently less than that of the sensitive factor, and the Michaelis constant with respect to the ribosomal dependent GTPase activity for the resistant factor was twice that of the sensitive factor. One simple, but unfortunately not unique, explanation for these findings assumes that G factor is an enzyme containing an active site for GTP hydrolysis. The mutation which specifically affects the G factor and results in fusidic acid resistance may affect a binding or catalytic site on the factor itself. Alternatively, the mutation may affect the interaction between G factor and the ribosome, having an indirect effect upon an intrinsically ribosomal activity. These possibilities cannot, as yet, be distinguished. The observation that both GTP hydrolysis and peptide chain elongation are inhibited by fusidic acid (8) and that both functions are affected by what is presumably a single mutational event further suggest that the uncoupled ribosomal dependent hydrolysis of GTP is relevant to the mechanism of protein synthesis.

Perhaps the most important consequence of this study, however, is that it provides firm biochemical evidence for a mutant which should prove very useful in mapping the genetic locus corresponding to G factor. It is possible that it will map close to other protein synthetic elements. Further, if we presume that the protein synthetic factors are subject to some form of coordinate regulation, it is likely that a mutant G factor, reduced in efficiency, might be overproduced and that this overproduction might also affect other, related soluble factors. Our current studies are directed toward determining which of the known antibiotic markers affecting protein synthesis cotransduce with fusidic acid resistance.[2]

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[2] *Note Added in Proof*—In collaboration with Dr. Max Gottesman, we have found that fusidic acid resistance frequently, but not always, cotransduces with streptomycin resistance. This is in accord with the idea, noted above, that the protein synthetic elements might form a coordinately expressed and closely linked group of cistrons.

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