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Comparative Metabolic Effects Of Chloramphenicol Analogues

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SUMMARY

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Two groups of chloramphenicol analogues have been examined with respect to their effect on mitochondrial and *Escherichia coli* protein synthesis and on DNA synthesis in cultured human lymphoblastoid cells; one group of analogues with substitutions in the *para* position of the ring and another in which the substitutions were in the propanediol and dichloroacetamide moieties of the molecule. In general, substitutions in the propanediol or dichloroacetamide portion of the molecule had profound effects upon its capacity to inhibit both mitochondrial and *E. coli* protein synthesis. Although the patterns of inhibition of both mitochondrial and *E. coli* protein synthesis were similar with most of the analogues, there were several exceptions, suggesting some basic differences in these two processes. Substitutions in the *para* position of the molecule did not influence in any recognizable pattern its effect on either protein or DNA synthesis.

INTRODUCTION

Chloramphenicol produces two types of hematological toxicity (1, 2): a common, dose-related, reversible bone marrow suppression involving primarily the erythroid elements, and a rare lesion [1/19,000 (3) to 1/40,000 (4)] characterized by marrow hypoplasia or aplasia, pancytopenia, lack of dose-effect relationship, and usually a fatal outcome. There is ample evidence indicating that reversible bone marrow suppression from chloramphenicol results from inhibition by the drug of mitochondrial protein synthesis (5–7). The mechanisms by which chloramphenicol produces bone marrow aplasia remain unknown. It has been postulated that aplasia occurs on the basis of a biochemical predisposition involving the pathway of DNA synthesis (2).

Thiamphenicol, an analogue of chloramphenicol, in which the *p*-nitro group is replaced by a methylsulfonyl group, has been widely used in Europe as a chloramphenicol substitute. The two drugs possess comparable antibacterial activity and are employed in similar dosages (8). Both chloramphenicol and thiamphenicol produce the dose-related reversible erythroid lesion. However, aplastic anemia after thiamphenicol has not been reported to date, although more than 12 million people have been treated with this antibiotic (8). This observation suggests that the *p*-nitro group of chloramphenicol may somehow be causally related to aplastic anemia

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and that a comparative metabolic study of the two drugs may provide an important clue to the mechanism involved. In a previous study (9) we have demonstrated that thiamphenicol is equally potent as an inhibitor of mitochondrial protein synthesis, consistent with its ability to produce reversible erythroid suppression. However, there was a distinct difference between the two drugs in their effects on DNA synthesis. Although chloramphenicol did not inhibit DNA syntheis when used in therapeutic levels, concentrations above 0.2 mM were progressively inhibitory (70% inhibition at 1 mM); comparable concentrations of thiamphenicol caused little or no inhibition. On the basis of this observation and in an attempt to look further into the structure-effect relationship in the chloramphenicol molecule, we have examined the effect of a number of analogues on protein synthesis in *Escherichia coli*, protein synthesis in rat liver mitochondria, and DNA synthesis in cultured human lymphoblastoid cells.

Two groups of analogues were used. In one group the substitutions were in the *para* position of the molecule; in the other they were made in the propanediol and dichloroacetamide moieties of chloramphenicol. In general, substitutions in the propanediol or dichloroacetamide portion of the molecule had profound effects on its capacity to inhibit both mitochondrial and *E. coli* protein synthesis. On the other hand, substitutions in the *para* position of the molecule did not influence in any recognizable pattern its effect on either protein or DNA synthesis.

METHODS

Materials. Bicine, ATP, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma Chemical Company. The amino acids were purchased from Sigma and Mann Research Laboratories. L-[*U*-^{14}C] Leucine (specific activity, 285–315 mCi/mmole) was purchased from new England Nuclear Corporation. All other chemicals used were analytic reagent grade. Crystalline chloramphenicol was provided by Parke, Davis and Company. All the analogues of chloramphenicol used, with the exception of thiamphenicol, were obtained from Dr. Robert Hans of Parke, Davis and Company. Crystalline thiamphenicol (Urfamycine) was a gift of Zambon, S.p.A., Milano-Vicenza, Italy.

All glassware, centrifuge tubes, and reagents used to measure amino acid incorporation into mitochondrial protein were sterilized either by autoclaving or by filtration through Millipore filters were a pore size of 0.45 μm.

Preparation of mitochondria. Rat liver mitochondria were prepared from overnight-fasted 250–300-g Sprague Dawley rats using 0.25 M sucrose as the homogenizing medium according to Schneider (10), at the centrifugation speeds recommended by Meyers and Slater (11). Liver slices were rinsed five times with homogenizing medium, followed by homogenization in a Teflon-glass Potter-Elvehjem motor-driven homogenizer. The homogenate was centrifuged twice at 800 × *g* for 10 min to remove unbroken cells and cell debris, and once at 7000 × *g* for 10 min to sediment the mitochondria. The mitochondrial pellet was resuspended in a homogenizer and finally recentrifuged at 18,000 × *g* for 10 min. The final mitochondrial pellet was suspended in 0.25 M sucrose to give a protein concentration of 45–75 mg/ml. Protein was determined by the method of Lowry *et al.* (12).

Assay of mitochondrial protein synthesis. The incubation medium contained Bicine buffer, pH 7.5, 50 mM; $MgCl_2$, 10 mM; KH_2PO_4, 10 mM; EDTA, 2 mM; ATP, 2 mM; phosphoenolpyruvate, 10 mM; pyruvate kinase, 8 enzyme units ; KCl, 50 mM; sucrose, 50 mM; a mixture of 19 [^{12}C]-amino acids, 2 μg each (13); and mitochondria, 2–3 mg, in a total volume of 0.6 ml. Incubations were carried out in duplicate in 25-ml Erlenmeyer flasks in a Dubnoff metabolic shaker at 37°. After incubation for 10 min, 0.7 μCi of L-[^{14}C]leucine (285–315 mCi/mmole) was added. At the indicated time intervals appropriate volumes of the incubation mixture were transferred to Whatman No. 3 paper discs, washed, and counted according to the method of Mans and Novelli (14).

Chloramphenicol, thiamphenicol, and most of the analogues used in the present

study were added to the incubation mixture as aqueous solutions. A few of the analogues used were not soluble in water. In these instances it was necessary to add a proportion of ethanol. However, in no case did the amount of ethanol added affect mitochondrial protein synthesis.

Any contribution by microsomes to amino acid incorporation into mitochondrial protein was ruled out by running the appropriate cycloheximide controls. Cycloheximide at a concentration of 300 μg/ml had no effect on amino acid incorporation into protein, thus indicating the absence of microsomal contamination (15, 16).

Assay of protein synthesis by E. coli B. *E. coli* B was grown in Difco nutrient broth and used in the midlogarithmic phase. The incubation mixture contained the test compound at the indicated concentration and 3–5 × 10^8 cells (but always the same number of cells per flask) in a total volume of 10 ml of the nutrient broth. After a 10-min incubation in 25-ml Erlenmeyer flasks at 37° in a Dubnoff shaking water bath, 1 μCi of L-[^{14}C]leucine (285–315 mCi/mmole) was added to each flask and the incubation was continued for 1 hr. The reaction was stopped by the addition of 1 ml of 50% TCA.[2] The TCA precipitates were washed, dried, dissolved in 0.5 N NaOH, plated on glass filter discs, and counted as previously described (17).

Assay of DNA synthesis. DNA synthesis in normal human lymphoblastoid cell lines, established in long-term cultures as described by Moore *et al.* (18), was assayed as previously described (9). Cells were grown in RPMI 1640 and studied in the logarithmic phase. Cells (5 × 10^6) were incubated with the appropriate analogue in 1 ml of Dulbecco's modified Eagle's medium at 37° for 15 min in a shaking water bath, after which 0.2–0.4 μCi of [^{3}H]thymidine was added (specific activity, 16 Ci/mmole). The cells were incubated for an additional 60 min. The reaction was stopped by the addition of 10 ml of ice-cold 0.15 N NaCl. The cells were centrifuged and washed twice with the cold 0.15 N NaCl, followed by the addition of 10% TCA to precipitate the proteins and nucleic acids. The pellet was washed three times in 95% ethanol, once in ethanol-ethyl ether (1:1), and finally once in ether. It was then dried, solubilized with Soluene (Packard Instrument Company), and counted using an organic fluor. Under the conditions described, DNA synthesis was linear for the 60-min incubation period.

[2] The abbreviation used is: TCA, trichloracetic acid.

RESULTS

Effect of para-substituted analogues of chloramphenicol on mitochondrial and E. coli protein synthesis. Sixteen analogues were examined, and the results are shown in Table 1. Most substitutions in the *para* position of the molecule did not alter the capacity of the drug to inhibit mitochondrial protein synthesis. Substitution with larger groups, such as phenylsulfonyl, ureidophenyl, and benzyl, appeared to render the molecule less effective. However, the possibility that this was related to decreased permeability could not be ruled out. Although the patterns of inhibition of mitochondrial and *E. coli* protein synthesis were similar with most of the analogues, there were several exceptions. Thus the methylbenzylsulfonyl, phenylsulfonyl, and dimethylsulfamoyl analogues were significantly inhibitory toward mitochondrial protein synthesis but had no effect on *E. coli* protein synthesis. Conversely, the benzyl and hydro analogues inhibited *E. coli* protein synthesis but not mitochondrial protein synthesis.

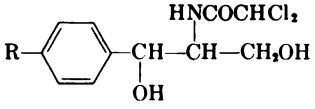
Effect of aliphatic substituted analogues. Among the five analogues examined, with one exception, all substitutions in the aliphatic side chain were associated with loss of inhibitory effect of *E. coli* and mitochondrial protein synthesis (Table 2). The 2-phenylthiourea derivative appeared to retain inhibitory activity toward mitochondrial protein synthesis.

Effect of analogues on DNA synthesis in lymphoblastoid cells. It is apparent from the data in Table 3 that the *p*-nitro group is not critical for inhibition of DNA synthesis. With few exceptions, notably thiamphenicol, most of the analogues with substitutions in the *para* position retained their capacity to inhibit DNA synthesis.

TABLE 1

Effect of para-substituted analogues of chloramphenicol on mitochondrial and E. coli protein synthesis

Analogues were added at a concentration of 93 μM, equivalent to 30 μg/ml in the case of chloramphenicol. Incubation time was 30 min using the conditions described in METHODS. All values have been corrected for zero time, and each individual value represents the average of at least two and usually four determinations. Agreement between determinations for a single analogue was within 10%.



Analogue	R	Inhibition of mitochondrial protein synthesis	Inhibition of *E. coli* protein synthesis
		%	%
Nitro	$-NO_2$[a]	90.2	99.2
Methylsulfonyl	$-SO_3CH_3$[b]	90.9	79.5
Methylthio	$-SCH_3$	86.5	98.2
Acetyl	$-COCH_3$	80.8	98.8
Methylsulfonylamine	$-SO_2NHCH_3$	75.2	92.4
Methylformyl	$-COOCH_3$	72.9	97.2
Trifluoromethyl	$-CF_3$	71.9	98.8
Bromo	$-Br$	50.9	93.6
Phenyl	$-C_6H_5$	50.3	98.6
Methylbenzylsulfonyl	$-SO_2(CH_2)_2C_6H_5$	49.5	6.0
Phenylsulfonyl	$-SO_2C_6H_5$	36.5	9.0
Dimethylsulfamoyl	$-SO_2N(CH_3)_2$	35.4	0
Chloro	$-Cl$	30.6	92.4
Ureidophenyl	$-NHCONHC_6H_5$	11.1	6.4
Benzyl	$-CH_2C_6H_5$	0	64.3
Hydro	$-H$	0	69.5

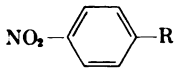
[a] Chloramphenicol.

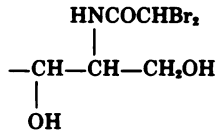
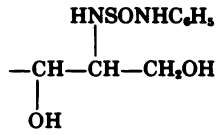
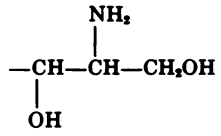
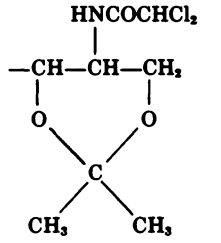
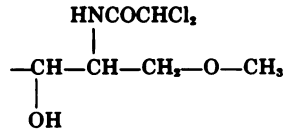
[b] Thiamphenicol.

TABLE 2

Effect of aliphatic substituted analogues of chloramphenicol on mitochondrial and E. coli protein synthesis

Methods were exactly as described in legend to Table 1.



Analogue	R	Inhibition of mitochondrial protein synthesis	Inhibition of *E. coli* protein synthesis
		%	%
N-Dibromoacetyl		90.6	98.8
2-Phenylthiourea		84.3	15.7
Free amine		19.8	12.4
Acetone adduct		21.3	8.2
3-Methoxy		11.3	8.2

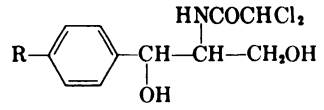
There was no recognizable pattern of relationship between the type of substitution and the degree of inhibition of DNA synthesis. Substitutions with large groups (e.g., ureidophenyl and methylbenzylsulfonyl) appeared to render the molecule slightly more inhibitory.

Substitutions in the aliphatic side chain likewise did not influence the inhibitory capacity of the molecule in a recognizable pattern (Table 4). The dibromoacetyl derivative was most inhibitory toward DNA synthesis, and the acetone adduct was without effect.

TABLE 3

Effect of para-substituted analogues of chloramphenicol on DNA synthesis in human lymphoblastoid cells

Analogues were added at a concentration of 1 mM, equivalent to 322 μg/ml in the case of chloramphenicol. DNA synthesis was assayed as described under METHODS.



Analogue	R	Inhibition of DNA synthesis
		%
Nitro	$-NO_2$[a]	52.0
Methylsulfonyl	$-SO_2CH_3$[b]	8.2
Methylthio	$-SCH_3$	28.5
Acetyl	$-COCH_3$	30.0
Methylsulfonylamine	$-SO_2NHCH_3$	16.5
Methylformyl	$-COOCH_3$	42.0
Trifluoromethyl	$-CF_3$	23.0
Bromo	—Br	53.0
Phenyl	$-C_6H_5$	35.0
Methylbenzylsulfonyl	$-SO_2(CH_2)_2C_6H_5$	55.5
Phenylsulfonyl	$-SO_2C_6H_5$	67.5
Dimethylsulfamoyl	$-SO_2N(CH_3)_2$	14.5
Chloro	—Cl	36.5
Ureidophenyl	$-NHCONHC_6H_5$	75.0
Benzyl	$-CH_2C_6H_5$	53.5
Hydro	—H	15.5

[a] Chloramphenicol.
[b] Thiamphenicol.

DISCUSSION

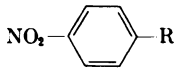
The present study on the structure-effect relationships of the chloramphenicol molecule was prompted by observations made with its analogue, thiamphenicol. Since the latter has not, so far, been associated with any reported cases of aplastic anemia, it was hoped that a comparative study of the two drugs might provide a clue to the pathogenetic mechanism(s) underlying this complication. Although the inhibition of DNA synthesis by inordinately high concentration of chloramphenicol (1 mM) is of uncertain significance, the lack of inhibition by similar concentrations of thiamphenicol could be significant. This difference raises some intriguing questions regarding the interrelationship of the inhibitory capacity of the chloramphenicol toward DNA synthesis, the *p*-nitro group, and the occurrence of aplastic anemia from this drug. Little can be said about the relationship of the *p*-nitro group to bone marrow aplasia, since other *para*-substituted analogues are not in clinical use, and hence a comparative toxicity study is not possible. The present study, however, indicates that the *p*-nitro group is necessary for inhibition of DNA synthesis, since other *para*-substituted analogues were equally inhibitory. Whether the capacity of chloramphenicol to inhibit DNA synthesis at high concentration is somehow related to its causing aplastic anemia cannot be determined at present.

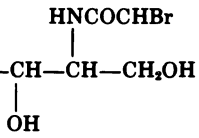
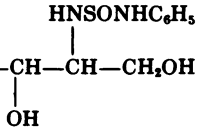
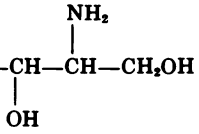
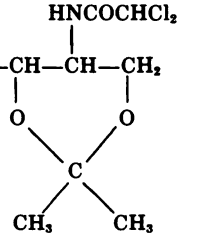
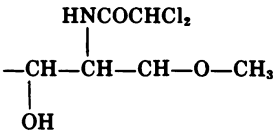
The data on *E. coli* protein synthesis confirm previous observations that an intact dichloroacetamide propanediol chain is critical for this biological activity (19, 20) and only minor changes can be tolerated. Since chloramphenicol inhibits peptide bond assembly on the 70 S ribosomes (21), an identical pattern of inhibition by the various analogues might be expected in *E. coli* and mitochondria. However, many exceptions were noted, suggesting that other determining factors must exist. One important factor might be related to differences in permeability. Lack of permeability must be considered a possibility for every analogue which is not inhibitory. Kroon and DeVries demonstrated that erythromycin and oleandomycin inhibit [^{14}C]leucine incorporation into protein of swollen mitochondria by 50–95% whereas no inhibition

TABLE 4

Effect of aliphatic substituted analogues of chloramphenicol on DNA synthesis in lymphoblastoid cells

Analogues were added at a concentration of 1 mM, equivalent to 322 μg/ml in the case of chloramphenicol. DNA synthesis was assayed as described under METHODS.



Analogue	R	Inhibition of DNA synthesis
		%
N-Dibromoacetyl		83.0
2-Phenylthiourea		61.0
Free amine		21.0
Acetone adduct		0
3-Methoxy		50.0

occurs in intact mitochondria (22). Similar date were obtained with lincomycin, indicating that the absence of inhibition in intact mitochondria is due to lack of permeability. In our studies two chloramphenicol analogues which were inhibitory in *E. coli* but not in mitochondria were also tested in swollen mitochondria, but no inhibition of mitochondrial protein synthesis could be demonstrated. This suggests that factors other than permeability must account for the difference between the sensitivities of *E. coli* and mitochondrial protein synthesis to these analogues. This intriguing observation deserves further study.

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