CHLORAMPHENICOL ACETYLTRANSFERASE: ENZYMOLOGY AND MOLECULAR BIOLOGY

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I. INTRODUCTION*

The isolation of chloramphenicol from a soil bacterium (*Streptomyces venezuelae*) was reported in 1947.[1] Its structure was soon revealed to be simple but novel in two respects and also interesting stereochemically. Not only was it the first natural product found to contain a nitro group, but it possessed a dichloroacetyl substituent. The two asymmetric centers in the propanediol side chain yielded four possible diastereoisomers but the antimicrobial activity was observed to be a property of only one, the naturally occurring compound D(-)*threo*-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol.[2] Figure 1 depicts the structure of the antibiotic using several conventions. A number of reviews and discussions of the chemistry and properties of chloramphenicol which have appeared during the past 2 decades may provide useful background on the drug and its mechanism of action.[3-8] Although details of the interaction of chloramphenicol with the bacterial ribosome have yet to be defined at atomic resolution, it can be said that the drug is both a specific and effective inhibitor of polypeptide chain elongation in prokaryotes by virtue of its affinity ($K_d \simeq 3\ \mu M$) for the peptidyl transferase center of the 50S ribosomal subunit (see Section V.).

The relative simplicity of the structure of chloramphenicol quickly led to economic routes of synthesis, and by 1950 the synthetic drug was in wide use clinically and had become accepted as a promising broad spectrum antibiotic. Ease of administration and its clinical efficacy in the era which preceded that of the semisynthetic penicillins and the newer aminoglycosides made it a favorite for clinicians. The reports of drug-induced bone marrow toxicity[9] and adverse reactions in newborn infants who had received chloramphenicol[10] led, however, to diminished enthusiasm for the antibiotic, and its use at present among informed clinicians is limited to life-threatening infections such as meningitis due to *Haemophilus influenzae*, certain infections due to anaerobic bacteria, and typhoid fever.[11,12] The advent of quick and reliable methods for monitoring the levels of the drug in body fluids, including one based on the enzyme which is the subject of this review,[13,14] suggests that the drug will continue to be used in man on a limited scale for

* Apart from standard abbreviations and those defined in the text, the following conventions and short notations are used: CAT refers to the polypeptide which in its tetrameric and catalytic form is chloramphenicol acetyltransferase. A subscript (e.g., CAT_I or CAT_C) indicates the naturally occurring enzyme variant (Type I or Type C) under discussion. CAT is the product of the gene *cat* which can be further designated *cat*^{I} if it refers to that specifying CAT_I. The expression k_{cat} is used in the general sense of the *cat*alytic constant familiar to kineticists. It is a complication rather than a convenience that it also refers in this review to the turnover number (sec^{-1}) for CAT.

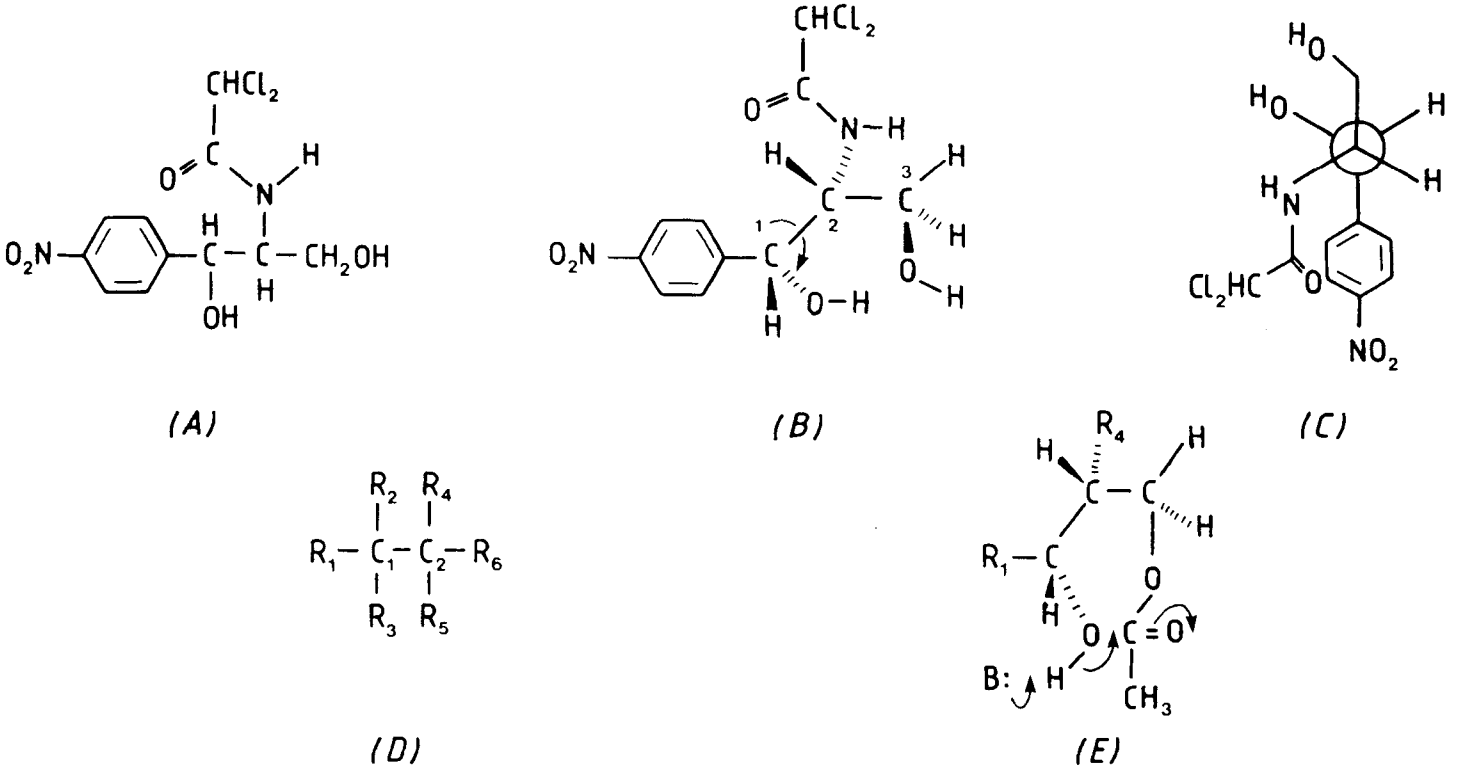


FIGURE 1. The structure of chloramphenicol has been depicted using several conventions. The biologically active compound is best illustrated by (B) and (C) wherein the stereochemistry of the propanediol side chain is unambiguous [1, (R)-2, (S)] and the effects of rotation along the C_1-C_2 axis can be visualized. Structure (C) gives the Newman projection of the preferred conformer of D, threo chloramphenicol. The scope for hydrogen bond formation between the hydroxyls is seen in both (B) and (C) whereas (E) stresses the possible importance of the favored conformer in the rearrangement of 3-acetoxy chloramphenicol to the 1-acetoxy derivative, a transformation which is base catalyzed and nonenzymic. The arbitrary numbering system for substituents at C_1 and C_2 has been used in Table 2 wherein the properties of a few selected analogues and isomers are summarized. The stereochemistry of chloramphenicol is that of (1)-nor-*pseudo* ephedrine.[2,3] More detailed accounts of the structure of chloramphenicol include the NMR studies of Jardetsky[4] and Tritton,[7] a theoretical conformational analysis by Bustard et al.,[5] and a recent synopsis by Nagabhusan et al.[89] in connection with the properties of biologically active 3-fluoro analogues of chloramphenicol.

selected infections in hospitalized patients. Chloramphenicol continues to be used in veterinary practice on a scale which is difficult to measure but likely to exceed human use. Taking a broader ecological view it should be noted that strains of Actinomycetes which produce chloramphenicol have been isolated frequently in many locales from soil samples, and bacteria have been described which produce analogues of chloramphenicol known as corynecins in which various nonhalogenated acyl groups replace the dichloroacetyl moiety of the parent antibiotic.[15] In short, there seems to be no basis for believing that environmental selection for chloramphenicol-resistant bacteria is either a new phenomenon or likely to be of limited duration on a biological time scale. As will be discussed subsequently, the persistence of chloramphenicol resistance and its appearance among many bacterial genera owes much to the linkage of the genetic determinant to other selectable markers and the frequency with which it is found to be part of transmissible and transposable genetic elements, plasmids, and transposons, respectively.

With the wisdom of hindsight, it is apparent that biochemical studies on the mechanism of resistance of bacteria to antimicrobial agents over the past 3 decades have been, until recently, both misleading and occasionally misguided because of the absence of appropriate genetic designations and controls. The literature on chloramphenicol resistance is no exception. A breakthrough came with the realization in the mid-1960s that accessory, cytoplasmic, and often transferable genetic elements (plasmids) were responsible for much phenotypic variation in bacteria and for clinically important antibiotic resistance in particular.[16-18] Even a summary of this important subject is not possible here, but a number of reviews and books describing the discovery and analysis of bacterial plasmids can be read with some pleasure and considerable profit.[19-30] The earlier (pre-1970) reviews are particularly valuable in stressing the development of concepts which have now become refined by the advent of powerful new techniques for the analysis of genetic structures. The picture which has emerged suggests that, while the ubiquity of drug resistance and the evolution of the determinants themselves can be explained in part by the promiscuity of the natural vector plasmids, the extreme mobility of some genes for antimicrobial resistance may be due to a phenomenon which has become appreciated only recently — the transposability of resistance markers themselves from one plasmid to another or to the chromosomes of host cells.[31-34]

With the above remarks in mind, it is not surprising to note that, notwithstanding the documented "emergence" of bacteria resistant to chloramphenicol in the years immediately following its introduction, real progress in an understanding of the genetics and biochemistry of resistance was not immediate. The appearance in 1955 of strains of dysentery bacilli which were resistant to chloramphenicol and three other drugs alerted Japanese microbiologists to the phenomenon which we now recognize as the plasmid-mediated *en bloc* transfer of several linked determinants.[16] The first mechanistic studies of chloramphenicol resistance specified by such plasmids were carried out with *E.coli* strains made resistant (R^+) by the conjugative transfer of a resistance plasmid from the epidemic strains of *Shigella* to familiar hosts more suitable for laboratory study. Cell extracts capable of supporting protein synthesis and prepared from R^+ *E.coli* K-12 strains were observed to be as sensitive to inhibition by chloramphenicol as were extracts from the R^- control strain.[35] This result was surprising since Miyamura reported in the same year (1964)[36] that *E.coli* strains carrying transmissible elements for chloramphenicol resistance were able to inactivate the drug rapidly and completely. The dilemma was resolved when it became apparent that the dialyzed cell extracts prepared for in vitro protein synthesis studies were necessarily deficient in acetate and acetyl-S-CoA. The latter was shown independently by the author[37] and by Suzuki and Okamoto[38] to be the cofactor required for the O-acetylation of chloramphenicol by the enzyme chloram-

phenicol acetyltransferase [EC 2.3.1.28] for which the abbreviation CAT has received general acceptance. Miyamura's important paper[36] had shown not only that *E.coli* and other Gram-negative bacteria with "R-factors" for the drug were able to inactivate chloramphenicol, but also that some naturally occurring isolates of the Gram-positive pathogen *Staphylococcus aureus* had this property. In fact, similar results with staphylococci were obtained independently by Dunsmoor et al.,[39] and the likelihood of plasmid linkage for chloramphenicol resistance in this microorganism was enhanced by transduction studies[40,41] and by the apparent instability of the genetic determinant.[42,43] It was less than surprising therefore to learn that such naturally occurring strains of chloramphenicol-resistant staphylococci also contained CAT.[44] The possibility that the gene for the enzyme and, by inference, its plasmid vectors might be the same in both Gram-positive and Gram-negative bacteria was eliminated by studies which showed that the staphylococcal system was inducible whereas the enteric "R-factors" specified a constitutive CAT system and, further, that the native proteins were different by electrophoresis and unrelated by immunologic criteria.

By the early 1970s it had become clear, both from direct evidence and by analogy with the picture emerging for resistance to the β-lactam antibiotics, that there were two quite distinct groups or families of CAT. The constitutive group[45] specified by plasmids in *E.coli* and related genera was associated with large (>20 kb) plasmids analogous to the F-factor of *E.coli* and was found linked to other drug resistance genes (including the β-lactamase effecting ampicillin resistance), whereas the inducible staphylococcal "family" of CAT variants[46] was almost invariably found to be associated with small (< 5 kb) plasmids which usually carried no other drug resistance markers. When the tetrameric structure of CAT became apparent from preliminary subunit studies on the purified proteins, it was possible to look for homologies in the structures which might permit the preparation of hybrid tetramers. As would have been predicted from the differences already cited for the two families of CAT variants, no interspecific heterotetramers could be prepared by hybridization in vitro from CAT variants purified from *S.aureus* and *E.coli*.[46,47] It was, however, possible to demonstrate genetic complementation between CAT point mutants in vivo[48] and to prepare hybrids in vitro between two members of any one family, and this approach was extended to the in vivo synthesis of heteromeric proteins by using a strain of *E.coli* carrying two compatible plasmids, each of which specified an identifiably different CAT polypeptide.[47]

The decade which has passed has seen remarkable developments and achievements in genetic manipulation and molecular biology which have accelerated the rate of progress in studies on the structure and organization of genes for antibiotic resistance. The special case of chloramphenicol resistance is no exception, and the theme which is now emerging is that the genetic studies will allow catalytic and chemical studies on the protein products to proceed more rapidly. The object of this review is to summarize the several aspects of the CAT system which are of interest to molecular biologists and microbiologists on the one hand, with the known features of the enzymes which invite thought and study by enzymologists and physical biochemists on the other. To a degree the structure of this summary is designed to meet these two objectives. Inasmuch as it is both undesirable and impossible to separate the biochemical function from genetic considerations, it is hoped that specialist readers will also be patient ones and bear with some overlap, repetition, and explanation of terms which may be familiar to some but almost unintelligible to others.

II. MICROBIOLOGY OF CHLORAMPHENICOL RESISTANCE

A. General Principles

Before proceeding to specific CAT variants and matters of catalysis and protein

structure there are points which can be made that may assist the reader through bacterial taxonomy and plasmid nomenclature which tend to obscure important observations and even principles. It may be recalled that it is a simple matter to find bacteria which are capable of growth in the presence of an antibiotic. Microbial colonies will appear on solid growth media containing chloramphenicol after the inoculation of Petri dishes with specimens as diverse as garden soil and hospital waste effluents. After colonies have been subcultured to yield pure clones which can be assigned to an appropriate taxonomic group on the basis of morphological, biochemical, and immunologic criteria, it is pertinent to ask whether the identified microbe, *Bacillus subtilis* for example, is *normally* able to grow in the presence of the drug. If, as in the case with *B.subtilis,* the answer is an unequivocal "no", one may assume that the clone in question represents a mutational variant which has "surfaced" during the selection processes or consists of progeny of a cell which, rather than lacking a function (because deleterious mutations far outnumber favorable ones), actually possess one or more *new* functions or properties conferred upon it by the presence of a resident bacterial virus (temperate bacteriophage), accessory cytoplasmic genome (plasmid), or "extra" fragment of DNA (transposon or insertion element) inserted in the chromosome. (The last of these examples also can cause mutations by inserting into a gene or cluster of genes, thus causing *loss* of function,[31,34] but, this important property is not germane to the present argument.) Most of the early studies of drug resistance either surveyed ecological sites (hospitalized patients and clinic washbasins) and sought to identify examples of "acquired" resistance without specification of cause or were of a different kind in which well-characterized and drug-sensitive type strains *(E.coli, S.aureus,* or *B.subtilis)* were screened for mutants, either spontaneous or induced by mutagens.

Although studies of mutation to chloramphenicol resistance have been of genetic interest, it is the author's view, albeit with the wisdom of hindsight, that they have been disappointing. For example, such mutations at two separate loci in *E.coli* K-12 yielded low level chloramphenicol resistance (still inhibited by as little as 15 $\mu g/m\ell^{-1}$ of drug), no evidence of CAT, and other phenotypic properties such as resistance to tetracycline.[49,50] The failure to obtain high-level resistance (growth in as much as 100 $\mu g/m\ell^{-1}$ of chloramphenicol) in mutants of strain K-12 has been confirmed by the author (unpublished) and by Sompolinsky and Samra.[51] The latter did, however, report on the isolation of mutants of *E.coli* B which grew in 100 $\mu g/m\ell^{-1}$ of drug and which inactivated chloramphenicol by a mechanism involving acylation.[52] This important observation has yet to be confirmed or extended and, until it is, it would be premature to say that chloramphenicol-resistant mutants of *E.coli* which produce CAT can be isolated from *E.coli.* Strains other than K-12 should be examined as there is mounting evidence that *E.coli* strains may differ widely in important respects which relate to mechanisms of genome evolution in general and genetic translocation in particular.[33,53,54]

At the risk of restating the obvious, it will be clear that it is improper or at least misleading to describe a microbial isolate as resistant to chloramphenicol (or any agent) if it is known that such resistance is generic and observed with all species of the genus. An extreme and trivial example is the resistance of yeast and fungi to chloramphenicol, a phenotype best described as tolerance or indifference rather than resistance in a specific and directed sense. This distinction can become blurred in certain special cases which are pertinent to chloramphenicol resistance and CAT. For example, the almost universal but variable resistance of *Pseudomonas* species to chloramphenicol may be due to a number of independent causes of which one is certainly a relative impermeability of the cell envelope to the drug. This barrier may be enhanced by the presence of a plasmid, the products of which lead to further exclusion of chloramphenicol from the cell interior.[55] (Such a mechanism, unrelated to CAT but plasmid-linked, can be studied in more detail after transfer of such plasmids to *E.coli* as has been done by several groups. The

property is inducible and may act in concert with CAT to yield even higher levels of resistance.[56-58])

B. Taxonomy, Plasmids, and Enzyme Types

The "seek and ye shall find" approach to identifying examples of chloramphenicol resistance and of CAT in particular yields isolates of bacteria which are, on taxonomic grounds, expected to be sensitive (for example, *Salmonella, Shigella,* and *Klebsiella* among familiar Gram-negative genera or the many Gram-positive micrococci of which staphylococci and streptococci are representative) but which are resistant and wherein, by genetic transfer to *E.coli* or physical means, one can demonstrate the presence of a plasmid. Only when such transfer (indirect) and physical (direct) studies are done on donor, recipient, and transcipient and when the resistance phenotype and CAT are present in both donor and transcipient can one say with confidence that a particular plasmid specifies the resistance gene. Complications arise when donor strains from the "wild" contain more than one plasmid, requiring segregation studies to relate gene products to a specific plasmid. With this point in mind, it is useful to recall that most of the enzymic and genetic studies to date with CAT have been done with a very few well-characterized plasmids or their derivatives. Some enzymes have been studied in the species wherein they were described originally and may be specified by a plasmid which has been investigated neither by genetic nor physical means. In other cases, a chromosomal locus for CAT seems certain, both on genetic grounds and from the absence of demonstrable plasmid DNA. Any CAT genetic variant which has been described might be designated by any of the following: (a) its original plasmid designation when such is known, (b) the species or strain of bacterium from which it has been extracted when plasmid linkage has not been established, (c) a description which indicates the presence of the structural gene on a novel plasmid constructed in vivo or in vitro, (d) its "current" association with a mobile genetic element such as a phage or transposon, and (e) when its structure and properties are known in detail, classification as a specific protein on catalytic or chemical grounds. The description of naturally occurring CAT variants has used, unfortunately, all six criteria. In the final analysis the last mentioned must be the most compelling. The summary of variants in Table 1 makes use of parameters such as electrophoretic mobility, kinetic data, susceptibility to inhibitors, reactivity with antisera, and the ability to form hybrid tetramers with other variants. As the primary structures become known, the limitations as well as the advantages of this approach will become clear. In general the author has used the convention of Roman numerals to categorize the CAT variants from Gram-negative bacteria and capital letters for those normally found in Gram-positive hosts. There is already evidence[57] of considerable heterogeneity among the most common (Type I) variant of CAT and this phenomenon will certainly be observed in other instances.

As may be apparent there are still some grounds for confusion. To give a specific example, it is known that the CAT specified by "Tn9" (the chloramphenicol resistance transposon) is a Type I enzyme.[59,60] The structural gene of Tn9 (or its product) has at various times in its laboratory life been studied in association with the genome of phage P1 (P1CM or P1 *cam*) and with phage λ (λ CM or λ *cam*)[61] while the lineage of this CAT determinant (*cat* has been proposed as the appropriate genetic designation) goes back to the generation of P1CM via a recombinational event between phage P1 and a plasmid (R14).[62] The *cat* of different P1CM later was incorporated in the vector plasmid pBR325[63,64] and *cat* is a determinant of the commonly used plasmid cointegrate originally designated pACYC184.[65] The properties of CAT specified by R6 and its derivatives are those of the Type I variant,[37] and the same may be said of the CAT specified by a plasmid variously known as R222, NR1, and R100[67] which, as with all

Table 1
CHLORAMPHENICOL ACETYLTRANSFERASE VARIANTS: ASSOCIATIONS WITH PLASMIDS AND BACTERIAL HOSTS

Enzyme type	Plasmid	Incompatibility group	Comments	Ref.
		Gram-Negative Bacteria		
I	R1	FII	Also R1 *drd*	23, 48, 102
	R6	FII	Origin of *cat* in pACYC184 (pAC184)	37, 66
	R14	FII	pSM14; origin of *cat* in Tn9	62
	R55	C	pIP55	57
	R57b	C		45, 57
	JR66b	FII	Total amino acid sequence determined	60, 110
	JR70	FII	Probable Type I; fusidate resistance	190
	R71	(Inc9)		57
	JR72	FII	Probable Type I; fusidate resistance	190
	JR73	FII	Probable Type I; fusidate resistance	190
	R100	FII	NR1; R222; many derivatives	24, 173, 191
	R390	N		45
	R429	FII	Hybridization studies with Type III	57, 60, 70
	R455	FI	Probable Type I	190
	R471	L		57
	R478	S		57
	R538	FII	Used for studies of transcription in vivo	179
	R724	B		57
	R726	H		57
	R16213	FII		57
	CsCol	1		57
II	R753	V		57
	R901	V		57
	RA3	W		45, 57
	RA4	W	Cloned via *Pst*I in pBR322 [=pKT242]	30, 45, 57
	S-a	W	Cloned via *Pst*I in pBR322 [=pKT205]	30, 45, 57
III	R387	K	Partial (>90%) amino acid sequence	45, 57, 70
	R621ala	I α	Cloned via *Pst*I in pBR322 [=pKT241]	30, 57
	R799	C		237
	R994	A-C		57

Enzyme from	Comments	Ref.
Haemophilus influenzae	Plasmid specified; very similar to Type II; also *H.parainfluenzae*	237, 239, 266
Proteus sp.	Similar but not identical to Type I; chromosomal	111
Agrobacterium sp.	Inducible CAT; in T_i^- *A. timefaciens*	111
Flavobacterium sp.	Constitutive	111, 141
Streptomyces sp.	Constitutive and chromosomal; novel CAT	111, 134
Bacteroides ochraceus (pGD10)	Constitutive, resembles Type I; FII plasmid	260
Bacteroides fragilis	Similar to Type II; no genetic studies	249
Pseudomonas aeruginosa	Novel type	261
	Gram-Positive Bacteria	
Staphylococcus aureus		
A	No genetic analysis	46
B	No genetic analysis	46, 72, 92
C (pC221)	Also pCW41 and other derivatives	72, 77, 79, 207
D	No genetic analysis	46
-- (pC194)	DNA sequence known (see also pCW8)	75, 207
Streptococcus agalactiae	Cross-reacts with antiserum to staphylococcal CAT	111

Table 1 (continued)
CHLORAMPHENICOL ACETYLTRANSFERASE VARIANTS: ASSOCIATIONS WITH PLASMIDS AND BACTERIAL HOSTS

Enzyme from	Comments	Ref.
Streptococcus faecalis	Plasmid-specified; may be Type D	226
Streptococcus pneumoniae	Plasmid linkage unlikely; similarities to staphylococcal CAT types	221, 223
Clostridium perfringens	—	111
Bacillus pumilus	CAT present but not characterized; chromosomal	231, 232

other plasmids of the FII incompatibility group, specifies the Type I enzyme. No exceptions to this correlation have yet been observed in the author's laboratory. The contrary, however, is not true as plasmids from other incompatibility groups can encode the Type I protein, although they need not and often do not.[57] Table 1 includes data for CAT variants specified by R plasmids and their derivatives in Gram-negative bacteria and examples from other microbial genera and species.

The above discussion and the summary of CAT variants given in Table 1 are meant to highlight the danger in associating a particular CAT gene or its enzymic product with a particular microbial genus, bacterial species, plasmid (natural or synthetic), or transposon. Whereas it may be appropriate to speak of "species-specific" enzymes (or an enzyme variant which is characteristic of a specific microbe) in selected instances, it is clear that such facile associations are not appropriate for the antibiotic-inactivating enzymes in general and CAT in particular. The problem is best illustrated by recalling (see Table 1) that a natural isolate of *E.coli* easily might harbor two compatible plasmids (for example plasmid R1 of the FII group and plasmid R387 of incompatibility group K), each specifying a different CAT (types I and III, respectively). But it must also be remembered that, to use the above illustration, plasmid R1 could just as reasonably be expected in an isolate of *Salmonella, Shigella,* or *Klebsiella.* By the same token, plasmid R387 (CAT_{III}) might be expected to reside stably in *Proteus mirabilis,* a species which is believed to contain a chromosomal locus for a CAT variant which is almost identical to the common Type I protein. The apparent ubiquity of the gene for the latter CAT may derive from the transposability of its gene (see Section IV.) rather than the properties of any of the many plasmids with which the CAT_I determinant is associated. It would be wrong therefore, given the present level of understanding of plasmid-related phenomena, to infer that plasmid carriage of a specific *cat* is other than coincidental. Although meaningful correlations may emerge, they are not yet apparent.

III. STRUCTURE AND FUNCTION OF CHLORAMPHENICOL ACETYLTRANSFERASES

A. General Properties

Only a few CAT variants have been studied in detail as catalytic proteins or mutimeric polypeptide assemblies. Since the constitutive enzymes synthesized from most R plasmid *cat* genes in Gram-negative species normally represent approximately 1% of the soluble intracellular cell protein,[68] even when made from single copy replicons, they continue to be the preferred candidates for protein chemistry[69,70] and biophysical work.[71] When such *cat* genes are present on high copy number plasmids and cells are grown on glycerol rather than glucose (see "Regulation of *cat* Expression"), the enzyme yield may approach or exceed 10% of cell extract protein. The staphylococcal *cat* variants[72] require induction

and have not been available in quantities approaching those of the Types I and III proteins specified by plasmids in *E.coli*. A positive attribute of the CAT system is the ease with which pure protein can be obtained in high yield by affinity chromatography on supports containing a portion of the chloramphenicol structure in the ligand bound via conventional alkyl spacers.[73,74] All CAT variants studied exist as tetramers consisting of identical subunits which, on electrophoresis under denaturing conditions, have been estimated at 22,000 to 26,000 daltons. The reason for the variability is not clear but may be due to a requirement for extreme conditions to fully reduce and unfold the polypeptide prior to electrophoresis. The polypeptide chains of the Type I *(E.coli)*[59,60] and staphylococcal (pC194)[75] enzymes are, respectively, 219 and 216 amino acids in length (see Table 4).

B. Substrate Specificity and Ligand Binding

The realization that chloramphenicol resistance was due to inactivation by acetylation led to several studies[37,76] of the specificity of th acyl acceptor using enzymes from *E.coli* which are now known to be Type I variants as well as the Type C staphylococcal CAT variant associated with plasmid pC221.[77–79] The results appear in Table 2 along with unpublished results from the author's laboratory. Also tabulated are estimates of the effectiveness of chloramphenicol analogues and isomers to inhibit polypeptide synthesis from synthetic polyribonucleotides in cell-free systems.[80,81] The comparisons are at best semiquantitative and are related in each case to results for the parent antibiotic. More useful would be the apparent K_m and relative V_{max} values for each analogue and enzyme but, notwithstanding this limitation of the data presented, the conclusion is that both families of CAT share with the ribosomal target(s) of chloramphenicol a nearly absolute requirement for the D,threo stereoisomer (Figure 1), a requirement for a substituent on the C_2-amino group, and the absence of substitution of the C_1 and C_3 protons. The structural requirements for inhibition of peptidyltransferase are more stringent than for CAT in that the nature of the electronegative halo-atom substitutions at the C_2 amino group influences effectiveness in inhibition of protein synthesis more than acetyl acceptor activity in the CAT assay. The CAT active site is also less discriminating than the 50S ribosomal target as regards the nature of the para-phenyl substituent. In fact, it should be noted that a critical analogue of chloramphenicol has yet to be tested in either the CAT system or the cell-free protein assay; the dichloroacetyl amide of L(-)-threonol wherein the stereochemistry of chloramphenicol is preserved but in which methyl replaces the p-nitro-phenyl substituent of the parent compound. However, apart from this caveat and the discrimination differences noted, the CAT variants studied to date recognize chloramphenicol and its congeners in a relatively specific manner. A large number of compounds related only superficially to chloramphenicol fail to serve as acetyl acceptors with CAT including D- and L-serine and homoserine (and their N-acetyl amides) and isopropyl-thiogalactoside, compounds tested because of the known microbial enzymes catalysing the acetyl-S-CoA-dependent O-acetylation of compounds involved in metabolism.[82–87] It is of interest in this connection that chloramphenicol is not a substrate for L-serine acetyltransferase from *S.typhimurium*[88] nor is it an inhibitor or substrate for thiogalactoside transacetylase, the mystery enzyme of the *lac* operon. The author has reviewed the partial (more than 70% complete) aminoacid sequence of the latter polypeptide as determined and kindly provided by A. Fowler and I. Zabin and has found as yet no obvious sequence homology with any of the CAT variants. The matter of the origin of CAT will be dealt with in more detail under "Evolution of Chloramphenicol Acetyltransferase".

As may be seen from Table 3 the apparent Michaelis constant (K_m) for chloramphenicol, determined in the presence of saturating levels of acetyl-S-CoA, varies

Table 2
EFFECTS OF CHLORAMPHENICOL ANALOGUES AND ISOMERS

Substituent at				Inhibition of protein synthesis (%)	Acetyl acceptor activity (%)			Induction of CAT_C (%)
R_1	R_2/R_3	R_4	R_6		CAT_I	CAT_C	(K_m)	
NO_2-Ph	H/OH	-$NHCOCHCl_2$	-CH_2OH	100	100	100	(3 μM)	100
NO_2-Ph	H/OH	-NH_2	-CH_2OH	0	0	0		<2
NO_2-Ph	H/OH	-NHCO(H)	-CH_2OH	—	55	20		5
NO_2-Ph	H/OH	-$NHCOCH_2OH$	-CH_2OH	10	60	40		5
NO_2-Ph	H/OH	-$NHCOCH_3$	-CH_2OH	50	120	75		100
NO_2-Ph	H/OH	-$NHCOCH_2CH_3$	-CH_2OH	—	175	110		22
NO_2-Ph	H/OH	-$NHCOCH_2Cl$	-CH_2OH	70	130	80		35
NO_2-Ph	H/OH	-$NHCOCH_2Br$	-CH_2OH	—	110	—		—
NO_2-Ph	H/OH	-$NHCOCHBr_2$	-CH_2OH (a)	—	90	95	(22 μM)	115
NO_2-Ph	H/OH	-$NHCOCH_2I$	-CH_2OH	—	80	n.d.		n.d.
NO_2-Ph	H/OH	-$NHCOCH_2CN$	-CH_2OH	10	75	100		16
H	H/OH	-$NHCOCHCl_2$	-CH_2OH (b)	<5	40	55	(68 μM)	35
CH_3SO_2-Ph	H/OH	-$NHCOCHCl_2$	-CH_2OH	85	65	105		125
CH_3CO-Ph	H/OH	-NHCOCHCl	-CH_2OH	80	100	110		100
I-Ph	H/OH	-NHCOCHCl	-CH_2OH	—	130	90		85
Ph-NHCONH-Ph	H/OH	-NHCOCHCl	-CH_2OH	<5	85	30		10
NC-Ph	H/OH	-NHCOCHCl	-CH_2OH	<5	95	110		85
NO_2-Ph	CH_3/OH	-NHCOCHCl	-CH_2OH	<5	25	22		5
NO_2-Ph	H/OH	-NHCOCHCl	-CH_3	<5	0	0		60
NO_2-Ph	H/OH	-NHCOCHCl	-$CH(OH)CH_3$	<5	0	0		10
L,threo chloramphenicol				<5	<1	0		20
D,erythro chloramphenicol				<5	<1	20		3
L,erythro chloramphenicol				<5	<1	0		20

Note: Selected analogues and isomers of chloramphenicol are compared with respect to their ability in inhibit a cell-free *E. coli* protein synthesis system, their effectiveness as acetyl acceptors for two CAT variants, and their ability to induce the CAT_C encoded by pC221 in vivo. The values given are at best approximate and meant only to emphasize the major structural determinants of each property. The acetyl acceptor assays were generally done at a concentration of 50 μM and the values tabulated are the relative rates of acetylation for each substrate with the CAT variant indicated. Induction rates for CAT synthesis in *S. aureus* (pC221) were determined as described previously.[79] The data on the effectiveness of analogues or isomers as inhibitors of an in vitro protein synthesis system[116] are from unpublished experiments by the author. The template in each case was poly UC (1:2), and the conclusions are based on the relative concentrations of each compound (compared with chloramphenicol) required for 50% inhibition of template-dependent [^{14}C] proline incorporation into a product insoluble in hot 5% trichloroacetic acid. A value of 10% in the table signifies that a concentration of the analogue 10-fold higher than that of chloramphenicol was required for comparable inhibition. A value of <5% indicates that no significant inhibition was observed at a concentration 20-fold higher than that giving 50% inhibition of polypeptide synthesis with chloramphenicol (15 μM). Apparent K_m values were determined for compounds (a) and (b) as well as for chloramphenicol for the Type C staphylococcal (pC221) enzyme and the Type I variant, and the values are given for each compound under the appropriate heading.

Table 3
SELECTED PROPERTIES OF CHLORAMPHENICOL ACETYLTRANSFERASE VARIANTS

Enzyme types	Binding to chloramphenicol-substituted agarose[a]	K_m^{app} (μM) chloramphenicol	K_m^{app} (μM) acetyl-CoA	Relative V_{max}[b] (%)	DTNB sensitivity[a]	Reaction with antiserum to CAT$_I$	Reaction with antiserum to CAT$_{III}$	Reaction with antiserum to CAT$_C$[c]
I	5	12	76	10—20	0	+	—	—
II	4	18	57	5—10	5	—	—	—
III	3	16	80	[100]	1	—	+	—
Proteus mirabilis	5	15	82	~8	3	+	—	—
Haemophilis parainfluenzae	3	18	53	5—10	5	—	—	—
Agrobacterium tumefaciens	3	21	133	<10	2	—	—	—
Flavobacterium sp.*	1	n.d.	n.d.	<5	n.d.	n.d.	n.d.	n.d.
*Streptomyces acrimycini**	3	17	143	<5	4	—	—	—
Bacteroides fragilis	2	5	n.d.	n.d.	5	n.d.	n.d.	n.d.
Staphylococcus sp.								
A	3	2.6	57	~4	0	—	—	+
B	3	2.7	56	~2	0	—	—	+
C	3	2.5	61	~2	0	—	—	+
D	3	2.7	46	~3	0	—	—	+
Streptococcus agalactiae	3	9.3	101	<5	1	—	—	+
Streptococcus faecalis	3	n.d.	n.d.	<5	1	—	—	+
Streptococcus pneumoniae	2	10	15	n.d.	1	—	—	(+)
Clostridium perfringens	2	22	85	n.d.	2	—	—	(+)

Note: The results of unpublished experiments in the author's laboratory are indicated by (*).

[a] Graded from 0 to 5 where 0 = no binding or no inhibition by DTNB (5,5'-dithio-bis 2-nitrobenzoic acid).

[b] Relative V_{max} is estimate of maximum catalytic rate in presence of saturating concentrations of both substrates. The turnover number (k_{cat}) for the Type III enzyme purified from *E. coli* (R387) is 1500 sec^{-1} and is taken as 100.

[c] The anti-CATc serum neutralized two streptococcal variants but did not give precipitation reaction. These results are indicated by (+). All others marked + give both precipitation and neutralization.

The data have been tabulated from References 57, 72, 111, and 249.

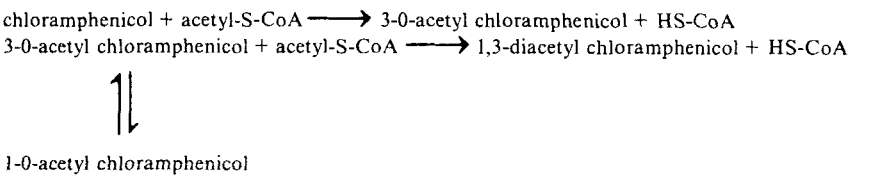
20-fold for the three analogues studied. In only one case has the equilibrium binding constant been determined for chloramphenicol and CAT; for a Type III enzyme studied in some detail because of encouraging X-ray diffraction data[71] on it and the ease with which it forms stable hybrids with certain other CAT variants.[47,70] The agreement between the result obtained (15 μM) by equilibrium dialysis (K_d) and from steady-state kinetics (K_m) is compatible with, but not diagnostic of, a mechanism (see below) in which the formation of an enzyme-substrate complex is not rate determining. Worthy of note is the recent synthesis of analogues of chloramphenicol wherein fluorine replaces the 3-hydroxyl group.[89,90] The 3-fluoro compounds are not substrates but are effective competitive inhibitors[91] (with respect to chloramphenicol only), allowing formation of nonproductive complexes of CAT with the acetyl donor and the analogue. Such complexes have been useful in chemical modification studies (see Active Site Topography) and may prove valuable for solution studies using [^{19}F]NMR spectroscopy to monitor the consequences of ligand binding. The antimicrobial activity of the 3-fluoro analogues of chloramphenicol and their recognition by CAT are surely reflections of the fact that fluorine is a close match for the van der Waals radius of the replaced hydroxyl group and may act as a hydrogen bond acceptor with the hydroxyl hydrogen at C_1. Although it has been argued[89] that the preferred conformer (Figure 1C) may not require such an interaction for stabilization, model building suggests that the proposed five-membered ring which would result might still be important for recognition by the ribosomal target[7] and CAT.

Rather less extensive studies have been done on the specificity of CAT variants as regards the acyl donor.[37] It is clear that the complete and unmodified coenzyme structure is required since acetyl-S-dephospho CoA, acetyl-S-pantetheine, and the S-acetyl derivative of the *E.coli* acyl carrier protein are not acetyl donors.[68] The size of the acyl group transferred also is critical as transfer rates drop markedly for substituents larger than propionyl.[92] Certain adenine nucleotides are effective as inhibitors of CAT_I and are competitive with respect to acetyl-S-CoA. Under conditions wherein the apparent K_m for the thioester was 0.1 mM, the K_i values for ATP and ADP were 2.2 mM and 3.0 mM, respectively.[93] Adenosine, pyrophosphate, and AMP were not inhibitory. Of the three physiological ribonucleotide triphosphates, only UTP gave inhibition approaching that of ATP. Although limited in scope these results suggest the importance of both the adenine and the phospho-diester portions of the CoA structure as determinants of coenzyme binding (see Figure 2). Selected analogues of the acyl moiety of acetyl-S-CoA may prove to be particularly helpful as they form specific but nonproductive complexes and appear to induce conformational changes at the catalytic center. The -S-acetonyl and -S-methyl derivatives are discussed in this connection (see Active Site Topography).

C. Mechanism of the Reaction

1. The Rearrangement of 3-0-Acetyl Chloramphenicol

Early studies with CAT showed that antibiotic inactivation in vivo proceeded rapidly to the formation of chloramphenicol monoacetate followed by the slow appearance of the diacetate and the same step-wise process was observed with cell extracts and purified enzymes. The first proposal was



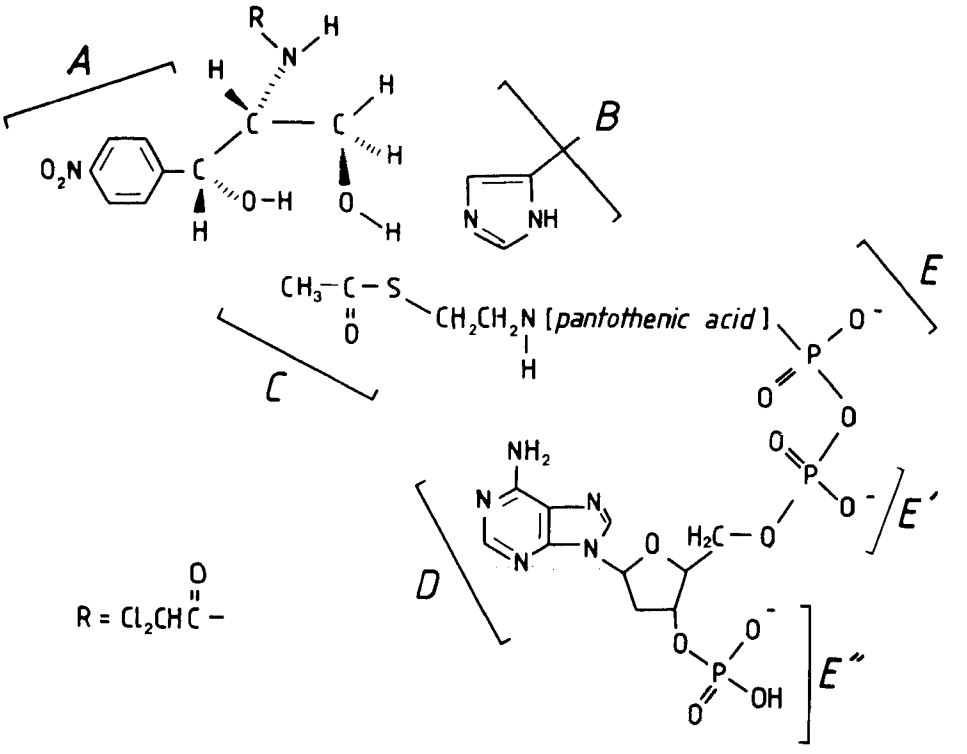


FIGURE 2. The catalytic center of CAT is likely to consist of hydrophobic (aromatic) functional groups interacting with the C_1-substituent of chloramphenicol (Site A) and several protonated amino or guanidino groups at sites E, E', and E'' which interact with the anionic backbone of CoA. Geometric constraints probably put an effective limit on the size of the acyl substituent of CoA (Site C), and there are specific interactions at Site D with the adenine ring of CoA. Site B corresponds to His-193 of the Type I structure which is highly reactive toward a number of alkylating and modifying reagents and which is unreactive when chloramphenicol is present. Not shown are the thiol groups which can be modified by iodoacetamide or chromogenic disulfides (Cys-31 and Cys-196 of Type I) or the carboxylate group believed to be present from the results of inhibitor studies. See text for details.

The 3-0-acetyl derivative of chloramphenicol is, in fact, a poor substrate but does rearrange in the absence of CAT to the 1-acetoxy compound which is then acetylated at the 3-hydroxyl to yield the diacetate.[94,96] The nonenzymic and base-catalyzed acetyl migration has not been studied in detail, but an analogous intramolecular succinyl migration has been observed in the rearrangement of 3-0-succinyl-chloramphenicol,[97] a pharmaceutical formulation which yields the free antibiotic in vivo. The proposed first step in the nonenzymic intramolecular acetyl transfer is shown in Figure 1E wherein deprotonation of the free hydroxyl by base accompanies nucleophilic attack at the carbonyl of the 3-acetyl group by the C_1 oxygen. The presence of the proposed tetrahedral intermediate has not been established. The equilibrium constant for the rearrangement of the acetyl mono-esters of chloramphenicol under the conditions used (pH 7.8; 37°) for the standard spectrophotometric assay is 0.41 and the rate of formation of the 1-0-acetyl ester from the enzymic product 0.11 min^{-1}.[98] The latter value is higher than that measured previously, but the explanation is likely to be a trivial one in that earlier studies[94] were performed at pH 7. The overall route to the diacetyl derivative now can be described with some confidence by the following scheme wherein the above rate

constant may be seen to be the sum of the forward (k_2) and reverse (k_{-2}) rate constants for the isomerization:

$$\text{chloramphenicol} + \text{acetyl-S-CoA} \xrightarrow{k_1} \text{3-0-acetyl chloramphenicol} + \text{HS-CoA}$$

$$\text{3-0-acetyl chloramphenicol} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{1-0-acetyl chloramphenicol}$$

$$\text{1-0-acetyl chloramphenicol} + \text{acetyl-S-CoA} \xrightarrow{k_3} \text{1,3-diacetyl chloramphenicol} + \text{HS-CoA}$$

What is still not clear is the extent of variation among the several CAT types in the rate of formation of the diester from 1-0-acetyl chloramphenicol and acetyl-S-CoA. It seems likely that some of the discrepancies in the literature on this point could be resolved by careful studies of the second step with purified enzymes and the presence of each substrate under defined conditions. The remaining differences would then represent biological variation in affinity for the 1-0-acetyl ester by each CAT variant. Experiments designed to measure the rate of formation of the diester from chloramphenicol and acetyl-S-CoA will necessarily include contributions from three rate constants, of which two (k_1 and k_3) are enzymic but not normally rate determining.[68,94,96]

It may be useful to stress a metabolic consequence of the monoester rearrangement; the microorganism need not diacetylate chloramphenicol to achieve the resistant state since the monoesters are also inactive as antibiotics and do not bind to *E.coli* ribosomes.[99,100] The energy cost in thioester bond equivalents ultimately expended for diacetylation is therefore twice that which is required for the expression of drug resistance. The acetate drain under normal circumstances may not be significant, but in strains limited in their ability to utilize or prodice acetate, the expression of resistance and growth rate can be affected. *E.coli* mutants in *ace*E or *ace*F, which cannot make acetyl-S-CoA from pyruvate but can from acetate, are chloramphenicol resistant only when acetate is supplied.[101]

An earlier experiment with a mutant requiring acetate for growth in the absence of drug was designed to estimate the stoichiometry in vivo.[52] The results suggested that the problem may be complicated by hydrolysis of the esters in vivo and the obligatory need for re-esterification, yielding values for acetate consumption well in excess of the two moles required per mole of antibiotic. This phenomenon may be useful in devising a selection against bacteria harboring CAT (for example, to isolate *cat* mutants or plasmid-free cells) by exposing cells to an analogue of chloramphenicol which is a good acetyl acceptor but not an antibiotic. The problem of the acetate load occasioned by a high demand for chloramphenicol acylation (high ambient concentration of drug analogue *and* high levels of CAT) is best illustrated by the observation that the level of chloramphenicol resistance does not increase linearly with CAT at very high concentrations of the antibiotic. Under conditions wherein high plasmid copy number leads to enhanced rates of synthesis of enzyme,[102] there appears to be a limit to the expression of resistance measured over time periods equivalent to several generations of normal growth. Very high CAT producers do eventually express levels of resistance to as high as 1 mg/mℓ^{-1} of chloramphenicol on solid media if given several days to do so.[103]

2. Mechanism of Catalysis

Until recently rather little attention has been given to the mechanism of the reaction catalyzed by CAT. Since the overall process for the critical first acetylation step couples the breaking of a high energy thiol-ester bond with the formation of an 0-acyl compound,

the energetic analysis is analogous to that of Jencks and co-workers[104] and discussed more fully in standard works on enzymic catalysis.[105,106] The difference in standard free energy $\Delta G^{\circ\prime}$ of hydrolysis of reactants and products, based on model compounds[107] is expected to be of the order of -2.8 kcal/mol^{-1} (-11.7 kJ/mol^{-1}) with a predicted equilibrium constant approximating 70. Published values for the equilibrium reached in the forward reaction in experiments with Type I variants of CAT are 15 and 17 for experiments with crude[108] and purified[96] enzyme, respectively. Such studies need to be extended by the enzymic approach to equilibrium from both directions with measurements of all reactants and under varied conditions before the conclusion can be reached that 3-0-acetyl chloramphenicol is other than a conventional oxy-ester with an expected value for $\Delta G^{\circ\prime} \simeq -4.7$ kcal/mol^{-1} (-19.7 kJ/mol^{-1}). The reverse reaction has now been studied in a coupled assay used successfully with choline acetyltransferase[109] and in which citrate synthase (CS) utilizes acetyl-S-CoA whereas malic dehydrogenase (MD) supplies oxaloacetate and provides a continuous spectrophotometric signal which is proportional to the oxaloacetate utilized.

$$\text{3-0-acetyl chloramphenicol} + \text{HS-CoA} \xrightarrow{\text{CAT}} \text{chloramphenicol} + \text{acetyl-S-CoA}$$

$$\text{acetyl-S-CoA} + \text{oxaloacetate} \xrightarrow{\text{CS}} \text{citrate} + \text{HS-CoA}$$

$$\left[\text{malate} + \text{NAD}^{+} \xrightarrow{\text{MD}} \text{oxaloacetate} + \text{NADH} + \text{H}^{+}\right]$$

The circumstantial evidence[45,57,110,111] favoring a central role for thiol groups at the active site of CAT variants from the Gram-negative family has not been observed in studies with staphylococcal variants.[72] In view of the known homologies in amino acid sequence (see Active Site Topography) between variants a unitary view of the catalytic mechanism would be both economical and attractive. Several acetyl-CoA dependent acetyl-transferase systems have been described in which an acetyl-enzyme intermediate can be implicated in a mechanism; homoserine-0-acetyltransferase[86] and arylamine N-acetyltransferase[112] being examples catalyzing ester and amide formation, respectively. Several kinetic analyses using steady-state methods are, however, inconsistent with a double displacement ("ping-pong") mechanism for CAT variants. The results of studies by Tanaka et al.[108] and Zaidenzaig and Shaw[113] are compatible with a ternary complex mechanism for Type I variants and a similar conclusion has been reached from studies in the author's laboratory on the Type III *E.coli* enzyme[98] and the Type C variant from *S.aureus*.[72] No exchange of the [^{14}C]-acetyl from 3-0-acetyl chloramphenicol into acetyl-CoA has been observed in the absence of free CoA with any CAT variant nor has it been possible to demonstrate enzyme-bound radioactivity after incubating CAT with stoichiometric amounts of [^{14}C]-acetyl-labeled substrate or product.

Recent steady-state kinetic studies with the Type III enzyme are also compatible with a sequential mechanism.[98] The picture emerging may be analogous to the more detailed mechanism proposed by Hersh and Peet[109] for the human placental choline acetyltransferase, but more systematic studies with at least two quite different CAT variants are required before a convincing and general mechanistic proposal can be made. In the light of evidence favoring the formation of an obligatory ternary complex prior to catalysis, the choices remaining which stress the likely importance of a unique histidine residue (see below) are broadly those in which it is central to a general base mechanism by which the proton of the C_3 hydroxyl is labilized (Figure 2) or a step-wise mechanism (not shown) with initial attack by the enzyme nucleophile at the acetyl C_2 carbon. Although there is little to favor the latter mechanism, it need not be ruled out by the steady-state kinetic results. Both mechanisms would allow for a very slow turnover of acetyl-CoA

seen when it is incubated with CAT in the absence of chloramphenicol. The putative, transient, and elusive acetyl-imidazolyl intermediate required by the second proposal would be formed at a significant rate only in the presence of enzyme-bound acyl acceptor, whereas abstraction of the C_3 hydroxyl proton in the general base mechanism would be enhanced markedly by the presence of the acyl donor. Both proposals require a degree of cooperativity between the respective substrate binding sites and the catalytic center, and there is circumstantial evidence that this may be the case from the results of chemical modification studies[110,114] (see below). Whatever chemical and kinetic mechanisms may be proposed ultimately, they must accommodate the very considerable variation in k_{cat} between variants which in selected cases can approach two orders of magnitude (Table 3). Using an analysis developed by Knowles and Albery[115] for evaluating "perfection" in enzymic catalysis the Type III variant yields a minimum value of k_{cat}/K_m of the order of 1×10^8 sec^{-1} M^{-1} when the apparent K_m for chloramphenicol (15 μM) is used in conjunction with representative turnover numbers for the pure protein.[114] The molecular means by which this very considerable rate enhancement is achieved for a two substrate reaction of considerable specificity may prove to be of interest beyond its obvious importance to chloramphenicol inactivation and resistance. It should be mention ed in passing that the apparent K_m values for chloramphenicol have been determined for a number of CAT variants (Table 3) and they are clustered around a mean of 16μM. The latter value corresponds to a concentration of 5 $\mu g/m\ell^{-1}$ which is a very rough approximation of the minimum concentration of chloramphenicol required to (a) inhibit growth of most bacteria which are sensitive to the drug[8] and (b) inhibit appropriate cell-free protein synthesis systems.[80,81,116]

3. Active Site Topography

a. Reactive Thiols

An estimate of the number and types of functional groups likely to participate in substrate binding and catalysis by CAT is available from a comparison of primary structures and from chemical modification studies. The first studies of the latter sort were done with Type I variants of CAT for which both the amino acid[60] and nucleotide sequences[59,117] have now been determined independently. Because of inhibition of activity following modification of thiol groups,[110] it was suggested that one or possibly two cysteine residues in CAT_I (now known to be Cys-31 and Cys-196) could be at or near the active site and might participate in the chemical mechanism. This approach has been extended to the Type II enzyme wherein it is clear that the role of Cys-31 is conserved in the primary structure and that it is so situated as to lead to ready modification by thiol-reactive reagents with loss of activity.[118] As noted above, however, there is no kinetic evidence for an obligatory acetyl-enzyme intermediate for any CAT variant, and there are reasons for inferring that Cys-31 in CAT_I and its equivalent in CAT_{II} are near the active site but probably do not participate in the catalytic mechanism. Although all three R factor-specified variants (Types I, II, and III) possess a cysteine residue corresponding to Cys-31 of CAT_I, the staphylococcal enzymes (from pC194 and pC221) have threonine at this position and are resistant to thiol-specific inhibitors. A second point favoring an "accidental" role for Cys-31 is that the Type III enzyme is inactivated by mixed disulfide formation with appropriate reagents, but the cysteine residue modified corresponds to position 57 (proline) in the prototype CAT_I structure (see Table 4).

b. A Very Reactive Histidine

The demonstrated reactivity of Cys-196 in CAT_I has not been confirmed in analogous studies using either the Type III (enteric)[114] or Type C (staphylococcal) variant.[72] As compared with CAT_I the latter show both (a) diminished sensitivity to inhibition by

Table 4
PRIMARY STRUCTURE OF CHLORAMPHENICOL ACETYLTRANSFERASES

```
Type          1    5    10   15   20   25   30   35   40   45   50   55
I (Tn9)       MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKF
II                 MNFTRIDLNTWN-          -SVAQC-
III                MNYTKFDVKNWVRREHFEFYVHFR?C??SLTSKIDITTL-   -FKDRHRL

A                  MTFNIINLETWDRKEYF-
B                  MTFNIINLETWDRKEYF-
C (pC221)          MTFNIIKLENWDRKEYFEHY(FNQ)QTTYSITKEINIT     DMIKNKGYEI
D                  MTFNIIELENWDRKEYF-
pC194              MNFNKIDLDNWKRKEIFNHY(LNQ)QTTFSITTETDISVLYRNIKQEGYKF

                   60   65   70   75   80   85   90   95   100  115  110
I                  YPAFIHILARLMNAHPEFRMAMKDGELVIWDSVHPCYTVFHEQTETFSSLWSEYH
III                PCGFIYLIAQAVNQFDELRMAIADNQLIVWDSVDPQFTVFHQETETFSALSCPYS

pC221              YPSLIRAIMEVVNKF MFRTG(IQS)DNKLGYWDKLDPLYTVFNKQTEKFTDIWTEFS
pC194              YPAFIFLVTRVINSNTAFRTG(NNS)DGELGYWDKLEPLYTIFDVGSKTFSGIWTPVK

                   115  120  125  130  135  140  145  150  155  160  165
I                  DDFRQFLHIYSQDVACYGENLAYFPKGFIENMFFVSANPWVSFTSFDLNVANMDN
III                SDIDQFMVNYLSVMERYKSNTLLFPQGVTPQNHINPLAPWVNFDSFDLNVANFDN-

pC221              NNFKQFYNNYKNDLLEYKDKEEMFPKNIIPESNTKM  PWIDFSSFNLNIANNSN
pC194              NDFKEFYDLYLSDVEKYNGSGKLFPKTPIPENAF(SLSII)PWTSFTGFNLNINNNSN

                   170  175  180  185  190  195  200  205  210  215  220
I                  FFAPVFTMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCEDWQGGA
III                -MAKYQQEGDRLLLPL(YLSY)QVHHAVCDGFHVARFINRLQELCNSKLK-

pC221              FLLPIITIGKFYSENNKIYIPVALQVHHAVCDGYHASLFMNEFQDIIHKVDDWI
pC194              YLLPIITAGKFINKGNSIYLPLSLQVHHSVCDGYHAGLFMNSIQELSDRPNDWLL
```

Note: Type I structure from Shaw et al.[60], Alton and Vapnek,[59] and Marcoli et al.[117] Partial sequences for Types II and III have been determined,[111,118] as have the amino-terminal sequences of the staphylococcal variants.[72] Extended regions of sequence for the Type III and Type C proteins have been deduced from studies (unpublished) in the author's laboratory by L. C. Packman, N. M. C. Kaye, J. E. Fitton. Compressions ( ) have been created in the sequences to maximize apparent homologies of flanking regions. The amino terminal methionine of Types A through D have been inferred from the nucleotide sequence of pC194.[75] Each of the four staphylococcal variants has threonine as the first residue of the protein purified from induced bacteria.[72] The alignment of CAT variants in Figure 4 uses a different convention wherein the amino terminus of each polypeptide in residue 1. The buried lysines (Lys-38 of CAT_{III} and Lys-136 of CAT_I) are indicated by crosses above each residue. Reactive cysteines are underlined. Italics are used to draw attention to those residues which are invariant. Note the conservation of proline residues at positions 90, 135, 149, 169, 186, and 56/57. The arrow points to the reactive His-193 at or near the active site.

thiol-specific reagents and (b) preferential alkylation of the histidine corresponding to His-193 of CAT_I. Although all three proteins (I, III, and C) share the sequence -His-His-Ala-Val-Cys-Asp-Gly-, it is possible and indeed likely that the discrepancies will be explained ultimately by rather small but critical differences in accessibility and reactivity of His-193 and Cys-196 among the three variants.

In view of the mounting evidence that Cys-31 and Cys-196 (or their equivalents) might be of less than central importance, the modification of a CAT_{III} by methyl

p-nitrobenzene sulfonate ("Bender's reagent),[119] iodoacetamide, and diethylpyrocarbonate (ethoxyformic anhydride)[120] have been examined as a function of pH and the presence of one or both substrates or "dead end" substrate analogues. The conclusions from these experiments[114] are that His-193 modification by these reagents is prevented by the presence of chloramphenicol but generally not by acetyl-S-CoA. The latter qualification arises from studies with CAT_{III} wherein modest protection against iodoacetamide can be achieved by both substrates. As might be expected from the identities of the nitrophenyl moieties of both Bender's reagent and chloramphenicol, the former has been seen to be an effective irreversible inhibitor at concentrations of the order of 0.1 m*M* while the antibiotic affords full protection. No protection against modification and inactivation was seen with either the S-acetyl or S-acetonyl derivatives of CoA whereas the methyl thio-ether of CoA accelerated inactivation and reduced substantially the protective effect of chloramphenicol. A working hypothesis which has yet to be substantiated is that binding by S-substituted CoA compounds leads to an important conformational change at the active site which, in at least one case, may both reduce affinity for chloramphenicol and expose His-193 to modification.

If the chemical mechanism of catalysis by CAT is a general one and involves His-193 rather than a thiol, it will remain a puzzle (rather than a problem) as to why some variants are remarkably sensitive to reagents which are specific for thiol groups yet are protected from covalent modification and inactivation by chloramphenicol.[110,111,118] This property is sufficiently marked in the case of the Type II variants as to preclude the standard assay for the enzyme in which a chromogenic disulfide is present with the enzyme prior to the addition of chloramphenicol.[68]

Before leaving the subject of location and possible importance of thiol or imidazole groups in CAT variants it should be noted that two analogues of chloramphenicol which are, in effect, substituted haloacetates have been found to be inactive as inhibitors. Both the N-bromoacetyl and N-iodoacetyl analogues of the parent antibiotic are O-acetylated by and fail to inhibit the Types I and III enzymes,[111,114] a result which contrasts with their reported efficacies as covalent modifiers and inhibitors of one or more proteins associated with the *E.coli* 50S ribosome.[121-123] Hence, although protected by chloramphenicol, the nucleophiles of neither His-193 nor any critical cysteine are suitably disposed to react with the haloacetyl side chain of the proposed affinity reagents. Finally, it can be said that the size of the group introduced at the thiol of Cys-31 may be of less importance than the local or transmitted structural changes which accompany modification. Cyanylation of Cys-31 in CAT_I is as effective in inhibiting activity as is the formation of mixed disulfides with the much larger thionitrobenzoate or thiopyridyl substituents.[110] Similar results have been observed in studies[118] with the Type II enzyme wherein the counterpart thiol to Cys-31 is cyanylated (Table 4).

c. Other Functional Groups

Circumstantial evidence suggests that one or more carboxylate anions reside at or near the active center of at least three CAT variants. Early experiments aimed at alkylation of thiols or the formation of mixed sulfides showed that iodoacetamide was 70 times more effective than iodoacetate inhibiting CAT_I and that nitropyridyl disulfides were far superior to Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) as inhibitors of the same enzyme.[110] The importance of possible charge repulsion in preventing the negatively charged dianion of the latter reagent to react with CAT was also shown by experiments which revealed that its methyl ester was at least 500 times more effective than the parent disulfide.[114]

The final functional group implicated in at least one variant (Type III) of CAT is the guanidino group of an arginine residue which is modified by diketone reagents in the

absence of both substrates. Chloramphenicol fails to protect whereas acetyl-S-CoA prevents the concurrent inactivation and modification seen in its absence.[114] Precisely which arginine reacts with the CoA is not yet clear, but electrostatic interactions and/or hydrogen bond formation with phosphates or the adenine moiety seem certain to be of importance.

Taking all the facts which are currently available from (a) comparisons of primary structure, (b) chemical modification, and (c) kinetic studies, a very schematic view of the substrate binding site (s) is depicted in Figure 2. The likely movement which probably attends the binding of both ligands (see above) is not shown but must be important both in catalysis and the "off" steps which, even if shown ultimately to be rate-determining for the overall reaction, are sufficiently fast to allow the rate of acetylation catalyzed by the Type III protein.

Before leaving the cartoon of the catalytic center of CAT depicted in Figure 2, it is worth noting that, although the para-nitro substituent of the benzene ring of chloramphenicol is not essential for substrate binding, there is evidence that the binding site for the acetyl acceptor consists in part of one or more hydrophobic and probably aromatic residues. Studies with solid supports designed for affinity chromatography[74] have revealed that elution of CAT may not require the authentic substrate but may be accomplished with less specific ligands such as the L-erythro analogue of chloramphenicol which lacks the correct stereochemical configuration in the propanediol side-chain. The striking superiority of methyl p-nitrobenzene sulfonate over iodoacetamide as an alkylating reagent for His-193 also reinforces the likely role of an aromatic "pocket" or "hole" at or near the chloramphenicol binding site.

For the sake of completeness it also should be stressed that, although each subunit of the active tetramer contains on average a single binding site for each substrate and a catalytic center, there is no information on whether the active site so defined is made up of residues which are part of the polypeptide chain of each monomer or are constructed from each of two contiguous domains of adjacent subunits.

D. Tetrameric Structure and Subunit Interactions

1. A Very Stable Tetramer

Efforts in the author's laboratory to dissociate the tetrameric structure of CAT_I into functional monomers and dimers by variations in pH, ionic strength, and change in solvent have been uniformly unsuccessful. The need for high concentrations of guanidinium chloride or urea to dissociate CAT has been confirmed more recently for both Type I and Type III variants[69,70] with the conclusion that extensive unfolding of the constituent polypeptides invariably accompanied the disruption of intersubunit bonds which were both strong and noncovalent. A unique unreactive residue was shown to be present in each variant by amidination with methyl acetimidate. Lys-136 of CAT_I and Lys-38 of CAT_{III} behave as inaccessible or "buried" residues which are only exposed to the imidoester on dissociation, but it remains to be shown unambiguously that they participate in salt bridge formation with putative counter anions of contiguous subunits. The extensive amidination of surface amino groups occurs with only partial loss of catalytic activity and no apparent dissociation, but amidination of all amino groups in the presence of high concentrations of guanidinium chloride prevents the effective reassociation of subunits after removal of denaturant.

2. Hybrid Tetramers In vivo and In vitro

The implications of variation in amino acid sequence for tetramer formation is well illustrated by the ease with which CAT variants within a given family associate to form functional tetramers.[46,47,70] By contrast, formation of heteromeric hybrids between, for

example, the CAT_C of *S.aureus* and the *E.coli* CAT_I or CAT_{III} polypeptides has not been demonstrated in vitro. The explanation for this failure may be trivial but at least is consistent with the view that the intersubunit contacts in any given family have evolved independently of the catalytic centers which show substantial homology (see Evolution of Chloramphenicol Acetyltransferase). The crucial test has yet to be done in that, until recently, in vivo hybrid formation between generically different CAT variants has been impossible. The probable importance of experimental design is best illustrated by comparing the results of hybridizaiton of CAT_I and CAT_{III} in vitro (dissociation of mixtures of homo-tetramers in guanidinium chloride followed by dialysis) with in vivo results obtained by purifying hybrid CAT from an *E.coli* strain which carries the two different *cat* determinants on distinct but compatible plasmids.[47,70] The characteristically low yields of active hybrid tetramers from reversible denaturation experiments in vitro is in contrast to the relative ease with which hetero-tetramers can be isolated from systems wherein hybrids have been formed from polypeptides of CAT_I and CAT_{III} as the latter are synthesized from their respective *cat* determinants in vivo. The latter approach was used to compare the equivalence of the buried lysine residues (see above) implicated in the association of sub-units of CAT but is likely to be of importance as a general method for testing monomer contact equivalence and symmetries between homologous oligomeric proteins.

An important caveat arising from the above studies is, however, that ratios of the expected multimers are other than predicted and may be a function of purification methods as well as structural constraints. A preliminary study[47] of CAT_I and CAT_{III} by hybridization in vivo used conventional purification methods and yielded only the α_4, $\alpha_2\beta_2$, and β_4 species whereas subsequent efforts[70] using a preliminary affinity chromatography step demonstrated the "missing" $\alpha_3\beta$ and $\alpha\beta_3$ asymmetric tetramers but did not provide all five predicted tetrameric species in the quantities predicted for a binomial distribution of four equivalent subunits. Explanations for these results include the obvious and trivial one that the rates of expression of cat^I and cat^{III} were different in vivo, but it seems equally likely that the reproducibly low yields of the $\alpha_3\beta$ and $\alpha\beta_3$ species reflects discrimination against the formation of asymmetric heteromers and/or their instability once formed. A molecular explanation for this observation must await X-ray diffraction studies on crystalline proteins, but it is at least likely that a general model for CAT tetramer assembly will include the features stressed in a proposal involving a "dimer of dimers" construction.

Efforts to refine the picture of the CAT tetramer by crosslinking studies using bis-imidoesters have been disappointing. Substantial intramolecular crosslinking of lysines occurs in each monomer, but less extensive reaction between subunits is observed.[124] The latter result is consistent with the observation that neither CAT_I nor CAT_{III} can be dissociated after the complete citraconylation of exposed amino groups, although both enzymes are inactivated. Removal of the citraconyl groups is, nonetheless, accompanied by a return to full activity. A conclusion which follows is that, while the interaction of strong repulsive centers at such positions can distort or disrupt the tertiary structure of individual subunits, such forces do not extend sufficiently far across the inter-subunit boundaries to overcome the strong attractive forces at the interfaces.

E. Evolution of Chloramphenicol Acethyltransferase

1. General Considerations

It will be clear from the remarks above that while the heterogeneity of structure among naturally occurring CAT variants complicates a study of the enzymology, it offers rich opportunities for studying protein and enzyme evolution. Nonetheless, the evidence for "horizontal" transfer between species and genera in microbial populations, effected by phages, plasmids, and transposable elements,[125-127] precludes a species-oriented analysis

of CAT along the lines adopted by students of the evolution of essential cell constituents such as the hemoglobins, cytochromes, and histones. Proteins such as CAT which are (a) specified by accessory genetic elements, (b) useful only under very special circumstances, and (c) function independently of other proteins, polynucleotides, or organelles seem likely to have evolved at greater rates and with constraints which may be different qualitatively.

It may be recalled that naturally occurring bacteria which are sensitive to chloramphenicol-resistant and free of plasmids usually contain no detectable CAT and that selection for chloramphenicol mutants from such strains invariably yields cells which do not contain CAT. (A preliminary report[128] to the effect that strains of *Proteus mirabilis* which show traces of CAT activity can be mutated to high level resistance and abundant enzyme production has not been confirmed. The matter of CAT in *Proteus* species is more complicated than was appreciated initially and is discussed below under Genetics and Molecular Biology. Since no naturally occurring substrate has been found which will substitute for chloramphenicol as an acyl acceptor, the matter of an "ancestral" acetyltransferase has not been explored directly. But it should be noted that a systematic search has not yet been made for components of bacterial cells which might serve as substrates in an assay system using pure CAT and [^{14}C] acetyl-S-CoA. The approach is straightforward, and the results could be most interesting. A directed effort at the forced evolution of CAT activity by analogy with the evolved β-galactosidase *(ebg)* of *E.coli,*[129] or the amidase pathway of *Pseudomonas*[130] seems less promising as the CAT reaction involves two very specific substrates and may therefore have evolved under more constraints than hydrolytic systems[129–131] wherein the second substrate is ubiquitous and at a concentration of 55 molar. The ultimate success of Hartley and his colleagues with the evolution of xylitol utilization in *Klebsiella*[132] via changes in the structure and function of ribitol dehydrogenase is closer to the matter of CAT in a formal sense (two substrates; sugar and coenzyme), but the precursor of CAT is neither at hand nor can it be imagined with any confidence.

2. Novel CAT Variants from Soil Bacteria

Several enzyme variants listed in Table 3 deserve special comment in the context of evolution of CAT. The CAT variants purified from several genera are sufficiently different in catalytic and structural properties to mark them as being of special interest and possible importance. Cases in point are the enzymes from several soil bacteria and the CAT found in certain strains of *Streptomyces*[133–135] and purified from *S.acrimycini.*[136] The *cat* of the latter is chromosomal[137] and the gene product is remarkable for an N-terminal sequence not seen with any other variant, a high proline content, and its failure to react with any of the available anti-CAT sera. It is noteworthy that microorganisms which produce chloramphenicol (or the related corynecins) do not contain CAT,[134,138] and it is still unclear how such prokaryotes, all of which contain 70S ribosomes, avoid autoinhibition of endogenous protein synthesis.[139]

Flavobacteria capable of growth on chloramphenicol as carbon source have been isolated[140–142] and shown to synthesize CAT in constitutive fashion. The enzyme has not been characterized in detail but appears to be distinct from all others examined.[103] The strains are taxonomically heterogenous and also versatile in that they appear to use both normal catabolic pathways and specifically induced steps to assimilate the inactivated antibiotic.[143] Reports of the antibiotic responsiveness of clinical flavobacteria confirm a uniform and high level of resistance to chloramphenicol,[144] but the biochemical phenotype has not been determined. Plasmid-linkage of CAT in *Flavobacterium* has yet to be demonstrated.

Agrobacterium tumefaciens has been the subject of intense study[145,146] because its

ability to cause crown galls in plants is due to the presence of the large Ti plasmid which holds promise as an intermediate in vector development for genetic manipulation in plants. *A.tumefaciens* is also chloramphenicol-resistant, but the determinant is not linked to the Ti plasmid.[103] The CAT found in *A.tumefaciens* is notable in that it possesses a lower isoelectric point than any other variant and is inducible, a property not often observed among Gram-negative bacteria.[111]

The last genus of bacteria which deserves special mention in the context of the evolution and diversity of CAT is *Myxococcus.* Myxococci have a complex developmental cycle involving both a vegetative phase with formation of fruiting bodies and a dormant phase with spore formation. Inducible chloramphenicol resistance has been observed in several species of *Myxococcus,* the phenotype involves CAT synthesis, and there is circumstantial evidence for plasmid linkage.[147,148] No information is available on either the genetics or the enzymology of the *cat* gene product in this intriguing organism.

3. Structural and Functional Consequences of Variation

Consideration of CAT variants for which amino acid sequence data are available reveals a striking variation among enzymes which all share a common tetrameric structure and appear to catalyze the acetylation of chloramphenicol by a similar mechanism. Table 4 and Figure 3 summarize the results of determinations of primary structure based on amino acid and nucleotide sequence analysis and also indicate the preliminary results of predictions of secondary structure for three very different types of CAT and by the use of six predictive methods. The result of the latter analysis[149] is particularly interesting because it may be a guide to tertiary structure homologies between variants commonly specified by plasmids in the Enterobacteriaceae *(E.coli* and "relatives") which have already been discussed in the context of subunit interactions, high catalytic competence, and surface immunologic determinants. Whatever the actual extent of ordered secondary structure in the proteins from Gram-positive and Gram-negative bacteria, it is clear that a matrix of predictive algorithms yields somewhat different results for each. Certain conserved regions can be noted but, apart from that found near His-193 (or its equivalents) which is likely to be of catalytic importance, even informed speculation is unconvincing.

What is clearly missing for any CAT variant is a few experimentally determined distance constraints to limit the number of permissible tertiary folds of the strongly predicted α-helices or β-strands. Analyses of myoglobin[150,151] and globular proteins with high content of β-structure[152] have suggested that reasonable predictions of unknown structures soon may be feasible. In the CAT system there are as yet no spectroscopic or chemical crosslinking data to prompt such an analysis. As more CAT variants are defined in terms of their primary structure it may be possible to refine further the secondary structure predictions for those known to be functionally homologous (e.g., Types I, II, and III of the enteric family and Types A through D of staphylococci).

4. Scope and Limitations of Experimental Evolution with CAT

A good deal has been learned from a detailed analysis of the experimental step-wise evolution in a few systems, but most of the information gained concerns either changes in catalytic specificity or efficiency on the one hand or gene expression on the other.[129-132,153] Neither has it been possible as yet to relate the changes in ligand binding or catalysis to a physical model nor has it been possible in most enzyme systems to look at the evolution of structural determinants, including both inter-subunit residues and those which interact with solvent. The ease of transfer of *cat* between divergent bacterial hosts and the relative stability of the genetic determinant in such instances suggests an interesting approach to answering at least two questions which are accessible experimentally.

Posed in the context of the Type C variant of CAT from *S.aureus* one may ask whether the surface amino acid residues of the enzyme which differ from corresponding ones of, for example, the CAT_I variant do so because of intrinsically different intracellular environments. Might it be possible, therefore, to demonstrate on the time scale of experimental evolution that substitutions in surface residues of the CAT_C would accompany the propagation of its structural gene in *E.coli*? Will maintenance of the *cat* specifying the Type I protein in *B.subtilis* (rather than *E.coli*, its usual host) lead to changes of primary structure which may ultimately be explicable by selection on the basis of differences in endogenous proteolytic activity? Such considerations impinge on the larger question of protein degradation and turnover which have been discussed recently,[154-156] but they can be seen to be distinct in the sense that intergeneric transfer may expose surface topographies (loops and susceptible peptide bonds) which may differ qualitatively from those which trigger host-specific degradation responses.

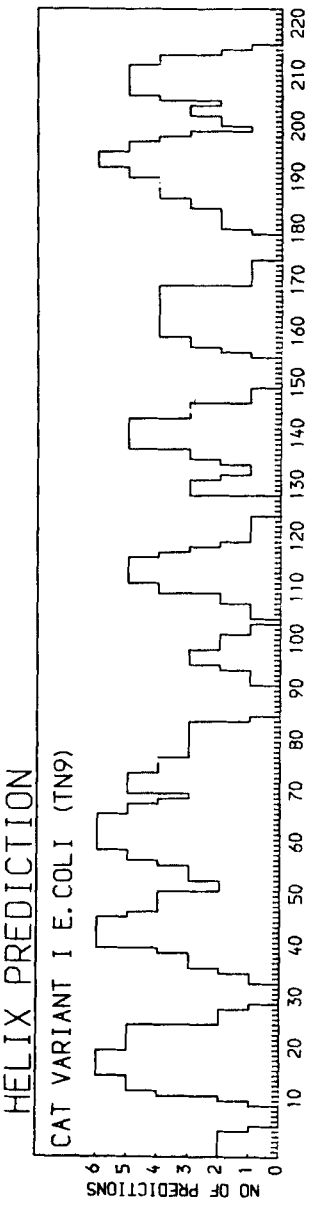
A second question bearing both on evolution in general and on the variety of CAT variants is that of the importance of genetic recombination between *cat* determinants which may differ substantially in structure. Yanofsky and his colleagues recently have demonstrated[157] the utility of a two-plasmid system for observing unselected recombination between deletion mutants in *trp*A using genes from *E.coli* and *S.typhimurium* which are 75% identical at the nucleotide level. The importance of recombination as a mechanism for evolution will not be enhanced by analogous and successful experiments with CAT variants, but the recombinant *cat* products may provide useful clues to rules of subunit association in the CAT families and the relative importance of regions of the structure(s) accounting for variations in heat stability, reactivity toward modification reagents, and immunological reactivity with monospecific antibody. The opportunities and prospects for the use of in vitro genetic recombination for such purposes are highlighted by the report of Betz and Sadler that a 13 amino acid insertion between residues 71 and 72 of CAT_I can be accomplished without loss of the chloramphenicol resistance phenotype and with retention of up to 15% of the wild-type enzyme activity.[262] Although the construction of the novel gene was indirect and arose from a study of *lac* operator insertions at the unique *Eco* RI site within *cat*[1] (see Section IV.), the experiment emphasizes the power which such methods will have when applied to specific questions of structure and function.

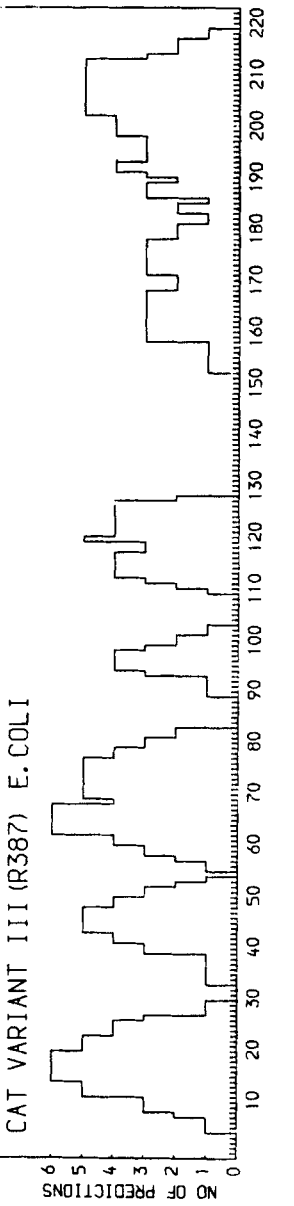
Finally, it must be stressed that the evolution of CAT encompasses more than the history of variation of the structural gene. As will be apparent in the following discussion, the nucleotide sequences of the *cat* genes for the very different Type I and Type C enzymes show few regions of identity, and the regulation of each is quite distinct. In particular, the open question of the mechanism by which synthesis of the Type C variant (and other staphylococcal *cat* products) is regulated has important implications for the evolution of the system. If, as has been proposed,[75] the CAT in staphylococci is autogenously regulated, it follows that the presence of a specific DNA binding region by the latter enzyme represents a very different and important structural constraint which is not shared by the constitutive variants more commonly found in Gram-negative bacteria.

IV. MOLECULAR BIOLOGY OF CHLORAMPHENICOL ACETYLTRANSFERASES

A. Data Limited to Only Two *cat* Types

Although some descriptive information is available on the regulation of expression of several CAT variants listed in Table 1, there are only two systems for which both nucleotide sequences and functional data are available. One is the Type I (Gram-negative) variant typified by the *cat* genes of Tn9, NR1 (alias R100 and R222), R6 (pAC184), and pBR325. The synthesis of CAT_I in *E.coli* is constitutive but subject ot





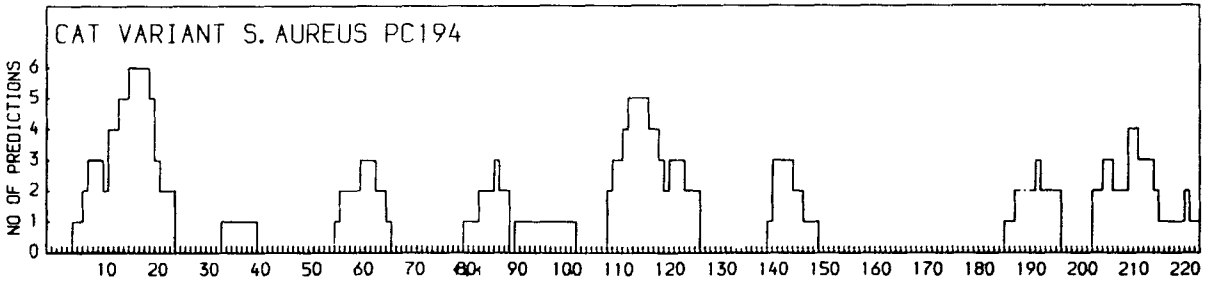


FIGURE 3. Six methods were used by Eliopoulos[149] to predict the most likely secondary structure for each of the three CAT variants.[250-259] As shown in Table 4 the primary structures of the Type I (*E. coli*/Tn9) and *S. aureus* (pC194) are known in their entirety whereas the Type III (*E. coli*/R387) is 95% complete. For predictive purposes the few missing residues of CAT_{III} were "replaced" by the corresponding residues in CAT_I, and residue 1 in each case is the amino terminal methionine of the polypeptide. Analogous histograms which were prepared (not shown) indicated the number of methods (ordinate) predicting β-structure or, alternatively, likely turns or coils. The latter predictions showed substantially less congruence among the three variants than was seen for α-helix or β-sheet. Comparison of the linked histograms for α- and β-structures for the three variants shows a similar alternation over the amino terminal half of the composite structure. As discussed in the text (Active Site Topography) there is circumstantial evidence from thiol modification experiments that Cys-31 (present in Types I through III), Cys-57 (Type III only), and Cys-196 (all variants) may occupy sites in the folded structure which are contiguous with or part of the binding site for chloramphenicol. Similar insights into the likely constraints on possible folding pathways could come from evidence that Lys-136 of CAT_I and Lys-38 of CAT_{III} may be functionally equivalent in forming salt bridges at the interface between subunits (see Tetrameric Structure and Subunit Interactions).

catabolite repression which is effected by 3′, 5′-cyclic AMP (cAMP) in concert with the apo-effector described as cAMP receptor protein (CRP) or catabolite gene activator protein (CAP). The other CAT system which warrants a detailed description and discussion is that found in *S.aureus* and which is induced by the presence of subinhibitory levels of chloramphenicol or the "gratuitous" inducer 3-deoxychloramphenicol. The most intensively studied examples of the *S.aureus* CAT are those specified by the small naturally occurring plasmids pC194 (2.9kb) and pC221 (4.2 kb), derivatives of which have attracted attention as cloning vehicles in Gram-positive bacteria.

B. Structure and Regulation of *cat* (Type I) in *E.coli*

1. Several cat Genes Have the Same Structure

It is fortunate that the CAT variant most frequently observed in natural isolates of enteric bacteria has come to be the most intensively studied by molecular biologists. Although the details need not be recounted, it may be noted that the first event which singled out chloramphenicol resistance (among several resistance determinants on enteric plasmids) for special attention was the chance isolation of a non-defective phage P1 derivative which had acquired a *cat* determinant from a plasmid known as R14 and now designated pSM14.[62,159] The *cat* involved is that now recognized as the gene for CAT_I which is encoded by Tn9, a distinct element which has maintained its physical and genetic identity through many transposition events which have been well summarized elsewhere.[158,160] The generation of such elements is discussed in a more general context by Chandler et al.[161] and by Iida and Arber.[162] Here it is sufficient to note that the "packaging" of *cat* between directly repeating 768 bp insertion sequences (IS1) which mediate transposition and which contain unique sites for cleavage by *Pst*I has greatly accelerated progress in the molecular biology and manipulation of *cat*. Its strong promoter (see below) and *Eco*RI site in the coding sequence for CAT_I have made it attractive as a target for cloning by insertional inactivation (pAC184 and pBR325).

In contrast to the wealth of detail available on Tn9 and its *cat* there is a paucity of genetic information relating structure to function for CAT_I. Early reports of intragenic recombination in *cat*[163] were followed by a more systematic inquiry into *cat* genetics.[164] The prospects for a genetic analysis of *cat* were reviewed briefly in the immediate pre-transposon era[165] but, apart from the importance of chain terminating and temperature-sensitive mutants of *cat* in analyzing fusidic acid resistance (see below), rather less has been accomplished than might have been expected.

2. Catabolite Repression in vivo

Harwood and Smith[166] showed that the synthesis of CAT could be suppressed by growth on glucose and that this effect was prevented by exogenous cAMP and failed to occur with glycerol as the carbon source. This examples of glucose or catabolite repression was studied subsequently with an in vitro transcription/translation system in which the *cat* gene was present in P1CM and wherein both cAMP and CRP (CAP) could be shown to be essential for maximal rates of CAT synthesis.[167,168] *E.coli* mutants lacking adenylate cyclase or the effector protein failed to support rates of CAT synthesis required for a high level of chloramphenicol resistance, and the addition of exogenous cAMP failed to restore CAT synthesis in *crp* (*cap*) mutants. After a hiatus which corresponded with the development of in vitro DNA manipulation and sequencing methods an elegant series of experiments have defined some details of the interaction of the regulatory region of the gene for CAT_I with RNA polymerase and the cAMP/CAP system.

3. The Expression of cat in vitro

Le Grice and Matzura first examined the transcription of *cat* borne by pAC184 and its dependence on both positive control elements[169] and then extended their analysis to the

cat shown to be associated with NR1 (R100).[170] A novel transposon generated by deletion from the r-determinant region of NR1 via phage P1[171] was cloned into the *Pst*I site of pBR322 by Iida as part of a study of the role of insertion sequence IS1 in the mechanism of transposition of drug resistance genes,[172-175] and this construct was used to determine the nucleotide sequence of the *cat* region of NR1.[117] Transcription in vitro was shown[169] to initiate with a G residue 39 base pairs removed from the methionine initiation codon and was seen to lead to a truncated mRNA product (12 residues) as well as the expected full length transcript. The synthesis of the short product, terminating in a GC-rich region, has not been studied further, but bears a superficial resemblance to the abortive transcripts observed in systems regulated by attenuation mechanisms[176,177] and could be of some importance in vivo. It should be noted that there have been few studies in vivo of the control of transcription of drug resistance genes and *cat* in particular. Studies with a plasmid likely to be identical to NR1 (R100) in all important respects revealed that the *cat* message is the main transcription product and that *cat* expression is enhanced by the presence of very high concentrations of chloramphenicol.[178,179] The in vitro transcription of *cat* in pAC184 has been shown by electron microscopy[180] to take place from a single strong promoter, but possible modulation of the process by translational effects could not be examined in this system.

Protection experiments in vitro[181] showed that the RNA polymerase binding site extends from +25 to at least −25 (relative to the transcription start) but that polymerase binding was poor in the absence of the cAMP/CAP complex. DNase digestion in the presence of the latter complex alone yielded protection centered around −35 and around −120 as well.[182] The complication of two cAMP/CAP sites appears to have been resolved by the observation that the −120 site is not essential for transcription,[183] but the remaining CAP site adjacent to or overlapping the promoter bears only an imperfect resemblance to the classic CAP sites of the *lac, gal,* and *ara* systems. Figure 4 compares the region in question of the Type I *cat* with its counterparts in each of the above genes. Although a detailed examination of the cAMP/CAP system is outside the scope of this review and has been recently discussed elsewhere,[184-186] it is clear that a general model accounting for its effects may have to accommodate increasing evidence placing CAP sites at or very near RNA polymerase binding sites in *cat* and other systems.[187] But whatever the mechanism may prove to be, it remains a puzzling fact that the gene specifying an antibiotic resistance gene should be subject to a form of regulation concerned with the availability of energy sources. Since the origin of CAT is equally obscure it may well be, as others have observed,[188] that the extreme natural environments where chloramphenicol and other antibiotics are found present special metabolic demands on microbes which inhabit such ecological niches. Martin and Demain recently have reviewed[189] the regulation of antibiotic synthesis and noted the frequency with which one or more steps in a pathway involving secondary metabolites may be subject to catabolite regulation. It would not be surprising if these two seemingly unrelated phenomena had a common explanation. The streptomycin adenylyltransferase which catalyzes the inactivation of streptomycin is both plasmid-borne and subject to catabolite repression[166] but has not been studied in detail. It also remains to be seen whether other R-plasmid *cat* genes are under cAMP/CAP control, and the same may be said of the variants synthesized by soil micro-organisms such as *Flavobacterium* and *Streptomyces*.

4. The Type I CAT and Resistance to Fusidic Acid

Before leaving the structure and functions of the *cat*^{I} it may be recalled that a number of workers have noted that plasmid-linked chloramphenicol resistance in *E.coli* is sometimes associated with the unexpected phenotype of resistance to fusidic acid,[172,190-194] a property which can only be observed when the *cat* gene is present in a host made sensitive to fusidate by virtue of one or more mutations conferring increased

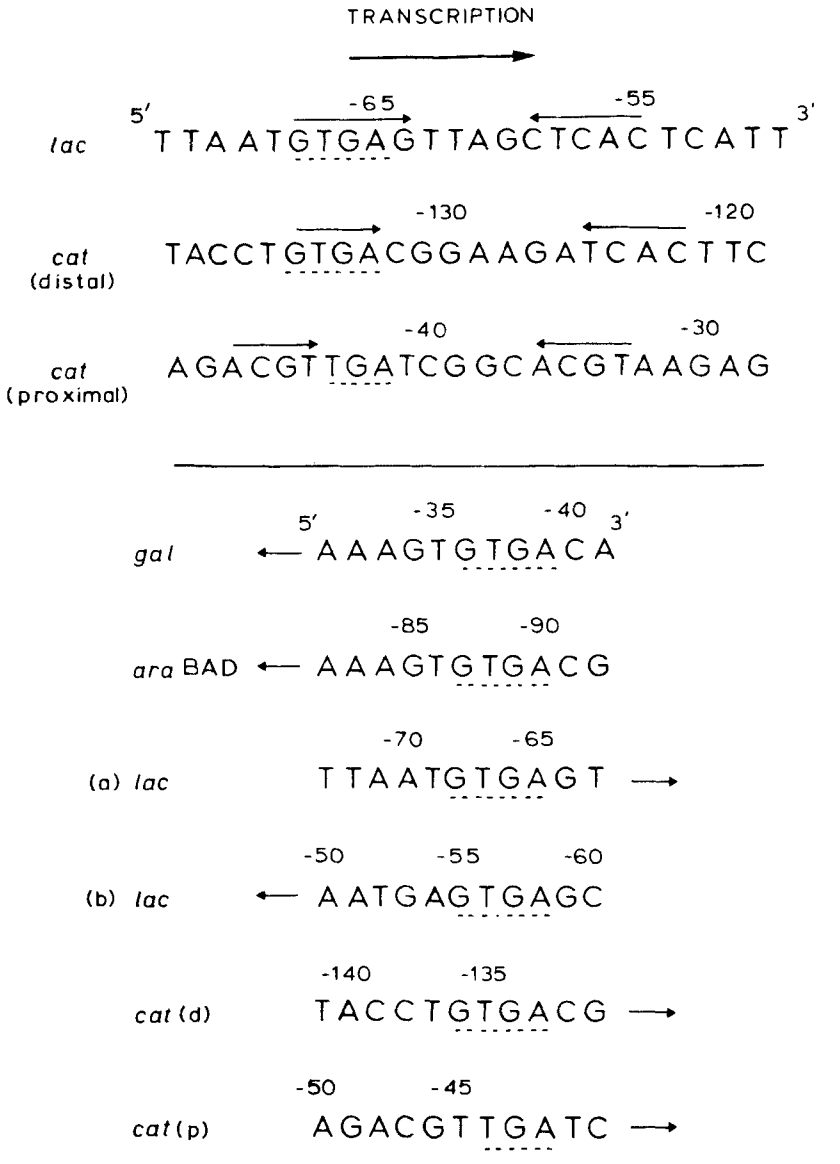


FIGURE 4. The structures of regulatory regions of genes under positive control by the cAMP/CAP complex with emphasis on segments shown to be protected from methylation or DNase digestion by the effector complex. Two conventions are used to emphasize different aspects of the structures being compared. In the top half, the *lac* site is shown in full to emphasize the dyad symmetry at -61/62 (converging arrows to indicate complementarity). The distal and proximal (with reference to the initiation of transcription) *cat* sites are compared to *lac* with respect to analogous regions of complementarity. The underlined bases are those which occur in all examples. In the lower half of the diagram the structures are compared in the fashion of O'Neill et al.[185] Arrows indicate the direction of transcription. The structures (a) *lac* and (b) *lac* refer to positions of the larger sequence wherein (b) *lac* can be seen to be the complement of the -60 to -50 sequence shown above. The latter convention was used to emphasize the factors which might favor the binding of two dimers of CAP (four subunits) to the *lac* regulatory site. The numbering in each case is with respect to the transcription start. The CAP sites of *gal* and *ara* differ from *lac* in that they fail to show dyad symmetry. The distal (d) CAP site is dispensible in that cAMP dependent transcription in vitro takes place in its absence from the alternative proximal (p) site.[181-183]

permeability to the drug. Dempsey and Willetts studied fusidate resistance *(fus)* specified by R100[191] and suggested that *cat* and *fus* could be separated genetically, but subsequent genetic studies by Iida and his collaborators clearly have demonstrated that *cat* and *fus* are colinear and that CAT_I must confer both resistance phenotypes.[172,188] Only the Type I enteric type of CAT specifies fusidate resistance, no *S.aureus cat* plasmids are resistant to the second antibiotic, and inactivation or modification of fusidate by CAT_I has not been demonstrated.[195] Since fusidate appears to be capable of inhibiting strains of fusidate-sensitive *E.coli* which harbor *cat* for the Type I enzyme,[188] the resistance mechanism will need to incorporate a very specific interaction between CAT_I and fusidic acid which serves to neutralize or prevent its effects on protein synthesis. The lack of evidence for inactivation and the high concentration of CAT_I in cells in vivo makes a "sponge" or "buffer" mechanism a tenable if undynamic mechanism. The ability of fusidate to inhibit the acetylation of chloramphenicol by CAT_I, the reversal of fusidate inhibition of cell-free protein synthesis by purified CAT_I, and the tight binding ($K_d = 25\ \mu M$) of [^{3}H] fusidate to the enzyme[195] are all compatible with this model.

The special properties of CAT_I in relation to the binding of ligands such as fusidate (a steroid with no obvious structural resemblance to chloramphenicol) has been observed as well with a number of dyes of the triphenyl methane series which were shown to be competitive inhibitors of CAT.[108] A recent report by Proctor and Rownd[196] has extended this observation by demonstrating that such rosanilin dyes are taken up by colonies containing CAT_I to such an extent that the phenomenon may be used to differentiate CAT^+ from CAT^- colonies under certain conditions. The latter may be an especially useful technique for molecular biologists cloning into the *Eco*RI site within the *cat* gene and wishing to identify directly clones which lack functional CAT_I. As no other CAT variants appear to bind the triphenylmethane dyes, the phenomenon will be useful only for *cat* specifying the Type I protein.[195]

Observations above on the diversity of CAT variants have already included the mention of the fact that *Proteus* species, members of the Enterobacteriaceae which differ in important respects from *E.coli*,[197] contain low level CAT activity. Exposure to chloramphenicol yields a population of cells with high levels of enzyme and full resistance to the drug, properties which decay exponentially following growth in the absence of chloramphenicol. The CAT in *Proteus* is of the Type I variety in all important respects.[111] Recent studies have shown that *Proteus* species are almost uniformly CAT^+ but no plasmids have been detected in the strains examined.[136] The *Proteus cat* has been cloned, and its structure is being studied with respect to targets for site-specific endonucleases, catabolite repression, and its ability to confer fusidic acid resistance in suitable *E.coli* hosts.

5. *Expression of cat (Type I) in Novel Hosts*

The rapid expansion of knowledge concerning the structure of *cat* and its usefulness in genetic manipulation has led to several efforts to use it as a probe to study gene expression in backgrounds other than *E.coli*. Cohen et al.[198] prepared a co-integrate plasmid from pBR325 and a yeast vector and sought the synthesis of CAT following transformation of *Saccharomyces cerevesiae* by selecting for resistance to chloramphenicol under aerobic growth conditions. Measurable levels of expression were observed, but it has not yet been shown rigorously that transcription is from the prokaryotic cat^I promoter. A different approach has been taken by others who have treated *cat* with site-specific endonuclease to generate a promoter-less structural gene which can be used as a cartridge to probe the genomes of other organisms for segments of DNA active in initiating transcription. This approach has been used with success in *B.subtilis*,[199,200] and fusion of *cat* with the collagen promoter has been used to monitor the

transcription of collagen following transfection of animal cells by Rous sarcoma virus.[201]

Synthesis of CAT_1 from an early SV40 promoter has been demonstrated in transfected monkey kidney cells, and selection for chloramphenicol resistance has been accomplished.[202] The attraction of the CAT system for animal cell biologists is that the enzyme and its resistance phenotype can be assayed or scored and further that there is no background enzyme activity from host cells to complicate such measurements. Cloning into the *Eco*RI or other sites will no doubt be feasible, and efforts to create novel endonuclease sites within *cat* could lead to its even greater utility as a cloning tool.

C. Structure and Regulation of Staphylococcal *cat*

1. General Features

Until relatively recently the CAT variants of staphylococci and related Gram-positive bacteria have been ignored by molecular biologists. A change came abruptly when Ehrlich demonstrated[203] that *B.subtilis* is readily transformed by small naturally occurring staphylococcal plasmids which usually specify one or (rarely) two resistance phenotypes. The likely importance of such plasmids for genetic manipulation in bacilli has led to a flood of information on the biology of such plasmids, their replication functions, and their resistance genes.

Much of what is known about *cat* in *S.aureus* comes from a study of the determinants found on two plasmids. The natural plasmid pC194[204] has been used widely for bivalent vector construction and the study of plasmid replication in bacilli and *E.coli* whereas the CAT of pC221 has been under study for a decade as the prototype inducible variant of Gram-positive bacteria.[72,77-79]

Before considering the nucleotide sequence[75] of the *cat* of pC194, it should be recalled that the staphylococcal CAT variants differ from their Gram-negative counterparts in being inducible by chloramphenicol and closely related compounds; they neither cross-react immunologically with the enteric enzymes[77] nor will they form mixed (hybrid) tetramers with them[46] and, generally speaking, the staphylococcal CAT variants are less sensitive to thiol specific reagents[72] and are less heat labile.[77] The *cat* plasmids of *S.aureus* do share similarities in structure (size, sequence homology, and restriction endonuclease sites)[205-207] and the CAT proteins show greater similarities in both structure and function than do the three enteric variants (see Tables 3 and 4).

2. Physiology of the Induction Process

The phenomenon of induction has been described and discussed for the CAT_C of plasmid pC221[77-79] and its derivatives,[207] and similar studies have been performed on other staphylococcal systems which have not been defined genetically.[208,209] In general terms the novelty of this process (and that of all inducible CAT systems) is that the inducer (chloramphenicol) is both a substrate for the enzyme and is itself the prototype general inhibitor of induced enzyme synthesis. The kinetics of induction are therefore predictably sigmoid in that the level of CAT increases within cells of *S.aureus* after a lag of 3 to 5 min following the addition of antibiotic at a subinhibitory concentration (5 μM or 1.6 $\mu g/m\ell^{-1}$) but rapidly reaches a plateau as chloramphenicol is acetylated and rendered ineffective as an inducer.[77,79] At a concentration of chloramphenicol 10-fold greater than that required to inhibit protein synthesis (approximately 10 μM in *S.aureus),* comparable induction is also observed but after a delay of an hour, during which time a slow rate of CAT synthesis can be detected and the concentration of active antibiotic falls slowly to a level which, although subinhibitory, still supports induction.[77] Such complications are avoided by the use of 3-deoxychloramphenicol which is effective as an inducer at concentrations (0.5 to 5 μM) where it has little effect on protein synthesis.[79] The induction process is inhibited by puromycin and by inhibitors of

transcription. Apart from the lack of a need for the 3-hydroxyl group, the structural specificity of the induction process mirrors in a general way the requirements for antibiotic activity and the CAT requirements for acetyl acceptor activity. The recently described 3-fluoro analogue of chloramphenicol[89] is an effective inducer at concentrations well below 5 μM, is an inhibitor of CAT_C (competitive with chloramphenicol; $K_i = 30\ \mu M$), and appears to be as active in antibiotic assays as the parent compound.[90] It may help to keep the physiology of the induction process in mind while considering the molecular biology.

3. Expression of the Staphylococcal cat in Other Genera

The chloramphenicol resistance plasmids of *S.aureus* replicate in *B.subtilis*[203,211,212] and *cat* is inducible.[213] Similarly, the staphylococcal *cat* of cointegrate bivalent replicons (capable of replication in either Gram-positive or Gram-negative hosts) is expressed in *B.subtilis*.[214,215] Synthesis of the staphylococcal CAT in *E.coli* is still enhanced by 3-deoxychloramphenicol, but the maximum levels of CAT achieved are substantially lower than those observed in *S.aureus* with *cat* on the parental plasmid, pC221. The full significance of this observation is not yet clear because of possible host-related variations in plasmid copy number. Further difficulties arise in interpreting the results of studies with *E.coli* on the expression of *cat* carried on small fragments (<1 kb) cloned into pBR322 from pC194[75] and pC221.[210] The absolute levels of induced CAT are low, but the fact remains that the only open reading frame in *cat* cloned from pC194 is that specifying CAT. Previously it had been demonstrated that the small (~ 1.9 kb) *cat* replicon pCW41 (derived from pC221) expressed a maximum of four polypeptides, including CAT, in an in vitro *E.coli* expression system.[216] One of the protein products of pCW41 was expected to be the putative regulatory apo-effector which has been so elusive. Efforts in the author's laboratory to isolate constitutive mutants for the *cat* of pC221 in vivo have been uniformly unsuccessful, and the hypothesis has been that a high plasmid copy number (>20 per chromosome)[217] insured that the mutation of a single copy of the regulatory locus would have little effect in *trans* on the many *cat* structural genes present. Our failure to isolate constitutive mutants after in vitro mutagenesis using purified plasmid DNA has cast doubt on such a facile explanation since special efforts were made to transform *S.aureus* with low ratios of DNA to bacteria and to allow for segregation of mutants.[218]

4. Nucleotide Sequence of a Staphylococcal cat

The structure of pC194 was determined by Horinouchi and Weisblum[75] as part of a study of the replication and regulation of small staphylococcal plasmids including pE194, a structurally distinct plasmid which specifies macrolide (e.g., Erythromycin) resistance.[219] The entire pC194 genome consists of 2910 base pairs and contains, in common with all staphylococcal plasmids conferring chloramphenicol resistance, a unique *Hind*III site which lies outside *cat*. The enzyme variant specified by pC194 has not been encountered in previous studies of CAT in staphylococci but has been observed to have an electrophoretic mobility[210] falling between those of the type B and C enzymes examined previously.[72] The complete nucleotide sequence of pC194 is of interest since it is an autonomous replicon, but the remarks which follow are directed at the structure and function of a 950 base pair fragment which carries *cat* and flanking regions and which when inserted in pBR322 yielded *E.coli* transformants which were observed to be chloramphenicol-resistant and in which the level of the resistance phenotype was enhanced by prior exposure of the cells to subinhibitory concentrations of the antibiotic.[75] Although determination of the nucleotide sequence of the *cat* region of pC221 (or its more convenient smaller derivative pCW41) has not been completed, it may be useful to compare both the structural and functional data for *cat* in pC194 with the analogous features of the *cat* originating in pC221. Figure 5 compares the detailed

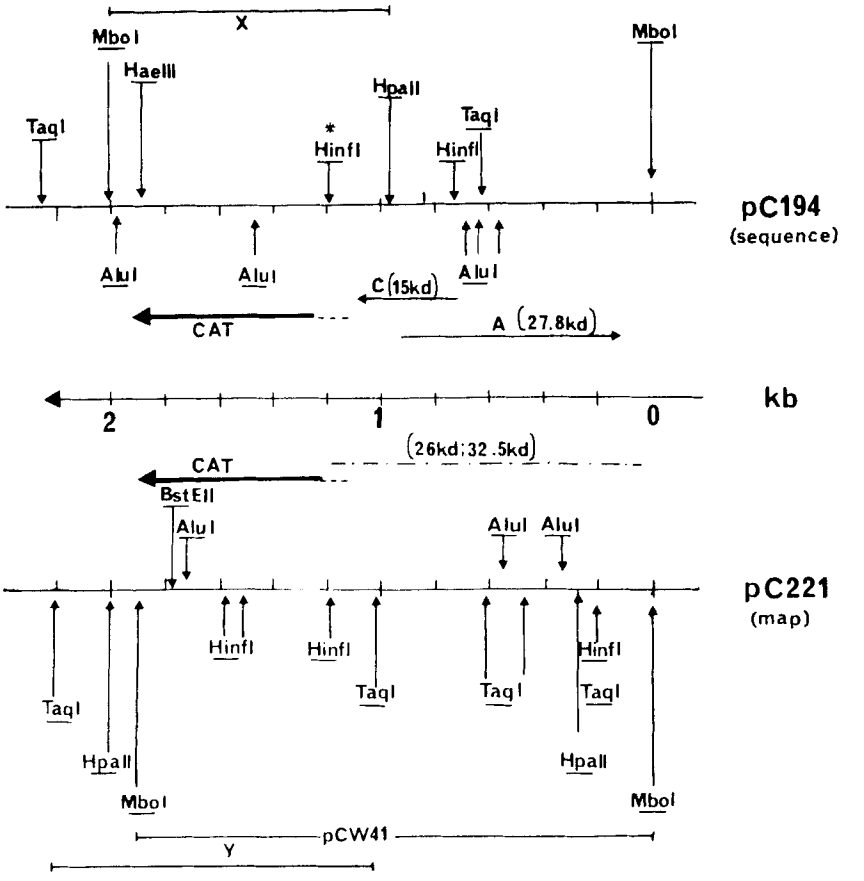


FIGURE 5. Comparison of restriction and functional map of pC221 with structure and predicted proteins of pC194 from DNA sequence.[75] *Mbo*I site at map coordinate O of pC221 in this diagram corresponds to site 1 kb from reference *Hin*dIII site on published map of pC221.[207] Position of CAT for pC221 determined from *Bst*EII site at residues 198—200 of amino acid sequence. Also pCW41 cut by *Taq*I still yields CAT in cell-free system.[210] Heavy arrows marked CAT represent structural gene region whereas dashed tail is 5′ regulatory region. Proteins A and C are predicted from nucleotide sequence.[75] Cell-free system *(E. coli)* yields CAT plus two other proteins (32.5 K and 26 K) from pCW41.[216] Solid lines marked X (top) and Y (bottom) indicate fragments cloned into pBR322 from pC194 and pC221 respectively to yield inducible *cat* expression in *E. coli*. The *Hin*fI site of pC194 marked with (*) lies in a region which is likely to be important for transcription, and this site appears to be conserved in the *cat* of pC221 (pCW41).

restriction map of the *cat* segment of pC221 with a comparable region of the structure of pC194. From the extent of divergence of amino acid sequences between their respective CAT products (Table 4) it is not surprising that there are substantial differences in restriction endonuclease targets between the two. Several similarities in structure are apparent, however, and best seen by noting the apparently identical targets at *Taq*I (616) and *Hin*fI (1190) and the position of the structural genes for CAT in each case. Two proteins are predicted from open reading frames of pC194 in this region, but neither are preceded by convincing ribosome binding sites on the putative mRNA. The use of supercoiled pCW41 in a cell-free *(E.coli)* expression system has led to the synthesis of CAT and two other radioactive polypeptides (apparent molecular weights 26 kd and 32.5 kd).[216] The significance or function of these in vitro products is not yet clear, but it is

interesting to note that the first four residues predicted for the 15 kd protein ("C") of pC194 are identical to the first four of the pC221 *cat* product (Met-Thr-Phe-Asn) while pC194 has Met-Asn-Phe-Asn at the amino terminus of CAT (see Table 4).

The most striking feature of the *cat* region of the structure determined by Horinouchi and Weisblum is a 37 base pair sequence which constitutes an inverted complementary repeat and which is located between the likely start of transcription and the ATG codon initiating translation. The 3′ end (sense strand) of the "hairpin" overlaps the proposed ribosome binding site. The authors have proposed that the *cat* of pC194 is controlled autogenously and that transcription is autoregulated by the interaction of the product (CAT) with this inverted complementary repeat in the presence of chloramphenicol. The evidence for this model for pC194 derives from the fact that (a) the *cat* segment cloned in pBR322 yielded inducible chloramphenicol resistance in *E.coli* and (b) the cloned segment X contained only one open reading frame, that yielding CAT (see Figure 5 and legend). Analogous experiments in *E coli* with a cloned fragment from pC221 (fragment Y in Figure 5) have yielded similar results; namely a five-fold increase in CAT when cells were exposed to 3-deoxychloramphenicol but no change in the level of expression of β-lactamase encoded by the vector pBR322. Attempts in the author's laboratory to extend these observations to an in vitro system have, however, yielded no evidence for autoregulation by CAT — in the presence or absence of inducer. That is, the addition of highly-purified CAT_C to an *E.coli* cell-free expression system using super-coiled pCW41 as template has not been observed to stimulate or inhibit the rate or the extent of CAT synthesis, and the addition of 3-deoxychloramphenicol is without effect.[210]

It remains to be seen whether the apparent induction in *E.coli* of staphylococcal CAT from the small fragment cloned into pBR322 is a faithful representation of the normal process seen in *S.aureus* wherein the increase in CAT level following induction can be on the order of 100-fold.[77] Indeed, both the expression and regulation of this *cat* highlight a problem which will become increasingly common as cloned bacterial genes from one genus are used to achieve expression in others. Recent studies of the expression of a β-lactamase specified by a plasmid in *S.aureus* have revealed a likely basis for the failure of genes from Gram-negative bacteria to be expressed in Gram-positive hosts such as *B.subtilis*.[263] Both the putative ribosome recognition site of the *S.aureus* β-lactamase and the CAT of pC194 contain five G-C base pairs and show a striking complementarity to the 3′ terminus of the 16S rRNA of *B.subtilis,* the latter observation suggesting an explanation for the diminished requirement for initiation factors when mRNAs of Gram-positive bacteria are translated in vitro with *B.subtilis* extracts.[264] The β-lactamase results not only mirror previous studies which showed that transcription and translation of pC221 (and its fragments) could be achieved in cell-free *E.coli* systems[216] but also reveal a striking similarity in the organization of the β-lactamase and CAT (pC194) genes in the regions which are likely to be active in initiating and regulating transcription. Both the β-lactamase[263] and the CAT of pC194[75] possess inverted complementary repeats, potential double-stranded stem structures which may play corresponding if not analogous roles in the regulation of synthesis of their respective enzymes. The type C variant of CAT (pC221) appears to be under transcriptional control in that (a) the presence of rifampicin during the induction period prevents the expected augmented expression of *cat* and (b) fully induced *S.aureus* cells contain increased levels of RNA which hybridizes specifically to a DNA fragment (0.9 kb) of pC221 (*Mbo*I to *Taq*I; see Figure 5) which contains only the *cat* region.[210] Circumstantial evidence therefore favors the view that the apparently autogenous regulation of CAT in *S.aureus* is at the transcriptional level, but the details are missing. If CAT does regulate its own synthesis, it seems likely that it does so as a positive apo-effector, acting in concert with chloramphenicol (or analogues with inducing potential). A repressor mechanism

(negative control) seems less likely *a priori* since substrate-induced derepression of enzyme synthesis would lead necessarily to concurrent repression, thus providing a negative feedback which is difficult to reconcile with the observed kinetics of induction.[79]

5. CAT Found in Other Gram-Positive Bacteria

Some of the problems and possibilities created by variety are well illustrated by a brief inspection of the instances wherein CAT has been detected in Gram-positive and chloramphenicol-resistant micrococci and bacilli. In epidemiologic terms, the recognition that a clinically important species such as *Streptococcus pneumoniae* may harbor resistance determinants to multiple drugs[220-222] *and* a CAT variant which resembles those of staphylococci[111] poses several questions. Studies to date with CAT have dealt with descriptive aspects of the problem. One awaits with interest detailed information on the comparative anatomy of *cat* and its associated structures from *S.pneumoniae*[223] and from other streptococci known to harbor the determinant.[224-227] The recent renaissance of interest in transformation of pneumococci by both homologous[228] and heterologous DNA[229] and the evidence that a conjugative process may play a role in transfer of the chromosomal *cat* of *S.pneumoniae*[223] both suggest that developments will be rapid. The evidence that erythromycin resistance[230] and kanamycin resistance (cited by Shoemaker et al.[223]) determinants of pneumococci are the same as their staphylococcal counterparts suggests that similar results will be found with *cat*. The situation in streptococci other than *S.pneumoniae* is more clear-cut in that the presence of plasmids are the rule rather than the exception, and some progress has been made on structural studies with plasmids bearing *cat*.[227] Since a process resembling conjugation in micrococci was first shown with *S.faecalis*[224] it will be instructive to pursue in more detail the horizontal spread of *cat* through it and related organisms.

Gram-positive bacilli have not been screened exhaustively for chloramphenicol resistance, but the common aerobic species (e.g., *B.subtilis, B.megaterium,* or *B.licheniformis* in laboratory use do not contain cat. Lovett and his colleagues have, however, observed that most strains of *B.pumilus* express resistance to low levels of chloramphenicol (10—20 $\mu g/m\ell^{-1}$) which correlates with the presence of a CAT which has not yet been characterized and seems likely to be chromosomal.[231] Fragments generated by *Eco*RI digestion have been inserted into an *S.aureus* plasmid (pUB110) specifying neomycin resistance which expresses high level inducible chloramphenicol resistance and CAT synthesis in *B.subtilis*.[232] Appropriate derivatives which are CAT^- by virtue of deletions involving the regulatory region have been used to screen for promoter functions in DNA segments from diverse Gram-positive sources, an approach which is technically different but analogous to the use of the Tn9 *cat* structural region as a probe.[199] It is of some interest that the cloned *cat* fragment derived from *B.pumilus* does not hybridize to pC194,[231] a result which suggests that CAT among bacilli may be more closely related to counterparts in other free-living soil bacteria than to clinical isolates of Gram-positive genera, an argument supported by the results of a recent study of tetracycline resistance plasmids[265] by Polak and Novick. Precisely where the CAT of *Clostridium perfringens* fits in is anything but clear, since only the protein itself has been characterized.[111] What does seem certain is that barriers to experimental gene transfer or expression among Gram-positive cocci and bacilli are not great and, further, that well-characterized *cat* genes from this amorphous group will play an increasingly important role as tools for in vitro genetic manipulation.[223-235]

V. PERSPECTIVES AND PROSPECTS

A. The Larger View

To a considerable degree the problems and questions posed by and with the CAT

system, as well as the strategies used to provide answers, are general ones. Rather little notice has been taken in this review of the vast literature on the organization and evolution of plasmids, the possible relationship of high gene copy number to rates of evolution and consequent diversity, or the fruits of three decades of study of the β-lactamases, the first antibiotic inactivating system to come under scrutiny. To a degree, much of what is now known of CAT and the methods used to explore its diversity and function have capitalized upon or recapitulated advances elsewhere. But it may be useful as well to summarize some aspects of CAT which make it an especially attractive system for a number of different kinds of inquiry.

B. CAT and Molecular Biology: Questions of Information and Conformation

An ideal system for probing the ultimate relationships of protein structure to function would possess attributes which should not be in dispute. First of all it should involve an effector protein of some size and structural complexity which has interacting ligands (substrates or modifiers) that are of some biological importance and chemical interest. It might be considered useful if the modes of regulation of both protein activity and gene expression were well established (or worthy of study in their own right), and it would be no mean advantage if the protein were to be stable, easy to purify, and synthesized in large amounts by a convenient micoorganism. The ideal system would involve a protein which readily yielded well-ordered crystals and was available naturally in many forms which were sufficiently different in structure to point to specific common segments or domains as determinants of function or organization. The ideal system would be readily amenable to genetic manipulation in vitro and site-directed mutagenesis and recombination. Spectroscopists would be grateful for the presence of intrinsic paramagnetic atoms or suitably disposed fluorescent "reporter" groups and be pleased if the protons of functional groups important in catalysis or ligand binding were readily resolved and assigned for study by high-resolution magnetic resonance techniques. Most but certainly not all of these features are potentially available within the CAT system, but it is equally clear that some areas have been neglected or examined only in cursory fashion.

C. Applied Microbiology and Molecular Epidemiology

The original rationale for studying chloramphenicol resistance in general and CAT in particular continues to motivate microbiologists. The scope for studies of inter-generic transfer, plasmid evolution, and the role of transposons seems almost unlimited, but it may well be that the apparent immense and even expanding diversity among *cat* determinants will disappear as catalogues of nucleotide sequences reveal that the heterogeneity of CAT phenotype is more apparent than real. The tedious but necessary "stamp collecting" which has been a characteristic of the first decade of studies has already given way to a more direct approach exploiting DNA biochemistry at an early stage. This has been noted already in connection with CAT among medically important Gram-positive cocci and promises to clarify the emergence of chloramphenicol resistance in *Haemophilus influenzae* and related species.[236-239,266]

But while practical answers of some interest and importance seem likely to come from studies of the horizontal movement of *cat* determinants through microbial populations, it is by no means certain that the characterization and even sequencing of novel *cat* genes or their products will yield data favoring specific vertical pathways for their evolution. Although common sense is on the side of those who argue that one must look to the soil for the origin of antibiotic resistance genes,[240] there are no hard data in the matter of CAT and it may be said to be disappointing in this connection that chloramphenicol producers do not contain CAT[134,138] although neighboring Actinomycetes[133-135] and other soil bacteria[140-142] may do so.[142] The strategy for avoiding suicide[139] among chloramphenicol

producers is uncertain whereas the mechanisms for resistance to thiostrepton and certain aminoglycosides or macrolides seem well established in their respective producing organisms (reviewed briefly by Thompson and Cundliffe[241]).

D. CAT as a Surrogate Receptor: Prospects for Drug Design

It has been scientifically sensible and even fashionable to argue and support the case that rational (and probably better) chemotherapy must eventually come from the custom design of drugs to fit targets[242] or avoid inactivating or modifying enzymes. Notwithstanding an immense global effort by academia and industry, the answers have not come easily even for the β-lactam and "lactamoid" antibiotics,[243] and it is fair comment to ask whether understanding (rather than necessity) actually preceded invention in this case. But to comprehend the structural and functional analogies of cell envelope transpeptidases and penicillin-binding proteins on the one hand and β-lactamases on the other is at present at least a realistic objective.[243] *Ab initio* considerations of the design of peptidyl transferase inhibitors which (a) make use of structure-activity considerations drawn from the action of chloramphenicol,[3-7] (b) are based on the structure of the 50S ribosomal catalytic center, and (c) take account of the enzymic properties of CAT seem rather more ambitious. The reason is, of course, not only the absence of an understanding at atomic resolution of peptidyl transferase; there is less than complete agreement as to where and what it is.[8,244] Given these limitations and granted that the chloramphenicol structural requirements of CAT for acetyl acceptor activity are an imperfect match for those of the ribosomal target, a case might be made for using CAT as a first approximation of the elusive peptidyl transferase. NMR studies by Tritton[7] suggest that the conformation of chloramphenicol when bound to the ribosome is not significantly different from the favored rotamer (Figure 1C) in solution, and there are reasons to believe (Section III.) that the structure is also favored at the CAT active site. The involvement of a histidine residue in the catalytic mechanism of both CAT and peptidyl transferase[245] seems likely, and the formal analogy in acyl activation between peptide bond formation and the synthesis of esters is striking. The ability of ribosomes to catalyze the synthesis of the latter[245,247] under special circumstances is, if not surprising, at least supportive of the argument. The object of the exercise would be to design an effective ribosomal inhibitor which need not resemble chloramphenicol in certain respects but would nonetheless bind with high affinity at the peptidyl transferase center. It may be argued that the 3-fluoro analogue of chloramphenicol[89] (and congeners) already satisfies the need for a new CAT-resistant peptidyl transferase inhibitor. But it is also possible that the bad behavior of chloramphenicol[9-11] will be recalled in the evaluation for clinical use of compounds specifically modeled after the parent drug for which there is no animal model to assess or predict lethal bone marrow toxicity.

E. First and Last Thoughts

The subject of resistance to chloramphenicol by enzymic acetylation was introduced by reference to the isolation of the antibiotic 35 years ago[1] and the discovery of transmissible resistance to it some 10 years later.[16] The account of a Ciba Foundation symposium on *Drug Resistance in Micro-organisms* held 25 years ago makes interesting reading by redirecting attention not only to the explosive developments and progress which have ensued but also to some problems which are still with us. In opening the proceedings, Harington observed[248] that ". . . the subject of chemotherapy remains distressingly empirical. The relationship between chemical structure and biological action in this field is still so ill-defined that we have only one significant guiding principle, based on biological theory, to help us in the search for new synthetic drugs for specific chemotherapeutic purposes".

The theme of the meeting was that developed in this review; namely, to see a specific

case of drug resistance as both a topic in its own right and a specific and illustrative example of more general phenomena. The "significant guiding principle" then as now must be that much in the way of hard data is needed on the macromolecules with which the antibiotics interact. Without the latter, the development of a new generation of effective agents would be, as Harington remarked, not unlike ". . . an operation such as oil prospecting . . . with no adequate background of geological information".

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