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A STUDY OF THE ENZYMATIC INACTIVATION OF CHLORAMPHENICOL BY HIGHLY PURIFIED CHLORAMPHENICOL ACETYLTRANSFERASE

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Summary

We report the purification of chloramphenicol acetyltransferase (acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) by a two-step procedure involving chromatography on a Sepharose 4B-reduced chloramphenicol matrix and DEAE-Sephadex A-50. This procedure resulted in a 120-fold purification with 50% recovery of the enzyme. Only one band of enzyme activity was present after electrophoresis on polyacrylamide gel. The enzyme is active over a broad pH range, maximal activity being observed near pH 7.6. Both chloramphenicol 1-acetate or chloramphenicol 3-acetate were found to be very stable in Tris-maleate buffer at pH 6.0 with negligible interconversion. The incubation at pH 6.0 of chloramphenicol 1-acetate with the purified chloramphenicol acetyltransferase yielded chloramphenicol 1,3-diacetate. These data indicate that the enzyme acetylates specifically at the 3-hydroxy position and the diacetylation is possible only because of non-enzymatic interconversion of chloramphenicol 3-acetate to chloramphenicol 1-acetate at higher pH values.

Introduction

Chloramphenicol acetyltransferase (acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) catalyzes the acetylation of chloramphenicol in the presence of CoASAc to yield 3-acetyl and 1,3-diacetyl esters [1,2]. The

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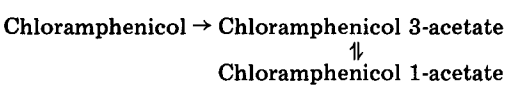
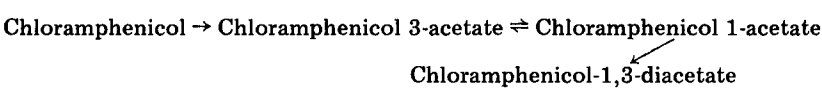
Abbreviations; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazolyl-2)-benzene.

enzyme is a tetramer composed of identical subunits of a molecular weight in the range of 20 000 [3]. In Gram-negative bacteria, three distinct types of chloramphenicol acetyltransferase (I, II and III) have been identified [4,5] based on their affinity for chloramphenicol, their electrophoretic mobility and their immunological properties.

Shaw [1,6] and Shaw and Brodsky [7] have proposed the following scheme for the enzymatic acetylation of chloramphenicol:



More recently [3], the following reaction scheme was postulated:



where the conversion of chloramphenicol 3-acetate to chloramphenicol 1-acetate would be a non-enzymatic process. While this mechanism appears plausible, it has never been proven experimentally.

We wish to describe the purification of chloramphenicol acetyltransferase to homogeneity by a two-step procedure involving affinity chromatography on a Sepharose 4B-reduced chloramphenicol matrix [8] followed by a chromatography on DEAE-Sephadex A-50. We will present evidence that the acetylation by chloramphenicol acetyltransferase is specific for the C-3-hydroxy position of chloramphenicol.

Materials and Methods

Reagents. Chloramphenicol, CoASAc (lithium salt) and 5,5′-dithiobis-2-nitrobenzoic acid were purchased from Sigma Chemical (St. Louis, MO). [^{14}C]-Chloramphenicol (13.3 mCi/mmol) came from New England Nuclear (Boston, MA).

Bacterial strain. Type I chloramphenicol acetyltransferase [9] was extracted from *Escherichia coli* W677/HJR 66, an R-factor-bearing mutant highly resistant to chloramphenicol (minimal inhibitory concentration of 1 mg/ml).

Culture method and preparation of cell-free extract. The culture of *E. coli* W677/HJR 66 and the preparation of the cell-free extract have been described previously [8]. The following modifications were made: the bacteria were collected at midlogarithmic phase (A = 1.5 at 490 nm) and they were disrupted at 0° C by continuous sonication for 15 min at 120 W with a Sonic Dismembrator (Artek System Corporation, Farmingdale, NY).

Chloramphenicol acetyltransferase was assayed by the dithiobisnitrobenzoic acid spectrophotometric method of Foster and Shaw [5] with a Beckman Acta III spectrophotometer [8]. A 0.2 M Tris-maleate buffer was used for the determination of the optimum pH of the enzymatic reaction.

Purification of chloramphenicol acetyltransferase. The first step in the purification consisted of an affinity chromatography using reduced chloramphenicol as the ligand [8]. The procedure was exactly as described previously except that the column was much larger (2.2 × 10 cm), 0.1 M Tris-HCl (pH 7.8)

containing 2 M NaCl was used as the washing buffer and the elution buffer contained 2 mg/ml of chloramphenicol. These modifications allowed the purification of a larger amount of enzyme in a shorter time. The eluted fractions containing more than 1 unit of chloramphenicol acetyltransferase per ml were pooled and dialyzed at 4° C for 24 h against 6 l of 0.1 M Tris-HCl (pH 7.8) containing 0.01 M 2-mercaptoethanol (buffer A) to remove most of the chloramphenicol which could interfere in the following precipitation step. The purified chloramphenicol acetyltransferase was then concentrated by precipitation at a final $(NH_4)_2SO_4$ concentration of 80%. The precipitate was collected by centrifugation, dissolved in the smallest possible volume of buffer A and dialyzed at 4° C for 12 h against 4 l of the same buffer.

The partially purified enzyme was then transferred onto a DEAE-Sephadex A-50 column (1.6 × 20 cm) equilibrated with 0.01 M Tris-HCl (pH 7.8) containing 0.01 M 2-mercaptoethanol at 4° C. The chloramphenicol acetyltransferase preparation (39 ml) was applied to the top of the column and eluted with a linear 0—0.5 M NaCl gradient. Fractions of 10 ml were collected. The linearity of the NaCl gradient was checked by monitoring Na on a flame photometer. Chloramphenicol acetyltransferase containing fractions were pooled and precipitated at 80% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in buffer A. The fractions were kept frozen at —80° C in 0.2 ml aliquots.

All protein determinations were performed according to the method of Lowry et al. [10] with bovine serum albumin as the calibration material except after affinity chromatography when chloramphenicol interferes with this procedure. In that case proteins were precipitated with trichloroacetic acid prior to their measurement.

Gel electrophoresis. We have used the method of Ornstein [11] and Davis [12]. The polyacrylamide gels were stained in Coomassie brillant blue R-250. The measurement of chloramphenicol acetyltransferase activity in the gels was made after the gels were frozen at —80° C and cut in 1 mm slices with a Mickle Gel Slicer (Brinkmann Instruments, Rexdale, Ontario, Canada). The enzyme was eluted from the slice after an incubation of 2 h at 4° C in 0.2 ml 0.1 M Tris-HCl (pH 7.8).

Thin-layer chromatography. Separation and quantitative analysis of and chloramphenicol, chloramphenicol 1-acetate, chloramphenicol 3-acetate and chloramphenicol 1,3-diacetate were performed by thin-layer chromatography (TLC) according to Shaw [1] using silica gel G plastic sheets (20 × 20 cm, 0.5 mm thickness, Mackerey-Nagel, Düren, F.R.G.). The solvent system consisted of chloroform/methanol (95 : 5). After migration, the spots were visualized at 340 nm in a Chromato-Vue (Ultra-Violet Products Inc., San Gabriel, CA) after spraying over the plate 15 ml scintillation cocktail (15.2 g PPO, 380 mg dimethyl POPOP in 3.8 l toluene). This allows us to visualize quantities of chloroamphenicol and chloramphenicol derivatives as small as 0.2 μg pure material. Each spot was localized, cut from the plates and transferred to a vial containing the scintillation cocktail. The radioactivity was measured with a Beckman LS-300 scintillation counter.

For purification and separation of larger quantities of products, 250 μg were applied to a single silica gel G plate. After migration and visualization at 340

nm with a mineral lamp without scintillation cocktail addition, the sheet was cut in strips along the width and each product was eluted with 10 ml ethyl acetate and diluted or evaporated until the desired concentration was obtained. The concentration of the various products was estimated from the initial specific activity of [^{14}C]chloramphenicol.

Chemical preparation of chloramphenicol derivatives. Chemically prepared chloramphenicol derivatives were used as reference material for TLC. They were synthesized according to Rebstock et al. [13]. The products were dissolved in 10 ml ethyl acetate. The chemical reaction produced about 20% of unreacted chloramphenicol, 15% of chloramphenicol 1-acetate, 40% of chloramphenicol 3-acetate and 25% of chloramphenicol 1,3-diacetate as demonstrated by TLC analysis.

Enzymatic preparation of chloramphenicol derivatives. [^{14}C]Chloramphenicol 1-acetate and [^{14}C]chloramphenicol 3-acetate were prepared by enzymatic acetylation at 37° C using the following reaction mixture: 2.2 μmol [^{14}C]chloramphenicol (1 mg/ml, 0.5 mCi/3.3 ml)/4.4 μmol CoASAc/250 mU of chloramphenicol acetyltransferase/320 μmol of Tris-HCl (pH 7.8) in a final volume of 4.4 ml. After 15 min, the reaction was stopped by adding 5 ml ethyl acetate in order to extract chloramphenicol derivatives. The extraction was repeated twice and the combined organic phases were evaporated under air. The radioactive products were dissolved in 0.4 ml ethyl acetate and analyzed using TLC as previously described.

This enzymatic acetylation resulted in approx. 4% of chloramphenicol, 15% of chloramphenicol 1-acetate, 77% of chloramphenicol 3-acetate and 4% of chloramphenicol 1,3-diacetate. The final concentrations of [^{14}C]chloramphenicol 1-acetate and [^{14}C]chloramphenicol 3-acetate were about 20 μg/ml based on the specific activity of the initial [^{14}C]chloramphenicol. The purity of these products is given in Table II.

The structures of the products obtained after chemical or enzymatic synthesis were not established since their R_f values and ultraviolet spectra corresponded to those found in the literature [1,14].

Mechanism of the reaction catalysed by chloramphenicol acetyltransferase. Studies of the enzymatic reaction using [^{14}C]chloramphenicol were performed with the same reaction mixture used for the preparation of chloramphenicol derivatives. After different incubation times, 0.1 ml of the reaction mixture was withdrawn and extracted twice with 0.1 ml ethyl acetate. Ethyl acetate was evaporated under air and the products dissolved in 0.05 ml ethyl acetate before application on TLC plates.

When radioactive acetylated chloramphenicol was used as substrate, the procedure was as follows: 1 ml (20 μg) radioactive chloramphenicol 1- or chloramphenicol 3-acetate in ethyl acetate was first evaporated under air. The products were then dissolved rapidly in the reaction mixture (final volume of 1 ml) consisting of 1 μmol CoASAc/225 mU chloramphenicol acetyltransferase/80 μmol of Tris-maleate (pH 6.0) or Tris-HCl (pH 7.8). A fraction of 0.1 ml of the reaction mixture was withdrawn at different time intervals and the products extracted and analysed using TLC as described previously.

Results

The purification of chloramphenicol acetyltransferase by affinity chromatography was first reported by Guitard and Daigneault [8]. We report the utilization of the same procedure for the preparation of large amounts of chloramphenicol acetyltransferase. By adding [^{14}C]chloramphenicol to chloramphenicol during the reduction procedure we could establish that 1—2 μmol of reduced chloramphenicol was bound per ml of Sepharose 4B gel suspension. The fixation of larger quantities of reduced chloramphenicol to the matrix could be achieved but the elution of the enzyme became difficult if not impossible. We have used eight different affinity columns over a period of 2 years. The best matrix were those capable of binding about 10 units of chloramphenicol acetyltransferase per ml of gel.

Table I summarizes the purification of chloramphenicol acetyltransferase. Based on 40 different separations, the recovery of the enzyme after affinity chromatography was always between 65 and 75%. The same column used over a period of 18 months kept the same capacity to bind the enzyme.

Fig. 1a shows an acrylamide gel electrophoretic pattern of purified chloramphenicol acetyltransferase after the affinity chromatography step. The preparation is highly homogeneous but a contamination band which represents 6% of the total proteins was always present. This minor band disappeared when chloramphenicol acetyltransferase was further purified on a DEAE-Sephadex A-50 column (Table I, Fig. 1b). Chloramphenicol acetyltransferase was eluted at a NaCl concentration of 0.33 M. No contaminant band was present though the preparation had roughly the same specific activity. The fact that specific activity was not improved might be explained by the lack of precision of the protein determination at low protein concentration and the possible interference by residual chloramphenicol.

The stability of chloramphenicol 1-acetate and chloramphenicol 3-acetate in different solvents is shown in Table II. These two products are stable for at least 3 months in ethyl acetate at 4°C. The dissolution or chloramphenicol 1-acetate in slightly alkaline solution such as 0.1 M Tris-HCl (pH 7.8) (solvent 2) leads to the direct transformation of chloramphenicol 1-acetate to chloramphenicol 3-acetate with a proportion of about 5 to 1 at equilibrium. However,

TABLE I

PURIFICATION OF CHLORAMPHENICOL ACETYLTRANSFERASE FROM *E. COLI* W677/HJR 66

	Enzymatic activity (U/ml) ***	Proteins (mg/ml)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification (-fold)
Sonic extract *	15.9	15.00	1.1	1590	100	1
Sepharose 4B-reduced chloramphenicol **	250.0	2.02	123.5	975	61.2	117
DEAE-Sephadex A-50 **	218.0	1.67	130.6	785	49.3	123

* From 7.5 g of bacteria (wet weight).

** After concentration of proteins by precipitation with 80% $(NH_4)_2SO_4$.

*** 1 unit of enzyme liberates 1 μmol of CoASH per min at 37°C.

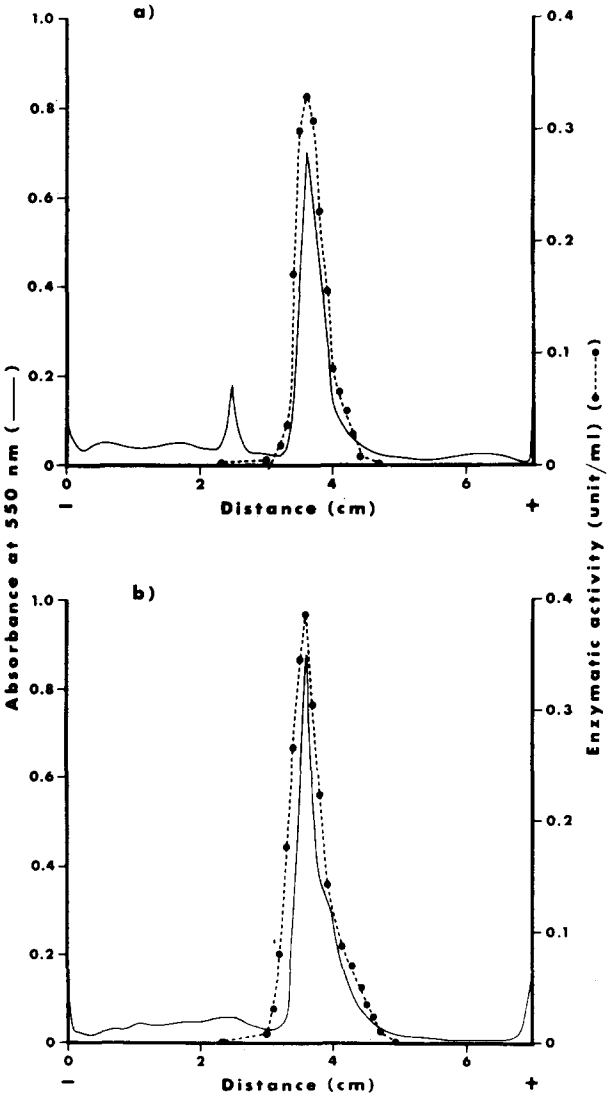


Fig. 1. Disc gel electrophoresis at pH 8.3 of the purified chloramphenicol acetyltransferase. (a) 8.1 μg (1 unit) of the enzyme preparation obtained after affinity chromatography or (b) 9.2 μg (1.2 units) of the enzyme preparation obtained after chromatography on DEAE-Sephadex A-50 are deposited onto the surface of the gel. Proteins are stained with Coomassie blue R-250 and scanned at 550 nm after decoloration. The activity of chloramphenicol acetyltransferase is measured by the spectrophotometric assay once the gel had been cut and the enzyme eluted from the gel.

when chloramphenicol 1-acetate or chloramphenicol 3-acetate were dissolved in a more acidic medium such as a 0.2 M Tris-maleate (pH 6.0) with or without CoASAc, the stability of each product was increased even after 45 min of incubation at 37°C, the equilibrium being near 90% of chloramphenicol 1-acetate and 10% of chloramphenicol 3-acetate when chloramphenicol 1-acetate was the starting material.

At pH 6.0, the remaining chlorampehnicol acetyltransferase is about 60% of

TABLE II

STABILITY OF CHLORAMPHENICOL 1-ACETATE AND CHLORAMPHENICOL 3-ACETATE

20 μg of [^{14}C]chloramphenicol 1-acetate or [^{14}C]chloramphenicol 3-acetate are dissolved in four different solvents and the stability of these products is investigated by TLC after 15 min of incubation at 37°C. The plates are cut and the radioactivity is measured in a scintillation cocktail. Quantitites of products are given in relative proportion of the total radioactivity. Solvents: I, ethyl acetate; II, 0.1 M Tris-HCl (pH 7.8); III, 0.2 M Tris-maleate (pH 6.0); IV, 0.2 M Tris-maleate (pH 6.0), 1 mM CoASAc.

Solvent	Starting material	Proportion of products			
		Chloramphenicol	Chloramphenicol 1-acetate	Chloramphenicol 3-acetate	Chloramphenicol 1,3-acetate
I	B	⩽0.005	0.960	0.040	⩽0.005
	C	⩽0.005	0.010	0.990	⩽0.005
II	B	0.030	0.100	0.870	⩽0.005
	C	0.030	0.080	0.895	⩽0.005
III	B	⩽0.005	0.920	0.070	⩽0.005
	C	⩽0.005	0.025	0.970	⩽0.005
IV	B	⩽0.005	0.935	0.060	⩽0.005
	C	⩽0.005	0.250	0.975	⩽0.005

the activity at pH 7.8 (unpublished data). It is therefore possible at this pH to acetylate chloramphenicol without any further transformation of the products of the reaction.

The normal acetylation reaction at pH 7.8 produces chloramphenicol 1-acetate, chloramphenicol 3-acetate and chloramphenicol 1,3-diacetate (Fig. 2a). These results are identical to those of Shaw [1] and Suzuki and Okamoto

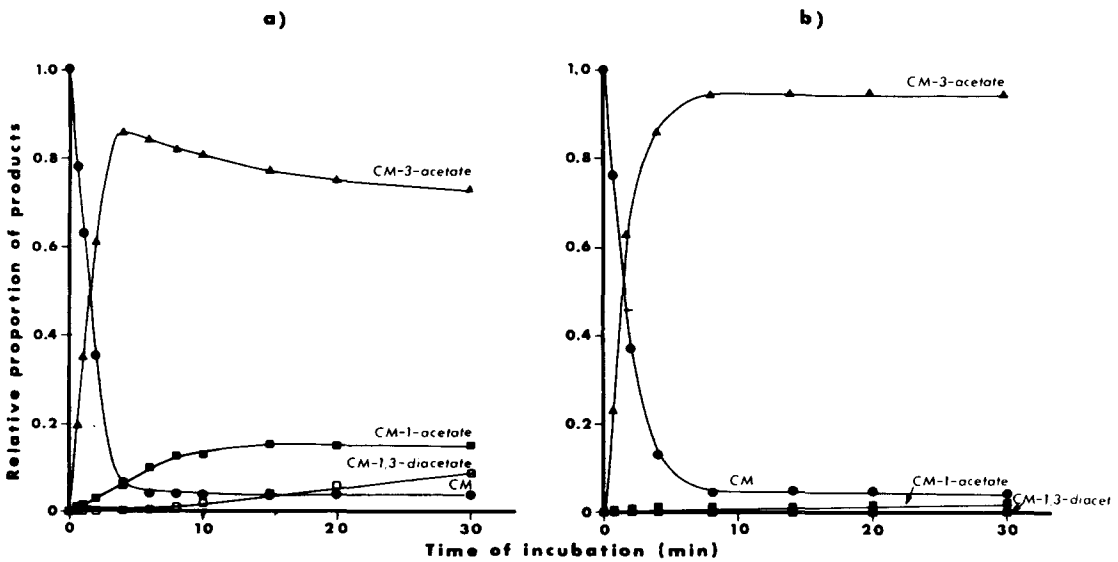


Fig. 2. Enzymatic acetylation of chloramphenicol by chloramphenicol acetyltransferase. 0.5 μmol [^{14}C]-chloramphenicol in a final volume of 1 ml is incubated with 1 μmol CoASAc and 60 mU chloramphenicol acetyltransferase in (a) 80 μmol Tris-HCl (pH 7.8) or (b) 80 μmol Tris-maleate (pH 6.0). At different time intervals, aliquots of 0.1 ml are withdrawn. The products are extracted and analysed by TLC.

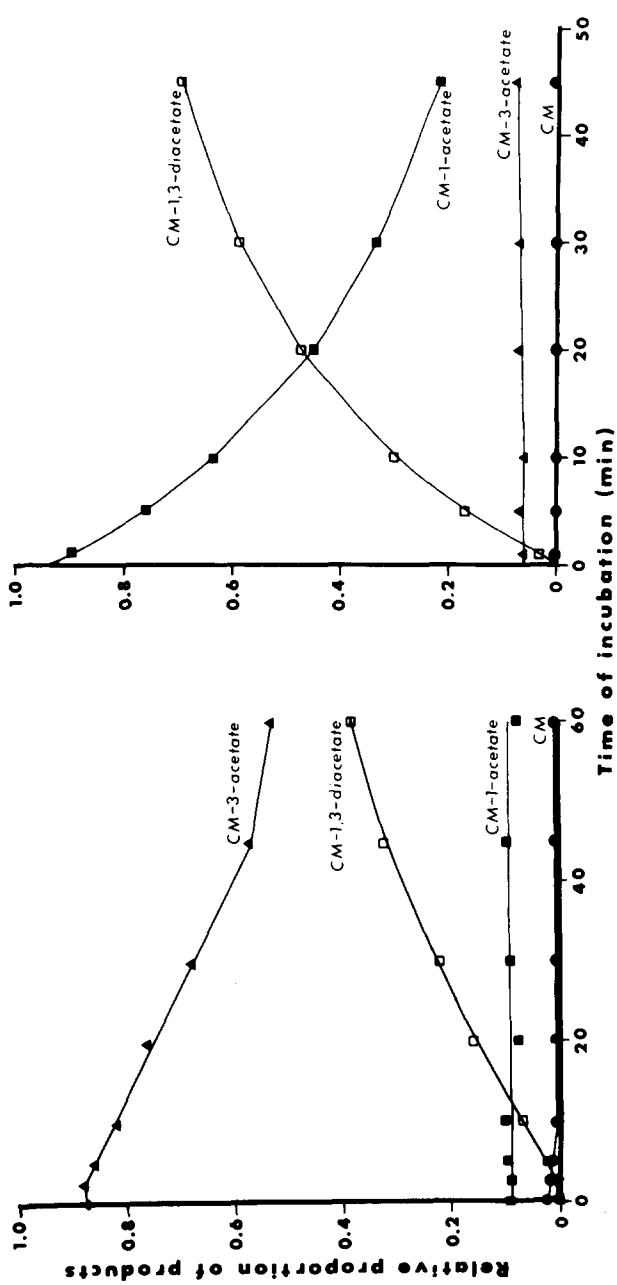


Fig. 3. Enzymatic acetylation of chloramphenicol 3-acetate and chloramphenicol 1-acetate by chloramphenicol acetyltransferase. (a) 20 μg [^{14}C]chloroamphenicol 3-acetate are dissolved in the reaction mixture (1 ml) containing 1 μmol CoASAc/225 mU chloramphenicol acetyltransferase/80 μmol Tris-maleate (pH 7.8). At different time intervals aliquots of 0.1 ml are withdrawn and the products extracted and analysed by TLC. (b) The same experiment was repeated with chloramphenicol 1-acetate as the substrate in Tris-maleate (pH 6.0).

[2], except that these authors could not identify chloramphenicol 1-acetate with the chromatographic solvent they used. If the same reaction is performed at pH 6.0 in Tris-maleate (Fig. 2b), only chloramphenicol 3-acetate is produced without appreciable formation of chloramphenicol 1,3-diacetate. The chloramphenicol 3-acetate is not transformed into another product. Chloramphenicol 1,3-diacetate is formed only at pH 7.8 (Fig. 3a). In contrast, when chloramphenicol 1-acetate is used as the substrate at pH 6.0 (Fig. 3b), there is an enzymatic transformation of chloramphenicol 1-acetate into chloramphenicol 1,3-diacetate. The presence of chloramphenicol 3-acetate in the reaction mixture is due to the contamination of the chloramphenicol 1-acetate used as the substrate.

The equilibrium constant of the first step of the enzymatic reaction can be established after 7.5 min (Fig. 2a).

$$K_{eq} = \frac{[\text{chloramphenicol acetate}]\,[\text{CoASH}]}{[\text{chloramphenicol}]\,[\text{CoASAc}]}$$

where chloramphenicol-acetate is the sum of the concentrations of acetylated products. The same calculation may be performed from the data of Fig. 2b after 10 min of incubation at pH 6.0. In both cases the K_{eq} is about 17, which is near the value of 15 reported by Tanaka et al. [15].

Discussion

Affinity chromatography has proved to be a very powerful purification procedure. This is exemplified by the present purification figures. The coupling of reduced chloramphenicol to Sepharose matrix and the elution pattern of chloramphenicol acetyltransferase demonstrate the high efficiency of the column. Our data compare favorably with chloramphenicol acetyltransferase purification achieved by conventional techniques [2,4,7,16,17].

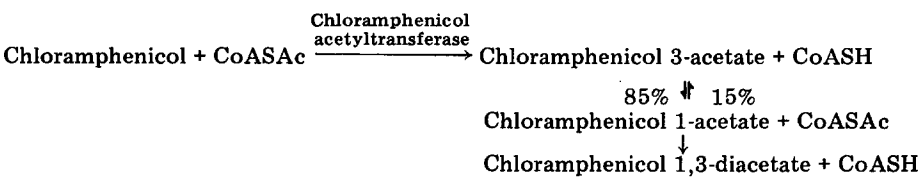
The nitro group of chloramphenicol is not essential for the enzymatic activity, as shown by Shaw [7]. Since the nitro group is reduced to an amino group before coupling, the reduced chloramphenicol serves as an excellent ligand. Chloramphenicol acetyltransferase is easily eluted with high concentrations of chloramphenicol but neither a linear 0.1—3 M NaCl gradient nor a 5.0—8.5 pH gradient elute the enzyme [8]. Zaidenzaig and Shaw [9] could not adsorb the type I chloramphenicol variant when using a reduced chloramphenicol affinity support. This discrepancy might be explained by the use of a different reduced-chloramphenicol gel coupling ratio.

The fact that chloramphenicol must be present in the elution buffer indicates a fairly specific chromatographic process and not simply an adsorption due to nonspecific factors. We did not attempt to bind other variants of chloramphenicol acetyltransferase. However we do recognize the advantage of using an appropriate spacer arm [9,18]. Based on the substitution of our matrix (1—2 μmol/ml) and the fact that only chloramphenicol can elute chloramphenical acetyltransferase, this suggests substrate-enzyme interactions for the retention of the enzyme on the column.

The affinity chromatography did not yield pure chloramphenicol acetyltransferase since contaminant proteins bind to the matrix and are eluted when

chloramphenicol is added to the elution buffer. Le Goffic and Moreau [19] and Le Goffic et al. [20] have also reported the presence of proteins adsorbed on the affinity matrix during the purification of aminoglycoside inactivating enzymes. This protein with an affinity for chloramphenicol might be involved in protein synthesis. Our results differ from those of Zaidenzaig and Shaw [9], who did not observe contaminant proteins after purification of chloramphenicol acetyltransferase by affinity chromatography.

The configuration of chloramphenicol in solution [21] shows the proximity of carbons 1 and 3. This proximity favors an exchange of acetate in alkaline solution, probably by nucleophilic attack. Since the C-3 position has less steric hindrance, the formation of chloramphenicol 3-acetate is promoted. In more acidic solution, the interconversion becomes impossible and chloramphenicol 1-acetate is stable. At pH 6.0, the only detectable product of the enzymatic reaction is chloramphenicol 3-acetate with no production of chloramphenicol 1,3-diacetate. On the other hand, the formation of diacetate is possible at this pH only when chloramphenicol 1-acetate is used as the substrate. Only the C-3 position can therefore be acetylated by chloramphenicol acetyltransferase at pH 6.0. This is in agreement with the results published by other investigators [22,23]. Presumably, at pH 7.8, chloramphenicol acetyltransferase catalyses the formation of the diacetate simply because chloramphenicol 3-acetate is slowly transformed chemically to chloramphenicol 1-acetate. The reaction cannot therefore proceed directly from chloramphenicol to chloramphenicol 3-acetate to chloramphenicol 1,3-diacetate as originally suggested [1,6,7]. The following reaction for diacetylation of chloramphenicol by type I chloramphenicol acetyltransferase can be proposed at pH 7.8:



This study demonstrates the important role played by the chloramphenicol 1-acetate derivative in the diacetylation of chloramphenicol. Our results explain why some workers did not observe the presence of the diacetate in slightly acidic medium during the study of the biotransformation of chloramphenicol [14,24,25]. The reaction catalysed by chloramphenicol acetyltransferase has not been studied in other R-plasmic bearing bacteria. It remains to be seen if all types of chloramphenicol acetyltransferase proceed according to this reaction scheme. Since chloramphenicol acetyltransferase can now be purified to a high degree of homogeneity with good yields, genetic and structural studies should progress rapidly [17,26].

Acknowledgement

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References

1 Shaw, W.V. (1967) J. Biol. Chem. 242, 687—693
2 Suzuki, Y. and Okamoto, S. (1967) J. Biol. Chem. 242, 4722—4730
3 Shaw, W.V. (1975) Methods Enzymol. 43, 737—755
4 Shaw, W.V., Sands. L.C. and Datta, N. (1972) Prot. Natl. Acad. Sci. U.S.A. 69, 3049—3053
5 Foster, T.I. and Shaw, W.V. (1973) Antimicrob. Agents Chemother. 3, 99—104
6 Shaw, W.V. (1971) Annu. N.Y. Acad. Sci. 182, 234—242
7 Shaw, W.V. and Brodsky, R.F. (1968) J. Bacteriol. 95, 28—36
8 Guitard, M. and Daigneault, R. (1974) Can. J. Biochem. 52, 1087—1090
9 Zaidenzaig, Y. and Shaw, W.V. (1976) FEBS Lett. 62, 266—271
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Ornstein, L. (1964) Annu. N.Y. Acad. Sci. 121, 321—349
12 Davis, B.J. (1964) Annu. N.Y. Acad. Sci. 121, 404—427
13 Rebstock, M.O., Grooks, H.M., Jr., Controules, J. and Bartz, Q.R. (1949) J. Am. Chem. Soc. 71, 2458—2462
14 Argoudelis, A.D. and Coats, J.H. (1971) J. Antibiot. 24, 206—208
15 Tanaka, H., Izaki, K. and Takahashi, H. (1974) J. Biochem. 76, 1009—1019
16 Winshell, E. and Shaw, W.V. (1969) J. Bacteriol. 98, 1248—1257
17 Shaw, W.V. and Bently, D.W. (1970) J. Bacteriol. 104, 1094—1105
18 Zaidenzaig, Y. and Shaw, W.V. (1978) Eur. J. Biochem. 83, 553—562
19 Le Goffic, F. and Moreau, N. (1973) FEBS Lett. 29, 289—291
20 Le Goffic, F., Moreau, N. and Chevereau, M. (1973) Biochimie 55, 1183—1186
21 Coutsogeorgopoulos, G. (1966) Biochim. Biophys. Acta 129, 214—217
22 Shaw, W.V. (1967) Antimicrob. Agents Chemother. 1966, 221—226
23 Ferrari, V. and Ella Bella, D. (1974) Postgrad. Med. J. 50, Suppl. 5, 17—22
24 Hoog, R., Siissmuth, R. and Lingens, F. (1976) FEBS Lett. 63, 62—64
25 El-Kersh, T.A. and Plourde, J.R. (1976) J. Antibiot. 29, 292—302
26 Liddell, J.M, Shaw, W.V. and Swan, I.D.A. (1978) J. Mol. Biol. 24, 285—286