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

W. V. Shaw‡

From the Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, New York 10032

**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.

Drug inactivation, altered cell permeability, and resistant target enzyme systems have been invoked as possible mechanisms underlying the drug resistance conferred by the presence of R factors (1, 2). The ability of chloramphenicol to inhibit the cell-free synthesis of polypeptides was used by Okamoto and Mizuno to test for the presence of subcellular resistance in R factor strains of *E. coli* (7). Although the initial studies suggested that extracts of such strains were fully sensitive to the antibiotic, a recent report indicated that resistant extracts were obtained if acetyl was present in the incubation mixture (8). The inactivation process was followed by bioassay of antibacterial activity, and the reaction was found to require adenosine triphosphate, Mg++, and coenzyme A as well as a protein fraction obtained after centrifugation of extracts at 100,000 × g. Furthermore, the above cofactor requirements could be replaced by the addition of acetyl-CoA. The present studies were undertaken to define more precisely the products of the inactivation process and to explore the enzymatic mechanisms involved.

**EXPERIMENTAL PROCEDURE**

*Bacterial Strains and R Factors—Resistance transfer factor Rs (CMR, TCR) (resistant to chloramphenicol and tetracycline) was obtained by segregation from a multiple resistant factor Rg and was provided by Dr. T. Watanabe. The factor Rs (CMR, TCR) was obtained in *E. coli* CSH-2, a methionine-requiring auxotroph of strain K-12. For the present studies, R factor was transferred by mixed cultivation to *E. coli* K-10 (9) by a modification of the methods described by Watanabe (1). Cultures of the donor and recipient strains were grown in nutrient broth to the midlogarithmic phase of growth, and equal aliquots were mixed and allowed to incubate 3 hours at 37° in the presence of an equal volume of unoinoculated sterile medium. The bacterial cells were collected by centrifugation and washed twice with 0.85% sodium chloride solution. An aliquot of the sodium chloride suspension was streaked on agar plates prepared from a mineral salts and glucose medium (10) containing 50 μg per ml of chloramphenicol. Colonies were picked from the chloramphenicol-minimal media plates and transferred to nutrient broth for further studies. When tested for their ability to grow in the presence of chloramphenicol, the wild type (K-10) and resistant strains (Rs) were inhibited 50% by 3 × 10⁻⁴ M and 3 × 10⁻⁴ M concentrations, respectively. All assays of antibiotic sensitivity and growth of the organism for enzyme preparation were carried out at 37° in Penassay (Difco) broth on a rotary shaker.*

Natural 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.

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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 × g for 30 min was used without further treatment (crude extract). For the spectrophotometric assay experiments, 1.5 ml of the crude extract was passed over a Sephadex G-50 column (20 × 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 extract 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 acetyl-CoA 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.

Isotopel Assay of Chloramphenicol Acetylation—The fate of chloramphenicol was studied initially by a modification of the incubation conditions described by Okamoto and Suzuki (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 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 monoacetyl and diacetyl derivatives of chloramphenicol. To test this possibility, [14C]-chloramphenicol was incubated with [2H]-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

1 W. V. Shaw, unpublished experiments.

2 Chloramphenicol refers to d-threo-2-dichloroacetamido-1-p-nitrophenyl 1,3 propandiol.
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-

![Diagram](https://via.placeholder.com/150)

**Fig. 1.** Autoradiographs showing the dependence of $^{14}C$-chloramphenicol acetylation on the presence of appropriate cell extracts and acetyl-CoA. The complete system consisted of 50 nmoles of Tris-HCl (pH 7.8), 0.1 n mole of acetyl-CoA, 0.05 n mole of $^{14}C$-chloramphenicol (0.8 $\mu$C per n mole), and 2 mg of the appropriate 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 Rsh. Incubation 4 contained Rsh enzyme which had been inactivated by prior heating at 90° for 10 min, whereas 5 included active Rsh 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, $^{14}C$-chloramphenicol; A and B, the two radioactive products formed in the complete system.

**Fig. 2.** Time course of the reaction. Crude extract from E. coli Rsh was incubated with $^{14}C$-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 $\mu$g per ml. Each incubation was stopped at the time indicated by extraction with ethyl acetate as described in "Experimental Procedure." The presence of $^{14}C$-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 I** Stoichiometry of $^{14}C$-chloramphenicol acetylation with $\alpha$H-acetate

<table>
<thead>
<tr>
<th>Source of crude extract</th>
<th>Radioactivity corresponding to</th>
<th>Radioactivity due to $\alpha$H-acetate recovered</th>
<th>Radioactivity due to $^{14}C$-chloramphenicol recovered</th>
<th>Ratio of acetate to chloramphenicol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive strain (K-10)</td>
<td>Chloramphenicol</td>
<td>2790</td>
<td>0.15</td>
<td>13,400</td>
</tr>
<tr>
<td>Resistant strain (Rsh)</td>
<td>Compound A</td>
<td>108,200</td>
<td>0.57</td>
<td>8,600</td>
</tr>
<tr>
<td></td>
<td>Compound B</td>
<td>139,600</td>
<td>0.73</td>
<td>5,800</td>
</tr>
</tbody>
</table>

Crude extracts of sensitive (K-10) and resistant (Rsh) strains were incubated with $^{14}C$-chloramphenicol (191,000 dpm per $\mu$mole) and $^{14}C$-chloramphenicol (15,000 dpm per $\mu$mole) for 20 min at 37°. Each incubation contained the following in a final volume of 1 ml: 100 nmoles of Tris-HCl (pH 7.8), 10 $\mu$moles of MgCl2, 60 $\mu$moles of KCl, 0.04 $\mu$moles of CoA, 4 $\mu$moles of ATP, 10 $\mu$moles of phosphoenolpyruvate, 0.05 $\mu$moles of pyruvate kinase, 0.1 $\mu$ moles of $^{14}C$-chloramphenicol, 2 $\mu$moles of $^{14}C$-acetate, and 5 $\mu$g of crude extract protein. The reaction mixture was extracted as described in "Experimental Procedure," and 0.02 aliquot 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 (Rsh) 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.
Silica gel in chloroform-methanol (95:5, v/v). The mobilities of the derivatives has remained unchanged. In which changes have been noted, the efficiency of separation of the 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 R5 was capable of activating 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 acetyl. 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

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### Table II

**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 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 0.66 0.79</td>
</tr>
<tr>
<td>Chloramphenicol monoacetate (enzymatic)</td>
<td>0.54 0.59 0.61</td>
</tr>
<tr>
<td>Chloramphenicol diacetate (enzymatic)</td>
<td>0.71 0.66 0.79</td>
</tr>
</tbody>
</table>

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ment of 14C-chloramphenicol and both radioactive products with dilute alkali in acetone at 0°C (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

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![Fig. 4. Absorption spectrum of the enzymatically prepared radioactive monoacetoxy derivative of 14C-chloramphenicol compared with the spectra of related reference compounds.](http://www.jbc.org/)

The monoacetoxy product was isolated from an incubation with R5 cell extract similar to that described in Fig. 1. After preparative thin layer chromatography on alumina sheets in System A, the appropriate area of radioactivity was located by autoradiography and was eluted with ethanol. The concentration of the product was adjusted to that of the reference compounds by comparing its radioactivity with the known specific activity of the substrate chloramphenicol. Spectra were determined in absolute ethanol with a Cary recording spectrophotometer.
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.

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.

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 μmoles of Tris-HCl (pH 7.8), 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 μmoles 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>365</td>
<td>68</td>
</tr>
<tr>
<td>p-CH &amp; O-</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; HOCH₂-CO- at 2-amino; none at 3-hydroxyl</td>
<td>350</td>
<td>73</td>
</tr>
<tr>
<td>None at 1-hydroxyl; none at 2-amino; none at 3-hydroxyl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CH₃CO- at 1-hydroxyl; CH₃CHCO- at 2-amino; none at 3-hydroxyl</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CH₃ &amp; O- at 1-hydroxyl; CH₃CHCO- at 2-amino; CH₃CO- at 3-hydroxyl</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>CH₂CO- at 1-hydroxyl; CH₂ &amp; O- at 2-amino; CH₂CO- at 3-hydroxyl</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.

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

Fig. 6 depicts the decrease in absorbance at 232 μm when
acetyl-CoA is incubated in the presence of Sephadex-treated enzyme and chloramphenicol. Under the assay conditions, there is negligible loss of acetyl-CoA in the absence of a suitable acceptor. The initial rates of acetyl-CoA disappearance have been calculated on the basis of the reported differences in the extinction coefficient at 232 nm of acetyl-CoA and its hydrolysis products (15). With the use of the spectrophotometric assay and conventional kinetic analysis (16), a $K_m$ of $1.5 \times 10^{-3}$ M was calculated for chloramphenicol. Table III summarizes the results obtained when a number of related compounds were substituted for chloramphenicol in the spectrophotometric assay. The specificity of the enzyme for the $\alpha$-three form of chloramphenicol is interesting since the three remaining stereoisomers are essentially devoid of antibacterial activity (17). The same observation may be made for the acyl requirement at the 2-amino position, as the free amine related to chloramphenicol is also inactive as an antibiotic (13). The correlation is not absolute, however, as the glycolic acid amide of chloramphenicol base is an effective acceptor of acetate but possesses only 4% of the activity of chloramphenicol (18). Although the earlier chromatographic data clearly suggested that the 1,3-diacyloxy compound might be formed from 3-acetoxy chloramphenicol, the data of Table III, Experiment C, indicate a substantial difference in the rate of acetylation of chloramphenicol and the 3-acetoxy derivative.

In contrast, reference to the time course experiment described in Fig. 2 indicates a less striking difference in the rates of formation of products from chloramphenicol and the monoacetoxy derivative, respectively. The experiments may not, however, be strictly comparable in that Fig. 2 depicts the synthesis of products from ATP, acetate, and chloramphenicol, whereas Table III summarizes kinetic data for the enzymatic acetylation of chloramphenicol congeners by acetyl-CoA. Subsequent experiments similar to that of Fig. 2 in which an excess of acetyl-CoA was added directly have suggested that the acetyl donor may have been limiting during the early phase of the experiment described in Fig. 2.

Each of the nonradioactive chloramphenicol analogues which appeared to serve as acetyl acceptors in the spectrophotometric assay was also tested by the chromatographic technique after incubation in the presence of $^{14}$C-acetate, enzyme, and the appropriate cofactors. In each instance, evidence was obtained for the formation of one or two radioactive products which were distinct from the nonradioactive products used, but the lack of appropriate acetoxy reference compounds has precluded the definitive identification of some of the products. A direct comparison of the acetyl acceptor activity of the isomeric monoacetoxy reference compounds is seen in Fig. 7. Incubation of $^{14}$C-acetyl-CoA with 1-acetoxy chloramphenicol failed to yield a diacetyl product, whereas the presence of the 3-acetoxy derivative led to the formation of the expected radioactive 1,3-diacyloxy 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$^2$, and 1,3-diacyloxy chloramphenicol (19), 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-diacyloxy chloramphenicol. The chromatographic and spectral studies suggest that it is the 3-acetoxy derivative which accumulates. Furthermore, the radioisotopic (Fig. 7) and spectrophotometric

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$^2$ When assayed against *E. coli K-10*, chloramphenicol and 3-acetoxy chloramphenicol inhibited the rate of growth by 59% at concentrations of $5 \times 10^{-4}$ M and $1.9 \times 10^{-4}$ M, respectively. Less than 45% inhibition was observed at $5 \times 10^{-4}$ M with 1,3-diacyloxy chloramphenicol. W. V. Shaw, unpublished experiments.
Chloramphenicol -> 3-acetoxy chloramphenicol

1,3-diaceotoxy chloramphenicol

Scheme 1

The second disturbing observation concerns the results depicted in Fig. 7 where it may be seen that when 14C-acetyl-CoA is incubated with enzyme, there is evidence for the formation of a small amount of the radioactive monoacetoxy derivative as well as the expected diacetoxy compound. No evidence has been obtained to suggest that the 3-acetoxy compound is enzymatically decacylated to chloramphenicol. The possibility that chloramphenicol might be generated from the monoester by a dismutation-like reaction (Equation 2) was examined with enzymatically prepared 3-acetoxy chloramphenicol-3-14C. When incubated with enzyme but in the absence of acetyl-CoA, there was no evidence for the formation of 14C-chloramphenicol or the radioactive diacetoxy compound resulting from such a dismutation.

2 Monoacetoxy chloramphenicol

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The high degree of specificity exhibited by the acetylating system of the Rf 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 3,3'-chloramphenicol. Of some interest also is the observation that the presence of an 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.

Chloramphenicol -> 3-acetoxy chloramphenicol

1,3-diaceotoxy chloramphenicol

Scheme 1

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 the 3-acetoxy compound 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 is the case 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 chloramphenicol congeners and for helpful advice regarding the identification of chloramphenicol derivatives. The encouragement and support of Dr. Donald Tapley enabled the author to undertake the present study, and they are gratefully acknowledged.

REFERENCES


4 The trivial name of chloramphenicol O-acetyltransferase is abbreviated by the acronym DATTA.

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