The Enzymatic Acetylation of Chloramphenicol by Extracts of R Factor-resistant Escherichia coli*

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SUMMARY

Chloramphenicol-resistant strains of Escherichia coli which carry the episomal resistance transfer factor R inactivate the antibiotic by enzymatic acetylation. In the presence of chloramphenicol and acetyl coenzyme A, cell extracts catalyze the formation of both a monoacetyl and a diacetyl derivative of chloramphenicol. Definitive identification of the reaction products as 3-acetoxy chloramphenicol and 1,3-diacetoxy chloramphenicol was possible with the use of thin layer chromatography and ultraviolet spectroscopy. A simple spectrophotometric assay of acetylation has been devised based on the disappearance of acetyl coenzyme A. Preliminary kinetic data for the acetylation of chloramphenicol and related compounds are presented.

Naturally occurring strains of Shigella which are resistant to multiple antibiotics have been shown to contain a nonchromosomal determinant of antibiotic resistance which may be transferred to Escherichia coli and certain other enteric bacteria by mixed cultivation of the resistant organisms with sensitive recipients. The general topic of the resistance transfer factors (R factors) and the justification for considering them to be episomes has been reviewed recently (1, 2). Bacteria resistant to streptomycin, chloramphenicol, tetracycline, and the sulfonamides were initially described, but subsequent reports from several laboratories indicate that determinants of resistance to kanamycin and neomycin as well as ampicillin may also be present in certain R factors (3-5). Conversely, strains of enteric bacteria carrying variants of such R factors have been isolated wherein one or more resistance characters have been lost (1). An analysis of the sedimentation properties of deoxyribonucleic acid from such strains suggests that the separate determinants of antibiotic resistance have characteristic buoyant densities in the ultracentrifuge and correspondingly distinct nucleotide base composition (6).

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† Recipient of a traineeship in metabolism under Grant TI-AM 5397 from the United States Public Health Service.
Preparation of Cell-free Extracts—One liter of culture medium was inoculated with the appropriate organism and grown to late logarithmic phase. The suspension was chilled rapidly and the bacterial cells were harvested by centrifugation. The cell paste was suspended in 50 ml of 0.01 M Tris-HCl (pH 7.8) and centrifuged again, and the final pellet was resuspended in 15 ml of the same medium. The bacterial suspension was subjected to three pulses of sonic oscillation of 20 sec each with the use of the Branson sonic oscillator. The supernatant fluid obtained after centrifugation at 30,000 x g for 30 min was used without further treatment (crude extract). For the spectrophotometric assay experiments, 1.5 ml of the crude extract were passed over a Sephadex G-50 column (20 x 1.4 cm) which had been previously equilibrated with 0.01 M Tris-HCl (pH 7.8). The protein-containing fractions were collected, pooled, and used without further treatment. The protein concentration of cell extracts was determined by the biuret reaction (11). All enzyme preparations were stored at -20° in small aliquots after it was observed that the crude extract showed a marked decrease in its ability to acetylate chloramphenicol after thawing and refreezing. Subsequent studies have indicated that only the ability to activate acetate is lost by such treatment since repeatedly thawed and frozen extracts are fully active in the spectrophotometric assay which is not dependent on the activation of acetate to acetyl-CoA.1

Radioisotope Assay of Chloramphenicol Acetylation—The fate of chloramphenicol was studied initially by a modification of the incubation conditions described by Okamoto and Susuki (8). Crude extract was incubated in the presence of ATP, CoA, an ATP-generating system (phosphoenolpyruvate, Mg++, K+, and pyruvate kinase), and 14C-chloramphenicol in a final volume of 0.5 ml contained in a 12-ml conical centrifuge tube. In some instances, acetyl-CoA was added directly in place of the reagents for the activation of acetate. In such instances, Mg++ and K+ were omitted from the system. At the conclusion of the incubation, the tube containing the reactants was immersed in an ice bath and extracted with 2 ml of cold ethyl acetate. The mixture was agitated with a Vortex mixer and the supernatant layer of ethyl acetate was aspirated after clarification by centrifugation. The extraction was repeated twice and the combined ethyl acetate fractions were pooled and taken to dryness in an air stream at room temperature. Control experiments revealed that no perceptible products were formed in the ethyl acetate extracts prepared from reactions incubated at 0°. The residue was dissolved in 0.20 ml of ethyl acetate and an appropriate aliquot was spotted at the origin of a thin layer chromatographic sheet. Chromatograms were developed in ascending fashion after the glass chromatographic vessel (20 x 20 x 8 cm) had been equilibrated for at least 1 hour with the appropriate solvent of Table I. 14C-Chloramphenicol and its radioactive products were located by autoradiography and were quantitatively determined by scintillation counting after the appropriate areas had been cut from the sheet and transferred to counting vials containing 10 ml of Bray’s solution (12). Alternatively, in some experiments, nonradioactive chloramphenicol congeners were incubated with 14C-acetate and treated similarly to yield 14C-acetyl derivatives which could be located autoradiographically. For spectral studies, the radioactive product was eluted from the appropriate area with ethanol and compared with authentic compounds treated in similar fashion.

1 W. V. Shaw, unpublished experiments.

Identification of Products of Reaction—A modification of the Kunz technique for the selective removal of O-acyl groups was used to show the susceptibility of the products of chloramphenicol acetylation to hydrolysis under conditions unfavorable for the cleavage of the dichloroacetamide moiety of chloramphenicol (13). Approximately 0.1 pmole of 14C-chloramphenicol or its radioactive products was isolated by thin layer chromatography, eluted with ethyl acetate, taken to dryness, and dissolved in 1 ml of an ice-cold solution of 0.05 N NaOH in acetone. The contents were kept at 0° for 1 hour and then adjusted to pH 7 by the addition of 0.1 ml of 1 M monobasic sodium phosphate. The mixture was then extracted with ethyl acetate and rechromatographed as described.

Chemicals and Materials—Except where specifically noted, chloramphenicol isomers, derivatives, and analogues were obtained from Parke, Davis, through the generosity of Drs. M. Rebstock and A. J. Glazko. All such compounds were crystalline materials except for 3-acetoxy chloramphenicol, which was chromatographically pure. The p-acetyl analogue of chloramphenicol was obtained from the Warner Lambert Research Institute, and the corresponding p-methylsulfonyl compound from the Sterling Winthrop Research Institute. Except for the stereoisomers, all compounds studied were of the D-threo configuration.

ATP, CoA, and acetyl-CoA were the products of P-L Biochemicals. 1-14C-Acetyl-CoA was synthesized by the method of Simon and Shemin (14). Sodium phosphoenolpyruvate and pyruvic kinase were obtained from Calbiochem. Sodium acetate-1,2-14C (2 mCi per mmole), sodium acetate-3H (25 mCi per mmole), and 1,4-14C-acetic anhydride (5 mCi per mmole) were purchased from New England Nuclear. p-threo-Chloramphenicol-3-14C (6.8 mCi per mmole) was purchased from Nuclear-Chicago, and was chromatographically pure in the solvent systems of Table II.

The materials for thin layer chromatography were Ladd thin films of alumina and silica gel as available from Packard, and were not activated before use. Thin layer sheets containing a fluorescent indicator were used to detect the nonradioactive chloramphenicol congeners by means of quenching when sheets were viewed under short wave ultraviolet illumination.

Results

Products of Reaction—The incubation of 14C-chloramphenicol with crude extracts of E. coli R4 yielded two radioactive products which were distinct from chloramphenicol. These products were not seen after incubation of the latter with heat-denatured R4 extract or with untreated R4 extract in the absence of acetyl-CoA (Fig. 1). To be noted, however, is the faint area of radioactivity which has been noted consistently when the wild type (K-10) extract is incubated with 14C-chloramphenicol (see “Discussion”). A time course of the reaction indicated that a precursor-product relationship probably existed between chloramphenicol, Compound A, and Compound B in the order indicated (Fig. 2). In view of the structure of chloramphenicol and the requirement for acetyl-CoA, it appeared likely that Compounds A and B were the monooacetyl and diacetyl derivatives of chloramphenicol. To test this possibility, 14C-chloramphenicol was incubated with p-acetate in the presence of crude extract and the cofactors for activation. As can be seen from the data in Table I, Compound A contains 1 mole of acetate per mole of

2 Chloramphenicol refers to D-threo-2-dichloroacetamido-1-p-nitrophenyl 1,3-propanediol.
chloramphenicol, whereas analysis of Compound B reveals 2 moles of acetate.

A consideration of the structure of chloramphenicol (Fig. 3) suggested that several possibilities existed for the attachment of acetyl groups to the chloramphenicol carbon skeleton. Although the most likely mechanism appeared to be the acetylation of one or both hydroxyl groups, it remained possible that 1 mole of acetate might be introduced in amide linkage in place of dichloroacetate by transacylation or via the prior hydrolysis of chloramphenicol to the 2-amino derivative. Advantage was taken of the lability of acetoxy derivatives of chloramphenicol to mild alkaline hydrolysis (13) to rule out the possibility that Compounds A or B might contain an N-acetyl substituent. Treat-

![Image](https://example.com/image.png)

**Fig. 1.** Autoradiographs showing the dependence of 14C-chloramphenicol acetylation on the presence of appropriate cell extracts and acetyl-CoA. The complete system consisted of 50 pmol of Tris-HCl (pH 7.8), 0.1 pmol of acetyl-CoA, 0.05 pmol of 14C-chloramphenicol (6.8 μC per pmol), and 2 mg of the appropriate enzyme enzyme protein in a final volume of 0.5 ml. Incubation was for 10 min at 37°. Enzyme was omitted from Reaction 1, whereas Reaction 2 contained cell extract prepared from the chloramphenicol-sensitive E. coli K-10. Experiment 3 was carried out in the presence of extract from the resistant strain R6. Incubation 4 contained E. coli enzyme which had been inactivated by prior heating at 90° for 10 min, whereas 5 included active R6 extract but no acetyl-CoA. In each case, the reaction was terminated by the addition of ethyl acetate and the products were extracted as described in “Experimental Procedure.” An aliquot representing one-twentieth of the extracted material was applied at the origin of alumina thin layer sheets for chromatography in System A of Table II. CM, X-chloramphenicol; A, B, the two radioactive products formed in the complete system.

![Graph](https://example.com/graph.png)

**Fig. 2.** Time course of the reaction. Crude extract from E. coli R6 was incubated with 14C-chloramphenicol as described in Fig. 1, except that acetyl-CoA was replaced by the following reagents: Mg-acetate, 10 mM; KCl, 60 mM; ATP, 4 mM; CoA, 0.04 mM; phosphoenolpyruvate, 10 mM; and pyruvate kinase, 30 μg per ml. Each incubation was stopped at the time indicated by extraction with ethyl acetate as described in “Experimental Procedure.” The presence of 14C-chloramphenicol and its radioactive products was determined by autoradiography. After scintillation counting of the eluted material, the yield of chloramphenicol or products was calculated from the known specific activity of the starting material. Chromatography was carried out with System A of Table II.

<table>
<thead>
<tr>
<th>Source of crude extract</th>
<th>Radioactivity corresponding to 14C-chloramphenicol</th>
<th>Radioactivity</th>
<th>Ratio of acetyl to chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive strain (K-10)</td>
<td>Chloramphenicol</td>
<td>2720 dpm</td>
<td>0.015 μmol 14C-acetate</td>
</tr>
<tr>
<td>Resistant strain (R6)</td>
<td>Compound A</td>
<td>108,200 dpm</td>
<td>0.57 μmol 14C-acetate</td>
</tr>
<tr>
<td></td>
<td>Compound B</td>
<td>139,600 dpm</td>
<td>0.73 μmol 14C-acetate</td>
</tr>
</tbody>
</table>

**Table I**

**Stoichiometry of 14C-chloramphenicol acetylation with H-acetate**

Crude extracts of sensitive (K-10) and resistant (R6) strains were incubated with H-acetate (191,000 dpm per μmol) and 14C-chloramphenicol (15,000 dpm per μmol) for 20 min at 37°. Each incubation contained the following in a final volume of 1 ml: 100 μmol of Tris-HCl (pH 7.8), 10 μmol of MgCl₂, 60 μmol of KCl, 0.04 μmol of CoA, 4 μmol of ATP, 10 μmol of phosphoenolpyruvate, 0.05 μg of pyruvate kinase, 0.1 μmol of 14C-chloramphenicol, 2 μmol of H-acetate, and 5 mg of crude extract protein. The reaction mixture was extracted as described in “Experimental Procedure” and 0.02 μmol of the extracted products was chromatographed on alumina thin layer sheets in System A of Table II. No radioactive compounds other than chloramphenicol were detected from the radioautographs prepared from the incubation containing the sensitive (K-10) extract, and no unreacted chloramphenicol could be detected after incubation with the resistant (R6) extract. The areas of radioactivity were cut from the thin layer sheets and eluted into Bray’s solution for scintillation counting in a Packard Tri-Carb scintillation spectrometer.
identification of products of chloramphenicol acetylation

Ascending chromatography was with the use of thin layer sheets as described under "Experimental Procedure" with the materials and solvents as follows: A, alumina in benzene-methanol (85:15, v/v); B, silica gel in water-saturated ethyl acetate; C, silica gel in chloroform-methanol (95:5, v/v). The mobilities indicated were determined under the same ambient conditions of temperature and humidity. The absolute RF values have been shown to be sensitive to extreme fluctuations of the latter, but, in instances in which changes have been noted, the efficiency of separation of the derivatives has remained unchanged.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RF in chromatographic system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>0.31 0.41 0.27</td>
</tr>
<tr>
<td>1-Acetoxy chloramphenicol</td>
<td>0.54 0.53 0.54</td>
</tr>
<tr>
<td>3-Acetoxy chloramphenicol</td>
<td>0.54 0.59 0.61</td>
</tr>
<tr>
<td>1,3-Diacetoxy chloramphenicol</td>
<td>0.71 0.60 0.79</td>
</tr>
<tr>
<td>Chloramphenicol monoacetate</td>
<td>0.54 0.59 0.61</td>
</tr>
<tr>
<td>Chloramphenicol diacetate</td>
<td>0.71 0.66 0.79</td>
</tr>
</tbody>
</table>

ment of 14C-chloramphenicol and both radioactive products with dilute alkali in acetone at 0° (see "Experimental Procedure") yielded in each case a single radioactive compound which was indistinguishable from chloramphenicol in the chromatographic systems of Table II. It seemed likely, therefore, that the acetates of Compounds A and B were in O-ester linkage.

Both chromatographic and spectral techniques were utilized to clarify the structures of Products A and B. When radioactive Product B was cochromatographed with authentic 1,3 diacetoxy chloramphenicol, the radiographic density corresponded with the location of the reference compound in all three solvent systems of Table II. The identification of Product A as 3-acetoxy chloramphenicol seemed likely when the radioactive monoacetoxy derivative cochromatographed with the authentic material in Systems B and C of Table II. The likelihood that the bulk of Compound A was the 3-acetoxy compound was enhanced by a comparison of the ultraviolet absorption spectra of the monoacetyl enzymatic product and the authentic compounds. As can be seen in Fig. 4, the enzymatically formed material exhibits the absorption maximum at 271 μm expected for 3-acetoxy chloramphenicol, whereas the 1-acetoxy isomer and chloramphenicol absorb maximally at 267 and 274 μm, respectively. Although the cumulative evidence suggested that Compound A was solely the 3-acetoxy derivative of chloramphenicol, it has been possible to show by autoradiography with more prolonged exposure times that small amounts of radioactive product with the chromatographic behavior of 1-acetoxy chloramphenicol have been formed in typical incubations such as that described in Fig 1. In no instance has such material accounted for more than 5% of the radioactivity attributed to Compound A. Variations in pH of the reaction over the range 6 to 9 (Tris-maleate buffer) have failed to influence the formation of the presumed 1-acetoxy compound, but have revealed that formation of the diacetoxy derivative (Compound B) is progressively enhanced by increases in pH above 7.

Specificity of Reaction—Since the crude extract prepared from E. coli R4 was capable of activating acetate to acetyl-CoA in the presence of the appropriate cofactors, a preliminary study was made of the ability of such a preparation to activate other fatty acids and transfer the corresponding acyl groups to chloramphenicol. Fig. 5 illustrates the thin layer chromatographic results of such an experiment. Both the expected monoacetyl and diacetyl derivatives of chloramphenicol are observed in the presence of acetate, and trace amounts of the monoacetate may be seen in the absence of added acetate. In the presence of propionate, however, two additional areas of radioactivity can be seen running ahead of the corresponding acetate derivatives. As in the case of the acetoxy chloramphenicols, the radioactive products
FIG. 5. Ability of other compounds to substitute for acetate as acyl donors in the enzymatic assay. Reaction conditions were identical with those of Fig. 2 except that MgCl₂ (10 mM) and a fatty acid (10 mM) replaced magnesium acetate in the incubation mixture. After 30 min of incubation, the products were extracted with ethyl acetate and chromatographed in System A as previously described.

![Diagram of CHLORAMPHENICOL-3-ACETATE](attachment:image)

**TABLE III**

Spectrophotometric assay of acetyl acceptor activity of chloramphenicol congeners

Various isomers and analogues were compared as to their ability to substitute for D-threo-chloramphenicol in the spectrophotometric assay of acetyl-CoA disappearance. The reaction was followed by the decrease in absorbance at 232 µm resulting from the presence of enzyme, acetyl-CoA, and a suitable chloramphenicol analogue. Both reference and sample cuvettes contained 100 pmoles of Tris-HCl (pH 7.5), 0.1 µmole of a chloramphenicol congener, and 0.1 mg of Sephadex-treated enzyme protein in a final volume of 1 ml. The reaction was carried out in a Beckman DB spectrophotometer at 37°C and was started by the addition of 0.1 µmole of acetyl-CoA to the sample cuvette only. In each experiment, a correction in rate has been made for any disappearance noted in the absence of substrate. In no experiment was the endogenous rate of hydrolysis in excess of 5 pmoles per min per mg of protein. Calculations were based on a decrease in optical density of 0.45 at 232 µm for the cleavage of 0.1 µmole of acetyl-CoA (14).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rate of disappearance of acetyl-CoA</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Stereoisomer of chloramphenicol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-threo</td>
<td>575</td>
<td>100</td>
</tr>
<tr>
<td>L-threo</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>D-erythro</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>B. Analogue with p-phenyl substitution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-NO₂(chloramphenicol)</td>
<td>525</td>
<td>100</td>
</tr>
<tr>
<td>p-CH₃SO₂⁻</td>
<td>355</td>
<td>68</td>
</tr>
<tr>
<td>p-CH₃CO⁻</td>
<td>425</td>
<td>81</td>
</tr>
<tr>
<td>C. Analogue with substitution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None at 1-hydroxyl; CH₃CHCO⁻ at 2-amino; none at 3-hydroxyl (chloramphenicol)</td>
<td>480</td>
<td>100</td>
</tr>
<tr>
<td>None at 1-hydroxyl; HOCH₂CO⁻ at 2-amino; none at 3-hydroxyl (chloramphenicol)</td>
<td>350</td>
<td>73</td>
</tr>
<tr>
<td>None at 1-hydroxyl; none at 2-amino; none at 3-hydroxyl (chloramphenicol)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CH₃CO⁻ at 1-hydroxyl; CH₃CHCO⁻ at 2-amino; none at 3-hydroxyl (chloramphenicol)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CH₃CO⁻ at 1-hydroxyl; CH₃CHCO⁻ at 2-amino; CH₃CO⁻ at 3-hydroxyl (chloramphenicol)</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>CH₃CO⁻ at 1-hydroxyl; CH₃CHCO⁻ at 2-amino; CH₃CHCO⁻ at 3-hydroxyl (chloramphenicol)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

formed in the presence of propionate were also cleaved by mild alkaline conditions and yielded unesterified ¹⁴C-chloramphenicol.

The specificity of the acetyl acceptor has been studied by two techniques: (a) the use of nonradioactive congeners of chloramphenicol and ¹⁴C-acetate in the chromatographic assay and (b) a more convenient spectrophotometric assay based on the chloramphenicol-dependent disappearance of acetyl-CoA described by Equation 1.

\[
\text{Chloramphenicol + acetyl-CoA} \rightarrow \text{acetoxy chloramphenicol + CoA-SH} \tag{1}
\]

Fig. 6 depicts the decrease in absorbance at 232 µm when
and conventional kinetic analysis (16), a rate constant has been calculated on the basis of the reported differences in the rate of formation of products (15). With the use of the spectrophotometric assay of Rg extracts, it was observed that the rate of acetyl-CoA disappearance was not dependent on the presence of chloramphenicol or its acetoxy derivatives as indicated. Tris-HCl (pH 7.8) was present at a concentration of 100 mM. The reaction was carried out in a final volume of 0.5 ml for 10 min at 37°. The reaction was extracted with ethyl acetate and chromatographed in System B of Table II. Under such conditions, two major products (3-acetoxy chloramphenicol and the 1,3-diacetoxy derivative) and a minor density (1-acetoxy chloramphenicol) may be seen after incubation of chloramphenicol with the complete system. The slow moving faint density seen in all incubations was not dependent on the presence of chloramphenicol or its derivatives and was not identified.

The demonstration that extracts prepared from chloramphenicol-resistant E. coli can acetylate chloramphenicol is of interest as the major products, 3-acetoxy chloramphenicol and 1,3-diacetoxy chloramphenicol, lack antibacterial activity. No information is available as to which derivative predominates in whole cells when resistant organisms are grown on chloramphenicol. Whether other modifications of the chloramphenicol molecule occur after acetylation has not been determined, but cleavage of the dichloroacetamide moiety and reduction of the aromatic nitro group have been reported under conditions of prolonged incubation (20).

The present studies indicate that the main pathway for chloramphenicol inactivation involves the stepwise synthesis of a monoacetoxy derivative prior to the formation of 1,3-diacetoxy chloramphenicol. The chromatographic and spectral studies suggest that it is the 3-acetoxy derivative which accumulates. Incubation of 14C-acetyl-CoA with 1-acetoxy chloramphenicol failed to yield a detectable product, whereas the presence of the 3-acetoxy derivative led to the formation of the expected radioactive 1,3-diacetoxy chloramphenicol.

**DISCUSSION**

The demonstration that extracts prepared from chloramphenicol-resistant E. coli can acetylate chloramphenicol is of interest as the major products, 3-acetoxy chloramphenicol and 1,3-diacetoxy chloramphenicol, lack antibacterial activity. No information is available as to which derivative predominates in whole cells when resistant organisms are grown on chloramphenicol. Whether other modifications of the chloramphenicol molecule occur after acetylation has not been determined, but cleavage of the dichloroacetamide moiety and reduction of the aromatic nitro group have been reported under conditions of prolonged incubation (20).

The present studies indicate that the main pathway for chloramphenicol inactivation involves the stepwise synthesis of a monoacetoxy derivative prior to the formation of 1,3-diacetoxy chloramphenicol. The chromatographic and spectral studies suggest that it is the 3-acetoxy derivative which accumulates. Furthermore, the radioisotopic (Fig. 7) and spectrophotometric
(Table III) assays of acetyl acceptor activity indicate a preference for the 3-acetoxy compound. Several observations suggest, however, that the mechanisms of the over-all reaction may be more complicated. The detection of small amounts of a radioactive derivative with the chromatographic characteristics of 1-acetoxy chloramphenicol raises the question of whether it represents the intermediate or is the product of a side reaction as described by the pathway shown in Scheme 1.

\[
\text{Chloramphenicol} \rightarrow \text{3-acetoxy chloramphenicol} \rightarrow 1,3\text{-diacetoxy chloramphenicol} \]

\[
\downarrow \text{1-acetoxy chloramphenicol}
\]

**Scheme 1**

The second disturbing observation concerns the results depicted in Fig. 7 where it may be seen that when \(^{14}C\)-acetyl-CoA is incubated with unlabeled 3-acetoxy chloramphenicol and enzyme there is evidence for the formation of a small amount of the radioactive monoester as well as the expected diacetoxy compound. No evidence has been obtained to suggest that the 3-acetoxy compound is enzymatically decarboxylated to chloramphenicol. The possibility that chloramphenicol might be generated from the monoester by a dismutation-like reaction (Equation 2) was examined with enzymatically prepared \(\alpha\)-acetoxychloramphenicol.\(^{14}\)

\[
\text{1-acetoxy chloramphenicol} \rightarrow \text{3-acetoxy chloramphenicol} + \text{chloramphenicol (2)}
\]

The high degree of specificity exhibited by the acetylating system of the R\(_a\) strain is striking and deserves more thorough study. It is clear from the data of Table III, however, that the optimal acetyl acceptors are closely related structurally and stereochemically to biologically active \(\alpha\)-threo-chloramphenicol. Of some interest also is the observation that the presence of an acetoxy substituent at C-1 adversely affects the enzymatic acetylation of the C-3 hydroxyl. Jardetzky (21) has presented evidence for the conformation of chloramphenicol in solution and has concluded that the hydroxyl groups are hydrogen bonded and must contribute to the stability of the preferred conformation.

The suggestion that propionate may serve as an acyl donor in the enzymatic reaction deserves further study. The limited ability of *E. coli* extracts to activate short chain fatty acids to the corresponding acyl-CoA derivatives (22) has limited the usefulness of experiments such as that described in Fig. 5. The spectrophotometric assay of acyl thioester cleavage in the presence of enzyme, chloramphenicol, and various acyl-CoA compounds should prove to be a more suitable technique.

The origin and mode of expression of transmissible drug resistance factors remain obscure although distinct mechanisms of resistance to several antibiotics are now well defined (8, 23). A recent report of Falkow *et al.* (6) has linked satellite bands of nonchromosomal DNA with specific epimodal determinants of resistance in R factor strains of enteric bacteria. Of special interest was the finding that chloramphenicol resistance is associated with a specific DNA band of a density of 1.716 g cm\(^{-3}\).

Although it is clear that the genetic information for expression of chloramphenicol resistance may be encoded in such DNA, at least two possible mechanisms may be suggested. Either the satellite DNA related to chloramphenicol resistance contains the information dictating the synthesis of *de novo* of chloramphenicol \(O\)-acyltransferase\(^{14}\) or its presence confers on the recipient cell the ability to produce an excess of enzyme over that normally synthesized. The possibility that the latter case is the example may be inferred from Fig. 1, wherein it may be noted that crude extract prepared from the chloramphenicol-sensitive (K-10) strain catalyzes the formation of small amounts of a radioactive compound having the chromatographic behavior of 3-acetoxy chloramphenicol. The material is not a radioactive contaminant as judged by its absence in incubations that contain no enzyme or heat-denatured extract. In any case, no further statement may be made regarding this observation until a survey of extracts from other sensitive strains has been carried out.

**Acknowledgments**—The author wishes to thank Dr. Mildred Rebstock for her generous gifts of chloramphenolic congeners and for helpful advice regarding the identification of chloramphenicol derivatives. The encouragement and support of Dr. Donald Topley enabled the author to undertake the present study, and they are gratefully acknowledged.

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