MODE OF ACTION OF CHLORAMPHENICOL

II. Inhibition of Bacterial D-Polypeptide Formation by an L-Stereoisomer of Chloramphenicol

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The antibiotic, chloramphenicol, has a selective inhibitory action on the synthesis of cellular proteins of susceptible microorganisms (Wisseman *et al.*, 1954; Gale and Folkes, 1953). While the drug fails to interfere with numerous cellular functions (Smith, 1953), some of them presumably prerequisites to protein biosynthesis (Wisseman *et al.*, 1953), the exact reaction which is directly affected by chloramphenicol remains unknown. Attempts to identify this cellular process and to determine the nature of the metabolic lesion induced by the drug are rendered difficult by the limitations of the present knowledge of the mechanism of protein formation.

Chloramphenicol is one of the four possible stereoisomers of 1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol (figure 1) (Rebstock *et al.*, 1949). Dramatic growth inhibiting properties towards microorganisms reside exclusively in the D(−)*threo* isomer which is the antibiotic while the three other isologues are largely devoid of antibiotic action (Controulis *et al.*, 1949).

It is this remarkable dependence of the growth inhibitory action on the stereochemical configuration of the drug molecule which has provided an approach, still indirect but most instructive, to the problem of circumscribing the site of action of chloramphenicol.

Bacterial cellular proteins are composed predominantly of amino acids of the L-series. Since only the D(−)*threo* isomer, i.e., chloramphenicol, inhibits the synthesis of such L-proteins, an antipodal relationship appears to exist between the steric configuration of the inhibiting drug and that of the proteins whose formation is inhibited. It seems logical to expect that an L-isologue of chloramphenicol might interfere with the formation of D-polypeptides by virtue of an analogous but converse relationship.

This, indeed, has been found to be the case. The present studies indicate that the formation of D(−)glutamyl polypeptide by *Bacillus subtilis* (Ivánovics and Bruckner, 1937; Bovarnick, 1942) is inhibited specifically by the L(+)*erythro* stereoisomer of chloramphenicol but *not* by the antibiotic itself. The same stereoisomer has little effect on the growth of the test organism while chloramphenicol completely suppresses its growth at a low concentration. The remaining two stereoisomers have no effect on either growth or polypeptide formation.

MATERIALS AND METHODS

Organism. Bacillus subtilis, strain 9945, was obtained from the American Type Culture Collection. Stock cultures were prepared using Sauton's medium agar slants, and 18 hour subcultures in nutrient broth from these original slants were used as inocula for the experiments on growth and polypeptide formation.

Experiments on growth and polypeptide formation. Two different types of experiments were carried out to study the effect of chloramphenicol and its stereoisomers on glutamyl polypeptide formation.

(1) *Determination of polypeptide and bacterial nitrogen in growing cultures.* In this first type of experiment the bacteria were grown at 37 C from a small inoculum in series of flasks, each containing 50 ml of Sauton's medium,[1] with or without added stereoisomers of chloramphenicol. At daily intervals over a period of six days

[1] Sauton's medium was prepared as follows: 4 g of L-glutamic acid, 2 g of citric acid, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4$, 50 mg of ferric ammonium citrate, and 16 ml of glycerol were dissolved in 1,000 ml of distilled water; the pH was adjusted to 7.4 with strong ammonia, and the medium was sterilized at 15 lb steam pressure for 20 minutes.

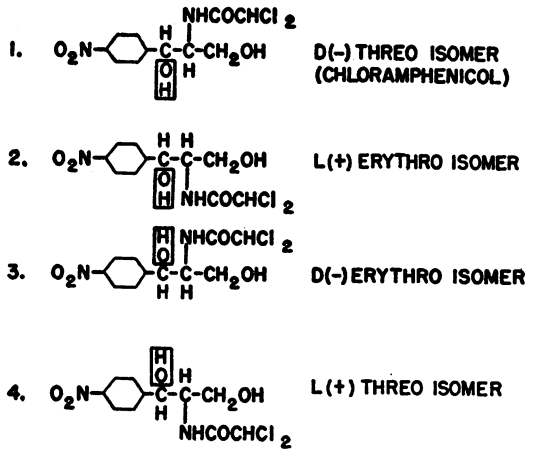

Figure 1. The four stereoisomers of 1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol.

following inoculation, one flask of each series was analyzed for polypeptide and bacterial nitrogen. Glutamyl polypeptide was determined as described by Bovarnick (1942). The cells were separated from their growth medium by centrifugation at 50,000 × G rather than by filtration through a Seitz filter as originally employed by Bovarnick. The sediments, which contained the pellicles formed during the growth of the organism, were hydrolyzed partly with 3 N hydrochloric acid for two hours in the autoclave at 15 lb steam pressure. Bacterial nitrogen was determined in sulfuric acid-hydrogen peroxide digested aliquots of these hydrolyzates, employing a modification of the nesslerization method described by Umbreit *et al.* (1951).

(2) *Determination of polypeptide elaborated by full grown cultures.* The type of experiment just described would not permit an examination of the action of chloramphenicol itself on polypeptide formation because growth of *B. subtilis* from the small inoculum is suppressed completely by low concentrations of the antibiotic. Therefore, a second type of experiment was designed in which the effects of chloramphenicol and its L(+)*erythro* isomer on the elaboration of polypeptide could be studied after the pellicles of *B. subtilis* were fully formed. Thus, the bacteria were grown first as a pellicle in stationary cultures at 37 C for four days in 500 ml of Sauton's medium. Specially constructed culture flasks were employed which had an outlet in the bottom and possessed a side arm, closed with a rubber vaccine bottle cap, originating near the bottom of the flask below the fluid level. After four days the bacteria had formed strong coherent pellicles. The medium then was drained off through the outlet in the bottom of the flasks and replaced aseptically with fresh medium. Three types of replacement media were employed: (1) Sauton's medium without additions, (2) Sauton's medium containing 50 μg per ml chloramphenicol, and (3) Sauton's medium containing 50 μg per ml L(+)*erythro* stereoisomer of chloramphenicol. If the replacement was done with care, the entire pellicles could be floated without fragmentation upon the surface of the new medium. Ten milliliter samples taken at daily intervals by means of a syringe and a needle through the side arms of the culture flasks were analyzed for polypeptide as described above.

Characterization of the polypeptide. The chemical nature of the polypeptide was studied in samples prepared by dialyzing the acid solutions of copper polyglutamate first against citrate buffer and then against distilled water as outlined by Bovarnick (1942). Hydrolysis of the polypeptide was carried out following the procedure of Housewright and Thorne (1950). A modification of the methods of Fowden (1951) and McFarren (1951) was used for the quantitative paper chromatography of the hydrolyzates. L-Glutamic acid was assayed manometrically with a preparation of L-glutamic acid decarboxylase from *Escherichia coli* (Umbreit and Gunsalus, 1945).

Sensitivity test. The bacteriostatic concentration of chloramphenicol and its L(+)*erythro* isomer against strain 9945 of *B. subtilis* was determined by incubating at 37 C for 24 hours serial dilutions of the drug or its isomer in inoculated Sauton's medium. The minimum bacteriostatic concentration as determined by visual observation of growth was 2 μg per ml of chloramphenicol and 125 μg per ml of the L(+)*erythro* stereoisomer.

The minimal concentration of the L(+)*erythro* isomer which would inhibit glutamyl polypeptide formation was determined, using the method of determining polypeptide in growing cultures which is described above. A dilution series of the isomer in flasks containing 50 ml of Sauton's medium, inoculated with the organism, was incubated at 37 C for four days and then analyzed for polypeptide. The minimum initial concentration which maintained inhibition over the four day experimental period was 33 μg per ml. It should be noted that suspensions of *B. subtilis*

will decompose readily considerable quantities of chloramphenicol within 24 to 48 hours (Smith and Worrel, 1950). It is reasonable to assume that in the present test experimental mixtures, containing lower concentrations of the isomer, might have escaped from the inhibitory action by virtue of an analogous decomposition. The actual inhibitory concentration of the L(+)-*erythro* isomer, therefore, should be less than the determined value of 33 μg per ml.

RESULTS

(1) *Inhibition of polypeptide formation in growing cultures.* The growing cultures of *B. subtilis*, strain 9945, which contained none of the isomers of chloramphenicol produced increasing amounts of polypeptide and of bacterial nitrogen throughout the six day experimental period following inoculation. The increase in the amount of each of these components followed a distinct course which is illustrated in figure 2. In this particular experiment, bacterial and polypeptide nitrogen values attained maxima of 6.0 and 12.0 mg, respectively, per 50 ml of culture fluid.

In contrast polypeptide formation was strongly inhibited in cultures containing 50 μg per ml of the L(+)*erythro* isomer of chloramphenicol while growth of the organism, as indicated by the increase in bacterial nitrogen, was not appreciably affected (see figure 2). In a 50 ml sample of culture the polypeptide never exceeded 0.5 mg of nitrogen while the bacterial nitrogen in the experiment illustrated, and others, attained 5.5 mg.

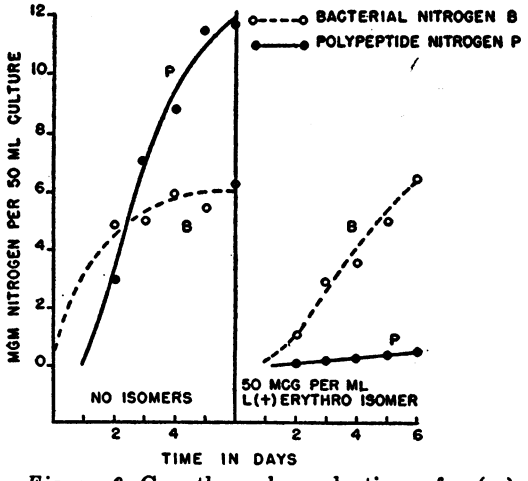

Figure 2. Growth and production of D(−) glutamyl polypeptide by *Bacillus subtilis* and inhibition of polypeptide formation by the L(+)-*erythro* stereoisomer of chloramphenicol.

One hundred μg per ml of the L(+)*threo* and 50 μg per ml of the D(−)*erythro* stereoisomers had no influence either on bacterial growth or on polypeptide formation.

(2) *Action of chloramphenicol and its L(+)-erythro isomer on polypeptide formation in full grown cultures.* Control cultures, in which four day old pellicles were floated on the surface of fresh Sauton's medium, continued to elaborate polypeptide into the medium over a period of an additional six days although the rate of polypeptide formation was somewhat lower than that observed in young growing cultures. The course of polypeptide formation in this kind of experiment is illustrated in figure 3.

Again, polypeptide formation was completely inhibited by the presence of 50 μg per ml of the L(+)*erythro* isomer of chloramphenicol in the medium after the nitrogen content of the polypeptide fraction had shown a slight rise during the first two days of the experiment.

In contrast, the D(−)*threo* isomer, i.e., chloramphenicol, failed to suppress the elaboration of polypeptide. When 50 μg per ml of the antibiotic

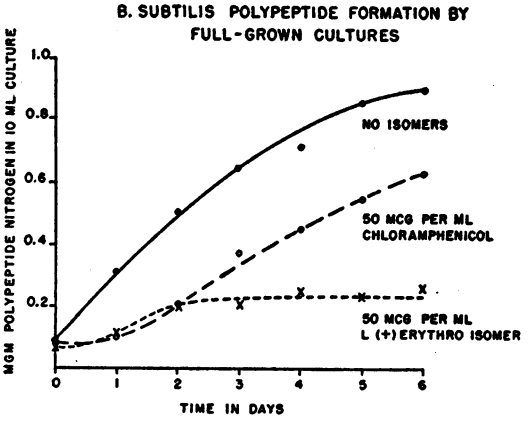

Figure 3. Production of D(−)glutamyl polypeptide by full grown pellicles of *Bacillus subtilis*. The presence of 50 μg per ml of chloramphenicol causes a delay after which polypeptide is formed at a rate similar to that of the control which does not contain the antibiotic; 50 μg per ml of the L(+)*erythro* isomer of chloramphenicol completely inhibit polypeptide formation after a similar delay.

were added to the medium, polypeptide formation was initially delayed but eventually attained a rate of formation similar to that exhibited by the control culture which did not contain the drug (see figure 3).

Characterization of the polypeptide. A qualitative chromatographic analysis of the hydrolyzate of the purified polypeptide revealed glutamic acid as the sole ninhydrin positive constituent. Quantitative ninhydrin tests on the hydrolyzates before chromatography as well as on the glutamic acid spots after the development of the chromatograms gave identical results which in turn were in good agreement with the value for total nitrogen in the hydrolyzates.

The crude precipitate of copper polyglutamate appears to be a mixture of substances; Bovarnick's analytical procedure (Bovarnick, 1942) requires the dissolution of this material in 1.5 N HCl. During this process a nonspecific protein fraction remained undissolved. Acid hydrolyzates of this "nonspecific protein" contained at least five different ninhydrin positive components on paper chromatograms, among them glutamic acid.

Upon refrigeration overnight a white precipitate frequently separated from the solution of copper polyglutamate in 1.5 N HCl. Hydrolyzates of this material contained chromatographically pure glutamic acid as the sole ninhydrin positive component. L-Glutamic acid decarboxylase assays as well as quantitative chromatography of the glutamic acid from both fractions before and after decarboxylase action indicated that most of the glutamic acid was the "natural" L(+) isomer.

These studies were carried out in order to control the analytical procedures for glutamyl polypeptide, to verify its nature, and to demonstrate the remarkable purity of the analyzed substance.

Attempts to demonstrate a capsule of B. subtilis. While D(−) glutamyl polypeptide is elaborated into the culture medium by *B. subtilis*, a serologically identical polypeptide, composed of D(−) glutamic acid residues, constitutes the capsular material of *Bacillus anthracis* (Ivánovics and Bruckner, 1937). Encapsulated variants of *B. subtilis*, however, are known to occur, and experimentally induced interchanges between variants of the organism have been described (Soule, 1928). The possibility had to be considered, therefore, that the suppression of the elaboration of soluble polypeptide by the L(+)-*erythro* stereoisomer might have been due to its retention as a polypeptide capsule induced by the isomer. Smears of *B. subtilis* from cultures elaborating polypeptide as well as from cultures in which polypeptide formation was inhibited by the presence of the isomer were stained with safranin-india ink and with the capsule staining methods of McFadyean and Hiss (McEwen, 1950). Microscopic examination did not reveal any significant differences among the organisms from both types of culture.

DISCUSSION

The prediction that an L-stereoisomer of chloramphenicol might interfere with the formation of polypeptides, composed of D-amino acids, has been proved correct by the present findings: the L(+)*erythro* isomer of chloramphenicol inhibits the synthesis of D(−) glutamyl polypeptide by *B. subtilis*. The action of the D(−)*threo* stereoisomer, i.e., chloramphenicol, on the other hand, is to exert a selective inhibition of the synthesis of proteins, composed of L-amino acids, in susceptible microorganisms (Wisseman *et al.*, 1954). It is the thesis of this discussion that both effects probably are brought about by analogous mechanisms of action which are characterized by the antipodal steric configurations of the reactants and inhibitors involved.

Inhibition of D(−) glutamyl polypeptide synthesis by the L(+)*erythro* stereoisomer occurs in growing cultures as well as in full grown bacterial cultures which have been elaborating polypeptide for several days prior to the addition of the isomer. This indicates that the isomer interferes with polypeptide formation *per se* and not with the formation of enzymes which might catalyze this process.

Although a step by step theory of the mechanism of polypeptide formation is still lacking, it is thought (Bovarnick, 1942; Rydon, 1948) to be comprised of the initial synthesis of D(−) glutamic acid followed by a polymerization which yields the polypeptide. Provided this hypothesis were correct, the first phase of these reactions probably would require the operation of the Krebs' cycle as well as the transfer of amino groups, processes which also are essential for bacterial growth. The failure of the L(+)-*erythro* isomer to inhibit the growth of *Bacillus*

subtilis apparently eliminates from consideration the possibility of an action by this substance on one of the cellular functions which bacterial growth and polypeptide formation might have in common. Similar considerations apply to the action of chloramphenicol since glutamyl polypeptide is formed in the presence of growth inhibiting concentrations of the drug.

It appears, therefore, that the action of the drug or its isomer on protein or polypeptide synthesis occurs on an advanced and stereospecific level of cellular functions which in the case of the D(−) glutamyl polypeptide has been termed rather vaguely a "polymerization". Such a polymerization of D(−) glutamic acid molecules, yielding the polypeptide, would not seem to require the same process of assembling specific sequences of large numbers of different amino acids as it is thought to occur in the biosynthesis of cellular proteins. It is, therefore, not the assemblage into definite patterns but rather a phase in the condensation of amino acids during protein or polypeptide synthesis which might be the reaction affected by chloramphenicol or its L(+)*erythro* isomer.

The idea that chloramphenicol might be an antagonist of some essential natural metabolite was introduced first by Woolley (1950) who conjectured that the drug was an antimetabolite of phenylalanine or a structurally related substance. Other workers have entertained similar hypotheses with respect to different amino acids (Mentzer *et al.*, 1950; Molho and Molho-Lacroix, 1952; Bergmann and Sicher, 1952). The present results are in discord with such an idea. If glutamyl polypeptide formation were inhibited by an amino acid antagonist, the inhibitory substance should be a structural analogue of glutamic acid, a condition which obviously is not met by the L(+)*erythro* stereoisomer of chloramphenicol.

The inhibition of L-protein formation and D-polypeptide synthesis by the sterically opposed D(−)*threo* isomer, i.e., chloramphenicol, and L(+)*erythro* isomer, can perhaps be explained on the basis of an antimetabolic type of action which differs in details from the one suggested by Woolley. According to this hypothesis the two inhibitory isomers should be regarded as structural analogues and biological antagonists of certain functional units, thus far unknown, which participate in protein synthesis.

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We are indebted to Parke, Davis and Company for the L(+)*threo*, L(+)*erythro*, and D(−)-*erythro* isomers of chloramphenicol and for the racemic mixture of the D and L *erythro* isomers.

SUMMARY

The L(+)*erythro* stereoisomer of chloramphenicol inhibits the formation of D(−) glutamyl polypeptide by *Bacillus subtilis* but does not affect the growth of this organism.

The D(−)*threo* stereoisomer, i.e., chloramphenicol, inhibits the growth of the bacilli but does not interfere with the formation of glutamyl polypeptide by the organism.

The L(+)*threo* and the D(−)*erythro* stereoisomers of chloramphenicol fail to influence either growth or polypeptide formation.

The hypothesis is advanced that the L(+)-*erythro* stereoisomer of chloramphenicol inhibits polypeptide formation through a mechanism analogous to that of the antibiotic on protein synthesis and that it operates as an antimetabolite of an unknown functional unit at an advanced stage of the synthetic process.

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