MODE OF ACTION OF CHLORAMPHENICOL

III. Action of Chloramphenicol on Bacterial Energy Metabolism

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The inhibition of protein synthesis in sensitive microorganisms (Wisseman *et al.*, 1953, 1954; Gale and Folkes, 1953*b*) stands out as one of the most striking effects of chloramphenicol on bacterial metabolism. It occurs at minimal growth inhibitory concentrations of the antibiotic and constitutes an action of the drug on a metabolic process of sufficient importance to cellular economy to place it in the foreground as a most likely explanation for the principal mechanism of antibiotic action of chloramphenicol. Indeed, the action on protein synthesis appears highly specific since no other major synthetic process tested seems to be affected to the same degree. Those already examined include nucleic acid synthesis (Wisseman *et al.*, 1953, 1954; Gale and Folkes, 1953*b*), polysaccharide synthesis (Hopps *et al.*, 1954), and D(−) glutamyl polypeptide synthesis (Hahn *et al.*, 1954), all processes tested in this laboratory in accordance with the systematic metabolic group analysis outlined in a previous publication (Wisseman *et al.*, 1954).

While the phenomenon of an action on protein synthesis is clearly established, the exact mechanism by which chloramphenicol exerts this effect has not yet been explained. There is indirect evidence that it may be through a specific interference with some phases of the final assemblage of building blocks into the finished proteins (Wisseman *et al.*, 1954; Hahn *et al.*, 1954). However, the possibility still remains, as has been discussed previously (Wisseman *et al.*, 1954), that an action on an essential contributory process might lead to inhibition of protein synthesis as a secondary phenomenon. Since protein synthesis is an endergonic reaction, interference by the antibiotic with "energy metabolism" could result in inhibition of protein synthesis.

Despite the growing body of evidence that a phosphate cycle involving adenosine triphosphate (ATP) is in some way associated with the synthesis of proteins (Chantrenne, 1953; Gale and Folkes, 1953*a*), the exact manner by which it participates in the assembly of amino acids into proteins is unknown and, hence, this particular segment of the pathway of energy flow in protein synthesis is not currently amenable to direct examination for chloramphenicol action. However, some segments of the energy flow pathway antecedent to this, namely, those which contribute to and culminate in the formation of ATP, can be tested for possible action of the antibiotic. Furthermore, widely divergent processes which depend upon utilization of metabolic energy can be examined for possible interference by the drug. This last named indirect approach, though not necessarily precisely representative of the advanced stages of protein synthesis, might uncover a general interference by chloramphenicol with utilization of metabolic energy. In this project, the following three different examples of bacterial energy metabolism were selected for systematic study with chloramphenicol to supplement the existing information (Smith, 1953) in this important category of cell function: (1) the luminescence of *Achromobacter fischeri* which presumably depends on a system of electron transport; (2) the adenylic acid-phosphate cycle in the glycolytic system of *Escherichia coli*, strain B, which appears to be a representative example of the transformation of energy through the synthesis of energy-rich phosphate bonds; and (3) the motility of *E. coli* which presumably reflects the conversion of metabolic energy to mechanical energy.

The current studies, of which representative examples are included in this report, indicate that none of these processes is influenced significantly by chloramphenicol even in concentrations which are 50 to 100 times higher than the minimal bacteriostatic levels for the test organisms.

I. Action of Chloramphenicol on the Luminescence of Achromobacter fischeri

Bacterial luminescence, long known to be dependent upon atmospheric oxygen, has recently been demonstrated in a cell-free system from *A. fischeri* to require coenzyme I and flavin mononucleotide for optimal light production (Strehler, 1953; Strehler and Cormier, 1953). Although the details are still unknown, it is likely that the light producing reaction is associated with the transfer of electrons from these cofactors to oxygen, probably through a special alternate carrier system (McElroy and Kipnis, 1947) which differs from the cytochrome system of the main respiratory pathway by its insensitivity to cyanide (Harvey, 1952).

MATERIALS AND METHODS

A. fischeri, strain 7744, obtained from the American Type Culture Collection, was maintained at 23 C in the basal medium of Farghaly (1950) fortified with 0.2 per cent peptone. For preparation of working suspensions, nutrient agar plates (Difco) containing 0.3 per cent glycerol and 3.0 per cent NaCl were inoculated with 0.5 ml of an 18 hour liquid culture and incubated overnight at 23 C. The surface growth from each plate was suspended in 10 ml of 3 per cent NaCl, washed, and resuspended in 7.5 ml of the sodium chloride solution. After aeration for 2 hours at room temperature these stock suspensions were used without further delay for the experiments on bioluminescence.

Luminescence was measured quantitatively in arbitrary units with a special photometer which employed a photomultiplier tube arrangement resembling that described by McElroy and Kipnis (1947) and which accommodated round cuvettes 19 mm in diameter.

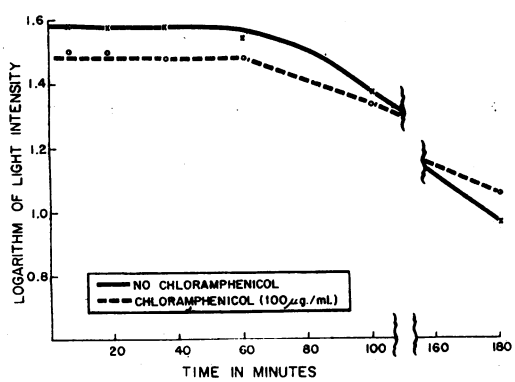

Figure 1. Failure of chloramphenicol to inhibit bacterial luminescence.

The action of chloramphenicol on bacterial luminescence was studied in quintuplicate samples of 5 ml volume containing 100 μg per ml of the antibiotic, 3.0 per cent NaCl, M/75 phosphate at pH 7.4, M/200 glucose, 0.2 per cent peptone, and usually 2 ml of a 1:500 dilution of the luminous bacterial stock suspension. A control experiment was carried out simultaneously without added chloramphenicol. The working concentration of bacteria was adjusted in each individual experiment to a level which emitted light of an intensity convenient for measurement in the photometer. The experimental tubes were aerated by continuous mechanical agitation which was interrupted only for the periodic measurement of luminescence at the indicated intervals.

The sensitivity of *A. fischeri* towards chloramphenicol was determined by inoculating the basal medium of Farghaly (1950) containing serial dilutions of the drug with the organism and examining bacterial growth visually after 24 hours' incubation at 23 C. The minimal bacteriostatic level of chloramphenicol was 1.5 μg per ml.

RESULTS

Figure 1 shows the results of a typical experiment; in all instances bacterial luminescence remained practically constant for the first 45 to 60 minutes of the experiments and then began to decline in an exponential manner. One hundred μg per ml of chloramphenicol did not influence significantly either the initial intensity of bacterial luminescence or the rate of its decay.

DISCUSSION

Since chloramphenicol is without influence on the emission of light by suspensions of *A. fischeri* which presumably depends on the transfer of electrons from flavin mononucleotide to oxygen through a special carrier, it may be inferred that the antibiotic does not interfere with this electron transport mechanism or with the series of reactions by which the flavin mononucleotide is reduced. The present findings corroborate and extend the observations reported by others that

reactions of this general type, such as those catalyzed by the enzymes xanthine oxidase, amino acid oxidase, and cytochrome oxidase, are unaffected by chloramphenicol (Smith, 1953). Finally, many bacterial oxidations which require the participation of complex sequences of redox reactions are not influenced by the antibiotic; hence, in all probability, this general category of reactions may be dismissed as the site of the growth inhibiting action of chloramphenicol.

II. Action of Chloramphenicol on Phosphorylation in E. coli

The molecule of chloramphenicol contains a *p*-nitrophenyl group, and the action of the drug on certain cellular processes involving protein formation shows a striking similarity to that of another aromatic nitro compound, viz., 2,4-dinitrophenol (DNP). Thus, incorporation of individual amino acids into the proteins of *E. coli* (Wisseman *et al.*, 1954; Melchior *et al.*, 1951) and of complex mixtures of amino acids into the proteins of staphylococci (Gale and Folkes, 1953*b*; Hotchkiss, 1947), formation of adaptive enzymes (Hahn and Wisseman, 1951; Monod, 1944), and multiplication of bacteriophages (Bozeman *et al.*, 1954; Spizizen, 1943) are inhibited by both substances alike.

The remarkable parallelism in the biological action of DNP and chloramphenicol suggested that chloramphenicol, like DNP (Hotchkiss, 1946; Loomis and Lipmann, 1948), might act on phosphorylation. Energy-rich phosphate bonds presumably provide the energy to "drive" protein synthesis (Lipmann, 1941; Borsook, 1950; Street, 1950; Chantrenne, 1953). Since the negative finding of Loomis (1950) dealt with the failure of chloramphenicol to act on a phosphorylating system in mitochondria, which were derived from animal cells not generally regarded as susceptible to the action of the antibiotic (Lépine *et al.*, 1950; LePage, 1953), it was considered essential to reinvestigate the action of chloramphenicol on a phosphorylating system from a microorganism whose growth is strongly inhibited by the antibiotic.

A glucose metabolizing system from *E. coli*, strain B, was chosen for this study since there is evidence that *E. coli* may metabolize glucose at least in part according to the Embden-Meyerhof scheme (Elsden, 1952). It was found that neither the turnover of radioactive phosphate by respiring whole cells of *E. coli* nor the esterification of inorganic phosphate by glycolytic enzymes in cell-free bacterial extracts of the same organism was influenced significantly by chloramphenicol.

MATERIALS AND METHODS

Organism. E. coli, strain B, grown with constant shaking in brain-heart infusion broth (Difco) overnight at room temperature, was harvested and washed with distilled water. For use as whole cells in the phosphorus P^{32}-turnover experiments, the organisms were resuspended in 0.85 per cent NaCl solution and aerated for 2 hours at room temperature.

Preparation of bacterial sonic extracts. Cell-free enzyme extracts were prepared by suspending the bacteria in 0.1 M potassium bicarbonate to form a thick paste, treating the suspensions for 45 minutes in a magnetostriction oscillator (Raytheon, Model S-102A), and centrifuging the sonic treated suspensions at 14,000 × G for 30 minutes. The clear yellowish and viscous supernatants were used for the experiments on esterification of inorganic phosphate and remained active for several months if stored at −20 C. Extracts containing 7 mg of total nitrogen per ml exhibited a high degree of phosphorylating activity.

Total nitrogen was determined with a modification of Johnson's procedure (1941) which employs sulfuric acid-hydrogen peroxide digestion followed by nesslerization.

Determination of P^{32} turnover. The turnover of inorganic phosphate by respiring whole cells of *E. coli* was studied with the aid of exogenous inorganic phosphate labeled with P^{32}. The experiments were carried out in series of conventional Warburg vessels, each containing in 3 ml of bacterial suspensions diluted 1:2, M/900 glucose and M/140 phosphate at pH 7.4, labeled with 7.5 μc of P^{32}. Two series of experiments were carried out simultaneously: (1) a control series without added chloramphenicol and (2) a series containing 100 μg of chloramphenicol per ml of experimental mixture. Each experiment extended over a total period of 1 hour during which time one flask from each series was removed every fifteen minutes for analysis.

Glucose was determined in 0.5 ml aliquots of the flask contents by the method of Folin and Malmros (1929). To the remaining 2.5

ml portions of experimental mixture was added enough strong formalin to make them 3 per cent with respect to formaldehyde. The bacteria were separated and washed twice by centrifugation at 10,000 × G for 30 minutes and finally suspended in 1 ml of water. Labaw *et al.* (1950) have shown that samples thus treated are remarkably free of adsorbed radioactivity. Aliquots of 0.1 ml of the suspensions were spread evenly to dry on copper planchets and then counted in a mica end-window Geiger counter (Tracerlab) with a window thickness corresponding to a weight of 3 to 4 mg per cm^2.

Determination of phosphate esterification by cell-free extracts. Phosphorylation by sonic extracts from *E. coli* was studied in conventional Warburg vessels, each containing 3 ml of 1:2 diluted bacterial extract with the following chemical additions: 25 μM of potassium orthophosphate, 50 μM of glucose, 50 μM of potassium fluoride, 1.5 mg of coenzyme I, and 1.2 mg of ATP. The pH was adjusted to 7.4 with potassium hydroxide.

The exclusive use of potassium salts was suggested by Utter's findings (1950) of an inhibition of glycolysis by sodium ions. Two series of experiments were carried out: (1) a control without added chloramphenicol and (2) experiments with 100 μg per ml of chloramphenicol added.

Experiments were run for 1 hour at 34 C with air as the gaseous phase. The contents of each vessel were then poured into 2 ml of ice cold 17.5 per cent trichloracetic acid (TCA); the mixtures were chilled, centrifuged, and free inorganic phosphate was determined in 0.25 ml aliquots of the supernatants by the method of Fiske and Subbarow (1925). The difference between the content of free phosphate in zero time controls and in the experimental mixtures was considered as phosphate esterified during the experiments.

Paper chromatography of phosphate esters. Samples for the paper chromatography of phosphorylated compounds were prepared in the following manner: Finely powdered barium hydroxide was added to the TCA supernatants from the Warburg experiments to bring the pH to 8.2 (distinctly pink with phenolphthalein); after addition of 25 ml of absolute alcohol to each 5 ml sample, the mixtures were refrigerated at −20 C for several hours. The precipitates were collected by centrifugation and dissolved in 2 ml of 0.1 N HCl; 0.55 ml of 2 N H_2SO_4 was then added to precipitate the barium ions. After centrifugation the supernatants were saved, the barium sulfate sediments were washed with 2 ml of water, and supernatants and washings were combined and made up to 5 ml.

Aliquots of 0.6 ml of these samples were chromatographed on Whatman no. 1 filter paper by the method of Bandurski and Axelrod (1951) using a methanol-formic acid mixture as the mobile phase. The developed and dried chromatograms were cut into serial consecutive 1 cm wide strips which then were eluted with 3 ml of 4 N H_2SO_4. Free inorganic and total phosphorus were determined in 1 ml aliquots of each of the eluates by the procedure of Fiske and Subbarow (1925) which for total phosphorus determination was preceded by evaporating the water from the samples and heating the acidic residues with hydrogen peroxide in order to digest organic matter. Esterified phosphorus present in the eluates was calculated from the difference between total and free phosphorus.

Determination of phosphoglyceric acids. Samples for the determination of phosphoglyceric acids by the polarimetric method of Meyerhof and Schulz (1938) were prepared in the same manner as for paper chromatography, and the specific rotation data of −650° to −670° for the equilibrium mixture of 3-phosphoglyceric and 2-phosphoglyceric acids were used since Utter and Werkman (1942) have shown that cell-free extracts from *E. coli* establish the same equilibrium between the two isomers as muscle or yeast.

Determination of individual glycolytic enzymes in sonic extracts from E. coli. For the following studies the sonic extracts from *E. coli* were dialyzed exhaustively against distilled water.

Hexokinase was demonstrated spectrophotometrically by an adaptation of Kornberg's method (1950) for the determination of ATP; the bacterial extract was substituted for the hexokinase fraction, and an excess of ATP was supplied, thereby making the bacterial hexokinase the rate limiting factor. The method of Somers and Cosby (1945) was used to show the presence of phosphohexose isomerase in the enzyme extracts; that of Dounce and Beyer (1948) for aldolase; and a modification of the same procedure for phosphofructokinase in which glucose-6-phosphate and ATP were substituted for fructose-1,6-diphosphate as substrate. With phosphohexose isomerase

and aldolase determined previously and individually the kinase became the limiting factor in the formation of phosphotriose.

A spectrophotometric application of the Thunberg technique (Tam and Wilson, 1941) was used for the determination of phosphotriose dehydrogenase (Still, 1940) and glycerophosphate dehydrogenase. The sodium glycerophosphate (Fisher Scientific Company, no. 644) contained 52 per cent α-glycerophosphate.

RESULTS

Phosphate turnover during aerobic dissimilation of glucose by E. coli. Glucose dissimilation by nonproliferating suspensions of *E. coli* proceeds in the presence of low orthophosphate concentrations without a detectable change in the concentration of free phosphate in the medium (Aubel and Szulmajster, 1950). However, P^{32} from labeled orthophosphate in the medium was taken up rapidly by cells of *E. coli* during glucose dissimilation in current experiments, indicating an exchange of intracellular phosphate with that of the medium. This ceased when all the glucose had disappeared from the experimental mixtures although oxygen consumption continued at a low rate. In control experiments without added glucose, oxygen consumption was small, and the uptake of P^{32} by the bacteria rose only to a level of about 30 per cent of that attained by cells which actively metabolized glucose. The presence of 100 μg per ml of chloramphenicol in the experimental mixtures had no significant influence on the consumption of oxygen, the disappearance of glucose, or on the uptake of phosphorus P^{32} by the bacteria as illustrated by the typical data presented in figure 2.

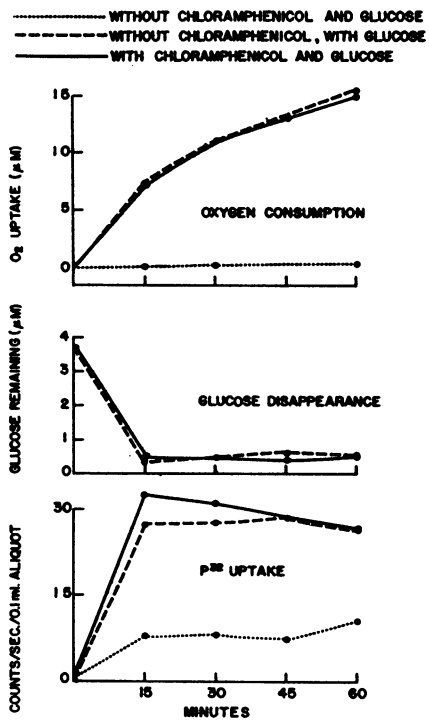

Figure 2. Failure of chloramphenicol to influence P^{32} uptake in *Escherichia coli*.

Esterification of phosphate and formation of phosphoglyceric acid during glucose dissimilation by cell-free extracts from E. coli, strain B. When glucose was dissimilated aerobically by sonic extracts from *E. coli*, strain B, in the presence of orthophosphate and fluoride ions, free phosphate disappeared and equivalent amounts of phosphoglyceric acid accumulated. No net phosphorylation could be demonstrated under anaerobic conditions.

Analyses for free phosphate and total phosphorus in eluates from series of consecutive strips from the chromatograms revealed the presence of a substance containing bound phosphorus which was not detectable by the acid molybdate spray reagent of Bandurski and Axelrod (1951). The resistance to acid hydrolysis, the chromatographic behavior, and the characteristic and marked change in optical rotation upon addition of ammonium molybdate (Meyerhof and Schulz, 1938) served to identify the compound as phosphoglyceric acid. No significant quantities of phosphorylated compounds other than phosphoglyceric acid were detected by the chromatographic methods employed.

Chloramphenicol, 100 μg per ml, failed to influence significantly the esterification of inorganic phosphate or the consumption of oxygen. Typical analytical data are presented in table 1.

Demonstration of individual glycolytic enzymes in cell-free bacterial extracts. The following enzymes were demonstrated to be present in the dialyzed sonic extracts from *E. coli* employed in these studies: hexokinase, phosphohexose isomerase, phosphofructokinase, aldolase, and phosphotriose dehydrogenase. A glycerophos-

TABLE 1

Failure of chloramphenicol to influence phosphorylation by cell-free extracts of Escherichia coli, strain B

Experimental Mixtures	μM Orthophosphate Esterified after 1 hr	μM Oxygen Consumed after 1 hr
Without glucose and chloramphenicol	5.3	16.5
With glucose and without chloramphenicol	22.9	27.9
With glucose and with chloramphenicol	23.9	25.1

phate dehydrogenase was also detected in the enzyme extracts by the Thunberg technique; additional coenzyme I did not increase its activity.

These enzyme studies were carried out in order to establish the presence of enzymes of the glycolytic system in the experimental mixture.

DISCUSSION

Chloramphenicol does not influence significantly the turnover of phosphate in whole cells of *E. coli*, strain B, and it does not inhibit the esterification of phosphate due to the operation of a part of the glycolytic system in cell-free sonic extracts from the same organism.

Juni *et al.* (1948) have shown that the turnover of phosphate in nonproliferating yeast cells, which dissimilate glucose in a nitrogen free medium, can be studied by determining the incorporation of P^{32}-labeled exogenous phosphate into the organisms. The same method appears to be applicable to the study of phosphate turnover in cells of *E. coli*, strain B. The incorporation of phosphorus P^{32} into the bacteria was not influenced by 100 μg per ml of chloramphenicol.

On the basis of the individual enzymes that have been demonstrated to be present, phosphorylation by cell-free extracts from *E. coli* in the present experiments appears to proceed through a part of the Embden-Meyerhof sequence of reactions; a metabolic block introduced by the inhibition of enolase by fluoride causes an accumulation of phosphoglyceric acid equivalent to the amounts of orthophosphate which disappear from the medium.

III. Action of Chloramphenicol on the Motility of E. coli

The motility of bacteria presumably provides an instance in which the energy derived from metabolic processes is ultimately converted into mechanical energy. The effect of chloramphenicol on the motility of a sensitive bacterium was investigated to supplement the other studies on bacterial energy metabolism.

MATERIALS AND METHODS

An actively motile culture of *Escherichia coli*, strain 67 L-15, obtained from the Bacteriology Department of the University of Maryland was grown in Difco brain-heart infusion broth for 4.5 hours at 35 C; the culture at this time was extremely motile and growing rapidly. Nine ml aliquots of the bacterial suspension were added to sterile cuvettes containing 1.0 ml of chloramphenicol and 2,4-dinitrophenol solution, respectively, 10 × the final desired concentration. Viable counts were made by the conventional plate count technique immediately after mixing and at the end of each experiment. Motility was observed by the hanging drop method and recorded at 30 minute intervals.

TABLE 2

Effect of chloramphenicol and dinitrophenol on motility and viable cell count of Escherichia coli, strain 67 L-15

Time after Addition of Compounds		Compounds Added		
		None (control)	Chloramphenicol (100 μg/ml)	Dinitrophenol (1×10^{-3} M)
min 0	Viable cell count	3.0×10^8	3.6×10^8	1.8×10^8
	Motility*	4+	4+	4+
30	Motility*	4+	4+	4+
60	Motility*	4+	4+	2+
90	Motility*	4+	4+	1+
120	Motility*	4+	4+	1+
	Viable cell count	1.1×10^9	2.2×10^8	2.8×10^8

* Motility was recorded in units of 0 to 4+ by comparison with the control which was arbitrarily assigned the value of 4+.

RESULTS

Table 2 presents the observations made in a typical experiment. Here, tubes containing added chloramphenicol or 2,4-dinitrophenol are compared with a control. The control culture remained actively motile and grew over the two hour observation period. Chloramphenicol, however, at a concentration 50 × the minimal growth inhibitory level had no detectable effect on motility even though growth was completely inhibited. In contrast, dinitrophenol produced a progressive decrease in motility which was noticeable after about 30 minutes. This occurred without a decrease in the number of viable cells.

DISCUSSION

Chloramphenicol does not interfere with bacterial motility at concentrations which exert a strong bacteriostatic effect. In contrast, 2,4-dinitrophenol causes a progressive reduction in motility even though the organisms remain viable. The metabolic requirements for motility in bacteria are not well known. However, on the basis of theoretical calculations, Morowitz (1954) suggested that the energy required for motility was of an order of magnitude which could be supplied by reasonable quantities of ATP. The action of 2,4-dinitrophenol on bacterial motility as well as ciliary action in animal cells (Seaman and Houlihan, 1951; Wernstedt, 1944) is compatible with such an hypothesis even though it does not necessarily constitute proof for the participation of a phosphate cycle.

The current experiments were restricted to short term observations in order to avoid as much as possible the operation of the unknown factors which influence motility, even in the ordinary growth cycle, and to avoid error introduced by any lethal action of the components.

GENERAL DISCUSSION

None of the three examples of bacterial energy metabolism investigated in the present studies is influenced significantly by concentrations of chloramphenicol up to 100 times higher than the minimal bacteriostatic levels for the test organisms.

While the exact mechanism of energy supply for protein biosynthesis remains unknown, some model reactions involving amide bond synthesis, e.g., formation of acetylsulfonamide, hippuric acid, glutamine, and glutathione, require ATP as a source of energy (Chantrenne, 1953). Furthermore, net protein synthesis in a cell-free system of bacterial origin (Gale and Folkes, 1953*a*) utilized ATP and hexose diphosphate as energy source.

The idea is widely accepted that protein synthesis utilizes phosphate energy which in turn is generated by electron transport involved in oxidations and stored by phosphorylation. Examples of these forms of energy metabolism and of the utilization of metabolic energy have been investigated in the present study. It is the opinion of the authors that failure of chloramphenicol to influence energy metabolism in the present examples strongly suggests that the drug does not inhibit protein biosynthesis by interfering with the generation of the necessary supply of metabolic energy and probably does not do so by a general inhibitory action on the utilization of such energy.

SUMMARY

Three different forms of bacterial energy metabolism have been studied for possible interference by chloramphenicol. These included (1) the bioluminescence of *Achromobacter fischeri* as an example of electron transport, (2) phosphorylation involved in the dissimilation of glucose in *Escherichia coli*, strain B, as a key process of synthesis of high-energy phosphate bonds and their utilization in glucose metabolism, and (3) the motility of *E. coli* as a special example of the utilization of metabolic energy.

None of these examples of energy metabolism is influenced significantly by chloramphenicol. The implications of these findings as regards the mode of action of chloramphenicol have been discussed.

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