The Enzymatic Acetylation of Chloramphenicol by the Multiple Drug-resistant *Escherichia coli* Carrying R Factor

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**SUMMARY**

The chloramphenicol-inactivating enzyme in the multiple drug-resistant *Escherichia coli* carrying R factor produced two products from chloramphenicol in a cell-free system as well as in whole cells.

Thin layer chromatography on silica gel, infrared spectroscopy, gas chromatography, and nuclear magnetic resonance analysis were carried out with the products. These products were identified as D-threo-2-dichloroacetamido-1-(p-nitrophenyl)-1-hydroxy-3-acetoxypropane and D-threo-2-dichloroacetamido-1-(p-nitrophenyl)-1,3-diacetoxypropane.

It was shown that a chloramphenicol-resistant *E. coli* B selected in vitro and a sensitive strain of *E. coli* K-12 have no capacity to produce acetyl derivatives of chloramphenicol.

Studies of the substrate specificity, heat stability, pH optimum, and chromatographic behavior of the chloramphenicol-acetylating enzyme from the multiple drug-resistant *E. coli* were carried out.

The chloramphenicol-acetylating enzyme from the multiple drug-resistant *E. coli* produces a 3-O-acetyl derivative of chloramphenicol and converts the former to a 1,3-O,0-diacetyl derivative. The enzyme which produces the monoacetetyl derivative was purified 120-fold, while the enzyme which produces the diacetetyl derivative from the monoacetetyl derivative was purified about 50-fold by the same purification procedures. It was not possible to decide whether one or two enzymes participate in the acetylation of chloramphenicol.

When the multiple drug-resistant *E. coli* cells were converted to spheroplasts by lysozyme and ethylenediaminetetraacetate, the chloramphenicol-acetylating enzyme was not released into the medium under conditions in which the bulk of ribonuclease was released but 8-galactosidase was not.

In our previous paper it was reported that the multiple drug-resistant *Escherichia coli* carrying R factor has drug-inactivating enzymes for chloramphenicol, dihydrostreptomycin, and kanamycin (1). We have also reported that the chloramphenicol-resistant strains of *Staphylococcus aureus* isolated from clinical materials have a similar chloramphenicol-inactivating enzyme (2). Based on the requirement of acetyl coenzyme A as a co-factor for inactivation of chloramphenicol, acetylation of the drug was proposed as the mechanism of inactivation (1). The present paper deals with the confirmation of the proposed mechanism by identifying the products of chloramphenicol inactivation, and reports on the purification and characterization of the chloramphenicol-inactivating enzyme from *E. coli* carrying R factor. A similar independent experiment on the identification of the products has been reported recently by W. V. Shaw (3).

**EXPERIMENTAL PROCEDURE**

**Bacterial Strains**—Details of *E. coli* K-12 CSH (4) and *E. coli* K-12 R5, a derivative of K-12 CSH made resistant to five drugs by transmission of an R factor from a naturally isolated drug-resistant strain of *Shigella sonnei* (5), have been described (1). *E. coli* B CM*, a chloramphenicol-resistant strain, was selected in vitro (6).

**Culture Medium**—The peptone-glucose medium contained 10 g of poly-peptone, 1.0 g of glucose, 3.0 g of NaCl, 0.1 mmole of CaCl2, 1.0 mmole of MgCl2, and 0.32 mmole of KH2PO4 in 1000 ml (pH 7.0). Medium A (7) was used in the experiment involving spheroplast formation. This medium contained 0.12 M Tris-HCl buffer, 0.08 M NaCl, 0.02 M MgCl2, 0.003 M Na2SO4, 0.001 M MgCl2, 2 × 10⁻⁴ M CaCl2, 2 × 10⁻⁴ M ZnSO4, 0.5% glucose, and 0.5% poly-peptone (Daigo Eiyo Company, Osaka, Japan) in a total volume of 1000 ml (pH 7.5).

**Cultural Conditions**—*E. coli* cells were grown in the peptone-glucose medium for 18 hours with shaking at 37°; the culture was diluted about 50-fold with the fresh peptone-glucose medium, and the cells were grown with forced aeration to the late logarithmic phase. The yield of cells was 2 to 3 g per liter, wet weight.

**Buffers**—The standard buffer contained 0.02 M Tris-HCl (pH 7.8), 0.01 M magnesium acetate, 0.06 M KCl, and 0.006 M 2-mercaptoethanol. Elution buffers contained 0.02 M Tris-HCl...
traction method. The heat-denatured sample mentioned above **phenicol** from chloramphenicol was assayed by a benzene extract.

0.40 ml of preheated water and heated for 5 min at 95°C to terminate the reaction. The reaction was carried out as follows:

\[ P = \frac{9B - H}{8} = \frac{10B - (R + H)}{8} = \frac{10R - T}{8} \]

where \( P \) is radioactive counts of inactivated products, \( D \) is the benzene-extractable counts ( = counts measured x 3 ), \( H \) is counts remaining in the reaction mixture after the benzene extraction, and \( T \) is the total counts contained in the 0.1-ml aliquot of the reaction mixture. Since the rate of diacetyl derivative formation by the chloramphenicol-acetylating enzyme is only 1 to 2% of that of monoacetyl derivative formation (see "Results"), an early period in the reaction we could assume the activity obtained to be that of monoacetyl derivative formation. Enzyme activity which converts the monoacetyl derivative to the diacetyl derivative (see "Results") was determined by either a paper or silica gel thin layer chromatographic technique. Ethyl acetate extraction (1.0 ml) from the heat-denatured sample was carried out twice. About 98% of both intact chloramphenicol and inactivated products was extracted by this procedure. The extract was spotted on a paper strip (Toyo Roshi No. 51A, 2 x 40 cm) or silica gel thin layer plate (Eastman Chromagram Sheet), and was developed with the solvents described below. After scanning of the radioactive peaks, the enzyme activity that produced the diacetyl derivative of chloramphenicol was determined by measuring the area under each radioactive peak.

Experiments following the incorporation of \(^{14}C\)-acetate into chloramphenicol and other substrates were carried out as follows. The reaction mixture, containing \(^{14}C\)-acetate, was incubated at 37°C with other cofactors, denatured by heat, and extracted with ethyl acetate. The extracted radioactivity was measured by a liquid scintillation counter, or fractionated by means of paper or silica gel thin layer chromatography, and scanned with the radiochromatogram scanner.

**Assay of Antibiotic Activity of Chloramphenicol**—This method has been described in a previous paper (1).

**Preparation of Spheroplasts**—Spheroplast formation was carried out by the method of Neu and Heppel (8) with a slight modification. An overnight culture of E. coli K-12 R5, 0.6 ml, was inoculated into 30 ml of fresh Medium A and incubated at 37°C with shaking for 3 hours. Thirty minutes before harvesting, isopropyl-\(\beta\)-thiogalactopyranoside was added to a concentration of 5 x 10^-3 M. Cells were harvested by centrifugation in the cold, washed twice with 4 ml of 0.01 M Tris-HCl buffer (pH 7.8), and suspended in 6 ml of 0.03 M Tris-HCl buffer (pH 7.8) containing 8% polyethylene glycol (average molecular weight, 1000) at 17°C (1.3 x 10^9 cells per ml). (These fractions are designated the supernatant, Washing 1, and Washing 2, respectively.) The suspension was supplemented with EDTA, pH 7.5, to a concentration of 0.001 M, followed immediately by sufficient 0.5% lysozyme to give 10 μg per ml. The suspension was kept standing for 25 min at 17°C and centrifuged at 10,000 x g for 10 min. The resultant supernatant (the released fraction) was kept, and the pellet was suspended in 6 ml of water, shaken vigorously to cause lysis of the spheroplasts. The lyso spheroplast suspension was centrifuged, the supernatant (the spheroplast fraction)
was saved, and the pellet was suspended in 2 ml of water and sonically disrupted at 10 kc for 5 min (the residue fraction). Each fraction was assayed for ribonuclease, β-galactosidase, and chloramphenicol-acetylating activity.

**Assay of Ribonuclease**—The method described by Neu and Hoppel (9) was used.

**Assay of β-Galactosidase**—The activity of this enzyme was measured according to the method of Horiiuchi, Tomizawa, and Novick (10).

**Determination of Protein Concentration**—Protein concentration was determined by the biuret method of Gornall, Bardawill, and David (11), and by the Folin method of Lowry et al. (12).

**Column Chromatography**—DEAE-Sephadex A-50 (Pharmacia) was suspended in water, and washed successively with 0.5 N NaOH solution, water, 0.5 N ICl solution, water, 0.5 N NaOH solution, water, and elution buffer. After the pH of the suspension buffer was adjusted and the column was packed, the column was washed with the elution buffer for 3 to 4 hours.

**Inactivation of Chloramphenicol by Intact Cells**—E. coli K-12 R5 cells or B CM1 cells (1 × 10⁹ cells per ml) were sonically disrupted at 10 kc for 5 min (the residue fraction). Each fraction was assayed for ribonuclease, β-galactosidase, and chloramphenicol-acetylating activity.

**Thin Layer Chromatography on Silica Gel**—The silica gel thin layer plate used was an Eastman Chromagram Sheet (100 μ), type K301R (silica gel with fluorescent indicator). The plate was activated by heating at 100° for 15 min, and the extract from the reaction mixture was spotted on it. Development was carried out with benzene-methanol-water (98:2:2, upper phase) for about 90 min. The chromatogram was surveyed for ultra-violet absorption and radioactivity.

**Paper Chromatography**—The paper strip used was Toyo Roshi No. 51 A (2 × 40 cm). The ethyl acetate extract from the reaction mixture was spotted on the paper and developed with a benzene-methanol-water (98:2:2, upper phase) for about 90 min. With this system, some precautions should be taken: all the procedures must be carried out at about 20°, since at higher temperature the monoacetyl derivative of chloramphenicol shows a very high Rf value; immediate use of the shaken solvent must be avoided, since it causes a forward tailing of intact chloramphenicol. The developed paper was scanned for radioactivity according to the method described in the following section.

**Scanning of Radioactivity on Chromatogram and Estimation of Amount of Products**—The chromatogram obtained by thin layer or paper chromatography was scanned for radioactivity with a radiochromatogram scanner of the windowless gas flow type. The percentages of intact chloramphenicol, monoacetyl derivative, and diacetyl derivative were estimated by measuring the area under each peak.

**Radioactivity Measurement with Liquid Scintillation Counter**—The solvent consisted of 9 g of 2,5-diphenyloazole (PPO), 0.2 g of 1,4-bis(2-(5-phenyloxazolyl))benzene (POPOP), 90 g of naphthalene, 100 ml of toluene, and xylene in a total volume of 1000 ml. Into 12 ml of the solvent, 0.1 to 0.3 ml of sample was dissolved and counted in a liquid scintillation counter.

**Analysis of Monoacetyl Derivative by Infrared Spectroscopy, Gas Chromatography, and Nuclear Magnetic Resonance Spectroscopy**—As reported under “Results,” an enzyme fraction A from DEAE-Sephadex column chromatography, Fig. 5b was obtained which has an ability to produce only the monoacetyl derivative from chloramphenicol. Five hundred milligrams of chloramphenicol were inactivated with this enzyme fraction in the complete system for chloramphenicol inactivation described above, and the reaction mixture was extracted with ethyl acetate. The extract was washed successively with 0.5% sulfuric acid solution, 0.5% sodium carbonate solution, and water, and dried over sodium sulfate. The residue obtained upon evaporation of the solvent was dissolved in water, and the solution was extracted with benzene. The benzene extract was washed with water and evaporated. By this procedure residual chloramphenicol that escaped inactivation was completely removed, and the residue was considered to be the monoacetyl derivative of chloramphenicol. Crystallization of the monoacetyl derivative was tried but was unsuccessful. With this sample infrared spectroscopy, gas chromatography, and nuclear magnetic resonance spectroscopy were carried out. Infrared absorption spectroscopy was carried out by the Nujol method in a Nippon Bunko apparatus. Gas chromatography was carried out by Yamanoto, Iguchi, and Aoyama according to their method (13). A Varian apparatus was used for nuclear magnetic resonance spectroscopy.

**Chemicals**—Chloramphenicol, 1-threo-2-dichloroacetamidol-1-(p-nitrophenyl)-1,3-propanediol, was generously supplied by Dr. T. Osono, Yamanouchi Pharmaceutical Company; it had a melting point of 150°. The stereoisomer of chloramphenicol, 1-erythro-2-dichloroacetamidol-1-(p-nitrophenyl)-1,3-propanediol, was generously supplied by Dr. M. Nagawa, Sankyo Pharmaceutical Company; it had a melting point of 172°. The 3-O-acetyl derivative of chloramphenicol, 1-threo-2-dichloroacetamidol-1-(p-nitrophenyl)-1-hydroxy-3-acetoxypropone, was generously supplied by Dr. K. Kasuya, Sankyo Pharmaceutical Company; its melting point was 80°; αₚ +3.48 (5% in dioxane). The 1,3-O, O-diaceetyl derivative of chloramphenicol, 1-threo-2-dichloroacetamidol-1-(p-nitrophenyl)-1,3-diaceotoxypropone, was generously prepared and supplied by Dr. Osono and Mr. K. Murakami, Yamanouchi Pharmaceutical Company; its melting point was 140.0-140.5°.

**Identification of Acetylated Products**

**RESULTS**

**Reversion of Inactivated Products to Antibiotically Active Substance by Hydrolysis**—Based on the acetyl-CoA requirement of...
the chloramphenicol-inactivating enzyme from E. coli K-12 R5, we supposed the mechanism of inactivation to be acetylation of the drug (1). If the supposition were true, we could expect the recovery of antibiotic activity by hydrolysis of the inactivated products.

Chloramphenicol (1 mg) was inactivated by the S 100 fraction of K-12 R5 in the complete system described in "Experimental Procedure." The residual chloramphenicol estimated by the assay of antibiotic activity was shown to be below 9% of the original activity. The inactivated products, together with the residual chloramphenicol, were extracted with ethyl acetate, and the solvent was evaporated. The residue obtained was dissolved in acetone. According to the method of Kunz and Hudson (15), the inactivated products were hydrolyzed, and antibiotic activity in the hydrolysate was measured. About 70% of the original antibiotic activity was restored. This result suggests that the inactivated products are mainly the O-acetyl derivative of chloramphenicol if the inactivation occurs through acetylation of the drug, since the N-acetyl derivative should be more resistant than the O-acetyl derivative to hydrolysis under the above conditions.

Separation of Inactivated Products from Chloramphenicol—\(^{14}C\)-Chloramphenicol was incubated with the R5 S 100 fraction and other cofactors, and extracted with ethyl acetate. The extract was fractionated by thin layer chromatography on silica gel. The chromatographic profile obtained displays three radioactive peaks (Fig. 1). The peak labeled CM in Fig. 1 represents residual chloramphenicol and shows antibiotic activity. Peak I and II do not show any antibiotic activity and represent inactivated products, which, as shown below, coincided with the authentic samples, the 3-O-acetyl- and 1,3-O, 0-diacetyl derivatives of chloramphenicol, respectively.

Determination of Amount of Acetyl Residue Incorporated into Inactivated Products—Under the same experimental conditions, two separate experiments were carried out. One system contained \(^{14}C\)-chloramphenicol and \(^{14}C\)-acetic acid with other cofactors in the reaction mixture, while the other contained \(^{14}C\)-chloramphenicol and \(^{14}C\)-acetic acid.

After incubation at 37°C, the reaction was terminated by heating, and extraction was carried out with ethyl acetate. The extractable counts from the latter system were assumed to be the counts incorporated into the inactivated products, since no significant radioactivity was detected in the extract when the reaction was carried out without substrate chloramphenicol or with the CSH S 100 fraction instead of the R5 S 100 fraction. This last assumption was supported by the following result, obtained with thin layer chromatography of the extract. The extract from the complete system containing chloramphenicol, R5 enzyme fraction, and \(^{14}C\)-acetic acid showed only two peaks which coincided with those (I and II in Fig. 1) of inactivated products.

The extract from the system which contained \(^{14}C\)-chloramphenicol was fractionated by thin layer chromatography, and the amounts of each product formed were calculated (Table I). The amounts of acetyl residue incorporated into each product were calculated and are shown in Table I.

These data lead us to the conclusion that the amount of acetyl residue incorporated into 1 mole of Product I is 1 mole, and that incorporated into 1 mole of Product II is 2 moles, indicating that Product I is a monoacetyl derivative and Product II is a diacetyl derivative of chloramphenicol.

Both the carbon atoms of acetic acid were incorporated into the chloramphenicol molecule, since experiments with either 1-\(^{14}C\)- or 2-\(^{14}C\)-acetic acid gave identical results.

Kinetic Studies of Formation of Monoacetyl and Diacetyl Derivatives of Chloramphenicol by Acetylating Enzyme—The enzyme from E. coli K-12 R6 cells produces both a monoacetyl and a diacetyl derivative of chloramphenicol. The relationship between the two products was investigated through kinetic studies of chloramphenicol acetylation.

Chloramphenicol was incubated in the complete system, and the time course of the product formation was followed by paper chromatography. The results are shown in Fig. 2.
experimental conditions, chloramphenicol was converted to the monoacetyl derivative within 1 min by the acetylating enzyme. Then the amount of monoacetyl derivative decreased, and the diacetyl derivative of chloramphenicol increased with time. The results indicate the following process: chloramphenicol → monoacetyl derivative → diacetyl derivative.

Another kinetic study showed that the rate of the reaction producing the monoacetyl derivative was 50 to 100 times greater than that producing the diacetyl derivative (see, for example, the specific activities of Line 1 in Table II).

Analysis of Monoacetyl Derivative by Infrared Absorption Spectroscopy, Gas Chromatography, and Nuclear Magnetic Resonance Spectroscopy—The monoacetyl derivative was prepared as described in “Experimental Procedure” and subjected to each analysis. The infrared absorption spectrum of the sample showed an additional band representing a carbonyl group at 1700 cm\(^{-1}\) compared to that of chloramphenicol. The over-all pattern was similar to that of the authentic 3-O-acetyl derivative of chloramphenicol. Although the result is not itself conclusive for the identification of the product, it is consistent with the above mentioned mechanism of the acetylation of the drug.

Gas chromatography showed that the product coincided with the authentic 3-O-acetyl derivative. The nuclear magnetic resonance spectrum showed an additional 3-proton signal at 7.9 ppm (\(\delta\)) compared to that of chloramphenicol, and all the signals observed were essentially the same as that of the authentic 3-O-acetyl derivative of chloramphenicol.

Detailed Analysis of Products with Thin Layer Chromatography—Using the thin layer chromatographic technique, we carried out a detailed investigation of the acetylated products. Fig. 3, c, d, and f, represents authentic samples of chloramphenicol, 3-O-acetyl derivative, and 1,3-G, O-diacyl derivative, respectively. The 3-O-acetyl derivative gives one major and two trace spots; one of the two traces corresponds to chloramphenicol, and the other is unknown. When the authentic 3-O-acetyl derivative is incubated in 0.10 M Tris-HCl buffer (pH 7.8) at 37°, the unknown spot increases nonsymetrically with time (Fig. 3, d and e) and takes about 5 min to reach a plateau level (about 20% of the major spot, measured spectrophotometrically). The chromatograms of the extract from the complete inactivation system (Fig. 3, a and b) show four spots, three of which correspond to chloramphenicol, 3-O-acetyl derivative, and 1,3-O, O-diacyl derivative. The remaining one corresponds to the authentic 3-0-acetyl derivative (about 20% of the major spot, measured spectrophotometrically).

Purification of chloramphenicol-acetylating enzyme—The reaction mixture contained 50 mg of chloramphenicol (3 X \(10^6\) cpm), 1.2 mg of protein of R5 S 100 fraction, and other co-factors as described in “Experimental Procedure” in a total volume of 0.5 ml. The reaction mixture was incubated at 37°, and 0.05-ml aliquots were taken for assay at the indicated times. The ethyl acetate extracts were fractionated by paper chromatography. The chromatograms were scanned for the radioactive peaks and the amounts of chloramphenicol and monoacetyl and diacetyl derivative were estimated. □, chloramphenicol; ○, monoacetyl derivative of chloramphenicol; ●, diacetyl derivative of chloramphenicol.

Fig. 2. Kinetic studies of the formation of monoacetyl- and diacetyl derivatives of chloramphenicol. The reaction mixture contained 50 mg of chloramphenicol (3 X \(10^6\) cpm), 1.2 mg of protein of R5 S 100 fraction, and other co-factors as described in “Experimental Procedure” in a total volume of 0.5 ml. The reaction mixture was incubated at 37°, and 0.05-ml aliquots were taken for assay at the indicated times. The ethyl acetate extracts were fractionated by paper chromatography. The chromatograms were scanned for the radioactive peaks and the amounts of chloramphenicol and monoacetyl and diacetyl derivative were estimated. □, chloramphenicol; ○, monoacetyl derivative of chloramphenicol; ●, diacetyl derivative of chloramphenicol.

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity forming the first product</th>
<th>Activity forming the second product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Specific activity</td>
</tr>
<tr>
<td>1. S 100</td>
<td>252</td>
<td>0.241</td>
</tr>
<tr>
<td>2. Heated</td>
<td>63.4</td>
<td>0.870</td>
</tr>
<tr>
<td>3. Ammonium sulfate</td>
<td>34.7</td>
<td>1.71</td>
</tr>
<tr>
<td>4. Ethanol</td>
<td>17.4</td>
<td>3.37</td>
</tr>
<tr>
<td>5. A-50</td>
<td>6.7</td>
<td>9.55</td>
</tr>
<tr>
<td>6. A-50 peak</td>
<td>0.2</td>
<td>29.3</td>
</tr>
</tbody>
</table>
derivative after prolonged incubation. The unknown spot is therefore thought to be a nonenzymatic product formed by incubation in the buffer.

From these results and the data described previously, we can conclude that the first product formed by the chloramphenicol-inactivating enzyme is \( \text{d-threo-2-dichloroacetamido-1-(p-nitrophenyl)-1-hydroxy-3-acetoxypropane} \) and the second is \( \text{d-threo-2-dichloroacetamido-1-(p-nitrophenyl)-1,3-diacetoxypropane} \) produced by way of the first.

**Lack of Chloramphenicol-acetylating Enzyme in E. coli K-12 CSH—**Various attempts to detect the chloramphenicol-acetylating activity in a cell-free preparation of E. coli K-12 CSH without R factor gave completely negative results.

**Products Formed by Intact E. coli K-12 R5, K-12 CSH, or B CM* Cells—**The R5 cells were incubated with \( ^{14} \text{C-chloramphenicol} \) under the condition described in “Experimental Procedure.” The ethyl acetate extract from a sonically disrupted culture was fractionated by paper chromatography. The result is shown in Fig. 4. There are two products which correspond to the 3-O-acetyl and 1,3-O,0-diacetyl derivatives of chloramphenicol. The second product observed on inactivation by the enzyme fraction is therefore not an artificial product produced only in a cell-free system.

The results of a similar experiment with a chloramphenicol-sensitive strain of K-12 (0.14 \( \mu \text{g} \) of \( ^{14} \text{C-chloramphenicol} \) and 1 \( \times \) \( 10^{10} \) or 1 \( \times \) \( 10^{14} \) cells per ml) and the resistant B CM* cells selected in vitro did not show any significant acetylated products. Therefore, these strains lack the chloramphenicol-acetylating enzyme and the mechanism of the drug resistance of B CM* is different from that of R5 cells.

**Purification and Characterization of Acetylating Enzyme**

All treatment of the enzyme was performed at 1–4°C unless otherwise noted.

**Simple Assay Conditions for Enzyme Activity That Produces Diacetyl Derivative from Monocetl Derivative—**From the results of the kinetic studies mentioned above, the substrate of the enzyme which produces the diacetyl derivative is assumed to be the monoacetl derivative of chloramphenicol. As we did not have a labeled monoacetl derivative, \( ^{14} \text{C-chloramphenicol} \) and an enzyme fraction which produces the monoacetl derivative only were utilized to supply the labeled monoacetl derivative. Such an enzyme fraction was obtained by ammonium sulfate fractionation (50 to 65% fraction) or column chromatography in the absence of 2-mercaptoethanol (Fraction A of Fig. 5b).

With this system, an assay of the activity producing the diacetyl derivative was possible.

**Partial Purification of Chloramphenicol-acetylating Enzyme—**By using the technique described in “Experimental Procedure,” partial purification of the chloramphenicol-acetylating enzyme was carried out with the S 100 fraction from K-12 R5 as a starting material. The degree of purification at each step is shown in Table II.

The recovery of the activity forming the first product (monoacetl derivative) remained constant throughout the purification procedure, while that of the activity forming the second (diacetyl derivative) decreased gradually. We could not, however, separate both activities even during the last column chromatography. The A-50 peak fraction of the last step in the purification showed a 121- and 47-fold increase in specific activ-
did not require Mg++ in the presence of acetyl-CoA for the acetylation of chloramphenicol.

Heat Stability of Acetylating Enzyme—The activity producing the first product of the 30 to 40% ammonium sulfate fraction (in the standard buffer) was stable up to 60° for 5 min, diminished considerably at 70° for 5 min, and was abolished completely at 80° for 5 min. The enzyme activity was stabilized against heating at 10° by the addition of the substrate chloramphenicol (2 mM).

In order to assay the enzyme activity producing the diacetyl derivative, the 50 to 65% ammonium sulfate fraction was supplied in addition to the CSH S 100 fraction and other required reaction components that were added to the heated enzyme fraction. We could not find any significant difference between the heat stability of the monoacetylated and diacetylated activities.

Substrate Specificity of Acetylating Enzyme—The reaction system is described in “Experimental Procedure.” The radioactivity extracted from the complete reaction system containing 14C-acetic acid was considered to be radioactivity incorporated into the substrate, since no significant radioactivity was extracted in the absence of the acetylating enzyme or a substrate (for example, chloramphenicol). The results of experiments of this type are shown in Table III and are expressed as a rough estimation of strongly positive, positive, or negative and as a percentage of control (choloramphenicol) when possible, because chloramphenicol the extraction efficiency of the various acetylated products of each substrate may be different.

The acetylating enzyme showed comparable activity toward chloramphenicol (D-threo), D-threo-1-(p-nitrophenyl)-2-acetamido-1,3-propanediol, and D-threo-1-(p-methylsulfonylphenyl)-2-acetamido-1,3-propanediol.

### Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degree of incorporation of 14C-acetyl residues into the compound</th>
<th>Percentage of control (chloramphenicol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol, D-threo-1-(p-nitrophenyl)-2-dichloroacetamido-1,3-propanediol</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>DL-erythro Isomer of chloramphenicol</td>
<td>++</td>
<td>100</td>
</tr>
<tr>
<td>D-threo-1-(p-Nitrophenyl)-2-acetamido-1,3-propanediol</td>
<td>++</td>
<td>55</td>
</tr>
<tr>
<td>D-threo-1-(p-Methylsulfonylphenyl)-2-dichloroacetamido-1,3-propanediol</td>
<td>+++</td>
<td>75</td>
</tr>
<tr>
<td>3-O-Acetyl derivative of chloramphenicol</td>
<td>+</td>
<td>1-2</td>
</tr>
<tr>
<td>D-threo-1-(p-Nitrophenyl)-2-amino-1,3-propanediol</td>
<td>+</td>
<td>&lt;5 (2)</td>
</tr>
<tr>
<td>D-threo-2-Amino-3-(p-nitrophenyl)-3-hydroxypropionic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenethyl alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3-Propanediol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ++++, strongly positive; ++, positive; -, negative.

2-dichloroacetamido-1,3-propanediol (Table III). The stereoisomer of chloramphenicol, D-erythro-1-(p-nitrophenyl)-2-dichloroacetamido-1,3-propanediol, was found to be a less active substrate of the acetylating enzyme than chloramphenicol (Table III). The 3-O-acetyl derivative of chloramphenicol was also found to be a substrate; however, the rate of incorporation of the acetyl residue into the compound was faster than expected from the result of the kinetic studies of the acetylation of chloramphenicol. Furthermore, the chromatographic profile of the ethyl acetate extract showed two peaks, one in the location of the 3-O-acetyl derivative and the other in that of 1,3,0-O-diacetyl derivative. These results were considered to mean that there were some contamination of chloramphenicol in the substrate, deacetylation and reacetylation of the substrate, or nonenzymatic migration of an acetyl residue from position 3 to 1 of the propanediol moiety of the substrate.

The radioactive acetyl residue was incorporated into D-threo 1-(p-nitrophenyl)-2-amino-1,3-propanediol at a rate of about 2% of that of chloramphenicol. We measured the ethyl acetate extraction efficiency of the substrate instead of the acylated products, which we could not do, and found it to be about 40% under the condition, described in “Experimental Procedure” at pH 7.8. Therefore, even after maximal correction, the data mentioned above (2%) will not exceed 5% (-2 x 100/40%), because we can expect that the acetylated products of the substrate would be more effectively extracted with ethyl acetate than would be the substrate. Thus, we can conclude that the activity of the acetylating enzyme for this substrate is below 5% of that for chloramphenicol (Table III).

D-threo-2-Amino-3-(p-nitrophenyl)-3-hydroxypropionic acid, phenethyl alcohol, and 1,3-propanediol did not show any sign that they are substrates of the acetylating enzyme (Table III). As for the first of these three compounds, paper chromatography (1-butanol-acetic acid-water, 4:2:1) was carried out after incubation in order to detect any acetylation of the compound in the reaction mixture itself, but we could not find any radioactive peak which was specific for the presence of the acetylating enzyme in the reaction mixture. Further negative checks were not carried out on the remaining two compounds, because it seemed unlikely that acetylated products of these compounds could not be extracted with ethyl acetate.

From these results we assume that the acetylating enzyme shows a considerable specificity for the chloramphenicol molecule. We think it appropriate to call the enzyme that produces the 3-O-acetyl derivative of chloramphenicol acetyl-CoA:chloramphenicol O-acetyltransferase.

Column Chromatographic Behavior of the Acetylating Enzyme on DEAE-Sephadex A-50 in Presence or Absence of β Mercaptoethanol—The 30 to 65% ammonium sulfate fraction dialyzed against the elution buffer containing 0.05 M KCl and 0.006 M 2-mercaptoethanol was loaded on a DEAE-Sephadex A-50 column. The enzyme was fractionated by linear gradient elution of KCl from 0.00 to 0.30 M in the presence of 2-mercaptoethanol. The resultant profile is shown in Fig. 5a. In the presence of 2-mercaptoethanol, both the mono- and diacetylated activities completely overlap each other.

However, in the absence of 2-mercaptoethanol the monoacetylated activity showed two peaks (Fractions A and B of Fig. 5b), of which only the latter (Fraction B) showed diacetylated activity (Fig. 5b). Separate rechromatography of Fractions A and B was carried out also in the absence of 2-mercaptoethanol.
Fraction B did not produce a new fraction corresponding to Fraction A in this rechromatography, and vice versa. Several trials to obtain Fraction B from Fraction A (and vice versa) were unsuccessful. The results of the gel filtration through Sephadex G-100 suggested that Fractions A and B have a similar molecular size.

Localization of Chloramphenicol-acetylating Enzyme—The surface localization of certain enzymes has been reported by Malamy and Horecker (16) and Neu and Heppel (8, 9, 17, 18). It was of interest to know if the chloramphenicol-acetylating enzyme is also located on the cell surface. Spheroplasts were prepared from K-12 R5 cells according to the method of Neu and Heppel (8).

The results are shown in Table IV. About 80% of the ribonuclease was released into medium from spheroplasts, while only 8% of the β-galactosidase and 13% of the chloramphenicol-acetylating enzyme was released. About 80% of these latter two enzymes were retained in spheroplasts. These results indicate that the chloramphenicol-acetylating enzyme is not localized on the cell surface.

**Discussion**

The products formed by the enzyme from the multiple drug-resistant *E. coli* carrying R factor were identified as the 3-O-acetyl and 1,3,6-O,6-diacyethyl derivatives of chloramphenicol. We could also find the 1-O-acetyl derivative of chloramphenicol in the products. These findings are consistent with those of Shaw (3). We may conclude that the 1-O-acetyl derivative is a nonenzymatic product, because an authentic sample of 3-O-acetyl derivative produced 1-O-acetyl derivative when the incubation was carried out in Tris-HCl buffer, and the formation of 1-O-acetyl derivative in an enzymatic system appeared to be independent of the amount of the acetylating enzyme, but dependent on the length of the incubation time. When the nonradioactive 3-O-acetyl derivative was incubated with the acetylating enzyme and labeled acetyl donor, the radioactive 3-O-acetyl derivative was produced as well as the expected 1,3,6-O,6-diacyethyl derivative (see “Results” and Reference 3). The results suggest that there is an acyl migration between the positions 3 and 1 of monoacetyl derivative, or a deacylation of the monoacetyl or diacyethyl derivative. The observation by Shaw (3) that 1-O-acetyl derivative did not accept acetyl residues makes the mechanism of over-all reaction difficult to understand.

We could not find a remarkable amount of other products, for example, a product formed by the reduction of the aromatic nitro group (19); however, we occasionally observed a trace amount of unidentified product, which appeared very near to the origin of the thin layer chromatograms.

The biological reason why the monoacetyl derivative is converted to the diacyethyl derivative by the enzyme from *E. coli* R5 is not yet known. The enzyme activity producing the second product is not essential for the expression of drug resistance. At present we cannot conclude whether one or two enzymes produce the two products in *E. coli* K-12 R5.

The acetylating enzyme showed about 50% specificity toward the DL-erythro isomer of chloramphenicol. According to Shaw (3), the enzyme showed only 1% specificity toward the D-erythro isomer. Thus we can assume that the enzyme has comparable specificity toward chloramphenicol and the D-erythro isomer. Investigations of other substrates have indicated that the sub-

**Table IV**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RNase</th>
<th>β-Galactosidase</th>
<th>Chloramphenicol-acetylating enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Washing 1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Washing 2</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Released</td>
<td>76</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Spheroplast</td>
<td>18</td>
<td>82</td>
<td>79</td>
</tr>
<tr>
<td>Residue</td>
<td>4</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

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![Figure 5](http://www.jbc.org/)

**Figure 5.** Column chromatographic behavior of the acetylating enzyme in the presence and absence of 2-mercaptoethanol. *a*, chromatographic profile of the acetylating enzyme in the presence of 2-mercaptoethanol (0.006 M). The 30 to 65% ammonium sulfate fraction (50 mg of protein) was loaded on a DEAE-Sephadex A-50 column (1 X 16 cm) and fractionated by the elution buffer containing 2-mercaptoethanol (0.006 M) and a linear gradient concentration of KCl from 0.05 to 0.35 M (total volume, 200 ml); 7-ml fractions were collected. *b*, chromatographic profile of the acetylating enzyme in the absence of 2-mercaptoethanol. The 30 to 65% ammonium sulfate fraction (50 mg of protein) was loaded on a DEAE-Sephadex A-50 column (1 X 16 cm) and fractionated by the elution buffer containing a linear gradient concentration of KCl from 0.12 to 0.30 M (total volume, 150 ml); 5-ml fractions were collected. O, activity producing the monoacetyl derivative of chloramphenicol; △, activity producing the diacetyl derivative of chloramphenicol from the monoacetyl derivative.
strate specificity of the acetyling enzyme is very specific for the chloramphenicol molecule (this paper and Reference 3).

We could not find any acetylated products either in cell-free preparations or in whole cells of the chloramphenicol-sensitive strain E. coli K-12 CSH, or in whole cells of B CM' under conditions which allowed the K-12 R5 cells to produce detectable amounts of the acetylated products. Shaw (3) reported an important finding that a chloramphenicol-sensitive strain of E. coli K-10 showed a weak acetylating activity. It will be worthwhile to clarify the situation regarding this point, since it may be relevant to the problem of the origin and mode of expression of the resistance genes of R factor.

It has long been known that E. coli produces another enzyme which can inactivate chloramphenicol by reducing the nitro group (19). This nitroreductase is not specific for chloramphenicol, its capacity to inactivate chloramphenicol is poor, and there is no definite correlation between the enzyme activity and chloramphenicol resistance. In contrast, chloramphenicol is rapidly inactivated by the cell extract as well as by whole cells of K-12 R5.

This chloramphenicol-acetylating enzyme is specific for chloramphenicol. According to our observations, the enzyme is completely absent in K-12 CSH and is only produced by cells with R factor (see Reference 1). Even in the observations of Shaw (3), the correlation of the enzyme activity and chloramphenicol resistance is quite clear. Therefore, it is probable that the chloramphenicol-acetylating enzyme is the basis of chloramphenicol resistance in cells carrying R factor. The results with B CM' indicate that the mechanism of chloramphenicol resistance of E. coli selected in vitro is quite different from that of E. coli K-12 carrying R factor.

The substrate specificity of the chloramphenicol-acetylating enzyme suggests that the origin of this enzyme may be related to the presence of chloramphenicol in natural circumstances, as suggested for penicillinase (20, 21).  

1 Note Added in Proof—It has been shown from our recent study on a temperature-sensitive mutant R factor that the chloramphenicol resistance gene of the R factor controls the synthesis of the chloramphenicol-acetylating enzyme directly and this enzyme is the cause of chloramphenicol resistance of the cell. The enzyme preparation obtained from a strain of E. coli carrying a mutant R factor, which showed temperature-sensitive resistance to chloramphenicol, also showed a heat-labile characteristic (K. Mise and Y. Suzuki, in preparation).

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Escherichia coli Carrying R Factor
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