STUDIES ON A CHLORAMPHENICOL ACETYLTRANSFERASE VARIANT

BY

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To my mother and father, Charlie, Susan, Stephen, John and Ian.

O Friends you came with me,

You helped me share my load,

From the dark end of the street,

To the bright side of the road.

(Van Morrison)

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Abbreviations

Non standard abbreviations are listed below.

Chloramphenicol acetyltransferase; CAT

$CAT_{subscript}$; for example subscript III, Type III enzyme

4-amino-7-oxa-bicyclo[4,10] hept-3-ene-2,5-dione-3-ene-2,5-dione-3-carboxamide; G7063-2

5,5 dithiobis(2-nitrobenzoic acid); DTNB

5,5 dithiobis(2-nitromethylbenzoate); DTNMB

5,5 dithiobis(2-nitrotoluene); DTNT

Methyl p-Nitrobenzene Sulphonate; MNBS

Diethylpyrocarbonate; DEP

TrisHCl (pH 7.8),50mM; chloramphenicol, 0.2mM; 2-mercaptoethanol, 0.1mM; TCM

TrisHCl (pH 7.8),50mM; 2-mercaptoethanol, 0.1mM; TM

TrisHCl (pH 7.8),50mM; standard buffer

NTCB; Nitrothiocyanobenzoate

Chapter One

Introduction

The antibiotic chloramphenicol was discovered as a bacteriostatic agent in cultures of Streptomyces venezuelae in 1947 (1,2). It was the first natural product found to contain a nitro group and also the first which was a derivative of dichloroacetic acid.

Since chloramphenicol (Fig.1) has two asymmetric carbon atoms four possible diastereoisomers exist. All four have been synthesized. The two erythro isomers are biologically inactive, whereas the L,threo isomer has less than 0.5% of the activity of the D,threo isomer (3).

Chloramphenicol is a specific and effective inhibitor of bacterial protein synthesis at low concentrations (4). Protein synthesis in mammalian cells, plant cells and in yeast is not sensitive to chloramphenicol, however (5).

Traut and Monro (6) were the first to demonstrate that chloramphenicol inhibits the peptide bond forming reaction. As a result of many studies (7,8,9,10), it is now clear that chloramphenicol allows the binding of aminoacyl-tRNA to the ribosomal A site but prevents recognition by the peptidyltransferase of its acceptor substrate. It is not clear how this is achieved. A high affinity site for chloramphenicol (K_{diss} $2x10^{-6}$M) (11) is located on the 50S ribosomal subunit (12) and a low affinity site (K_{diss} $2x10^{-4}$M) (11) on the 30S ribosomal subunit (13), although it is not clear whether the latter is important for the primary action of the drug. Components involved in the binding of chloramphenicol to ribosomes have been examined by affinity labelling of derivatives of the drug, (for example using monoiodochloramphenicol or monobromochloramphenicol) and by the splitting and reconstitution technique (14,15,16). Using these techniques proteins L2, L16, L27, L6, L24, S3 and S6 are labelled. Proteins L2, L27, L6, L16 and L24 possess antigenic determinants clustered within the peptidyltransferase region of the 50S subunit (17). There is, therefore, reasonable agreement between the data obtained with affinity analogues and that from immunogenic studies. What remains to be elucidated is which

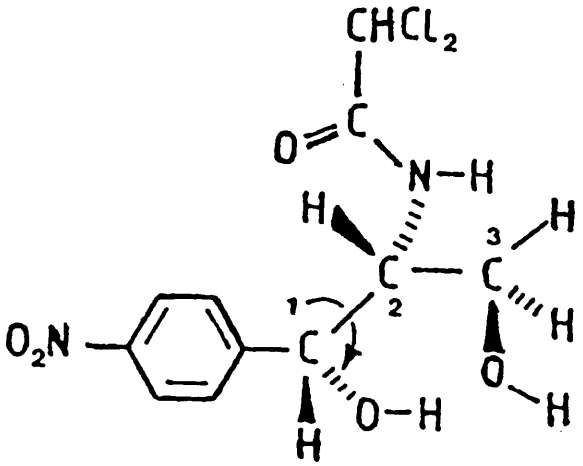


Fig.1.

The structure of Chloramphenicol.

proteins is (are) the peptidyltransferase and which are present in the P and A sites where the transferase recognizes its donor and acceptor substrates.

Chloramphenicol was the first antibiotic released for clinical use as a wholly synthetic product (18) and has been widely used as a broad spectrum antibiotic. Reports of drug induced bone marrow damage and adverse reactions (19,20) have led to a considerable decrease in its use. It is, however, still an extremely useful drug in life threatening infections such as menigitis due to Haemophilus influenzae, certain infections due to anaerobic bacteria and typhoid fever (21,22).

Chloramphenicol resistant strains were first isolated in 1954 (23). Apart from intrinsic tolerance of certain microorganisms (fungi, mycobacteria and Pseudomonas), virtually all examples of high level (and hence clinically important) resistance to chloramphenicol are the consequence of inactivation by the enzyme chloramphenicol acetyltransferase (CAT) (E.C. 2.3.1.8) (24).

CAT recognizes chloramphenicol and its analogues with a similar specificity to that of the ribosomal peptidyltransferase and acetylates the primary hydroxyl group at the 3 position of chloramphenicol using acetyl-S-CoA as the acyl donor (Fig.2). The O-acetylated drug no longer binds to the 50S subunit and protein synthesis is no longer inhibited (25).

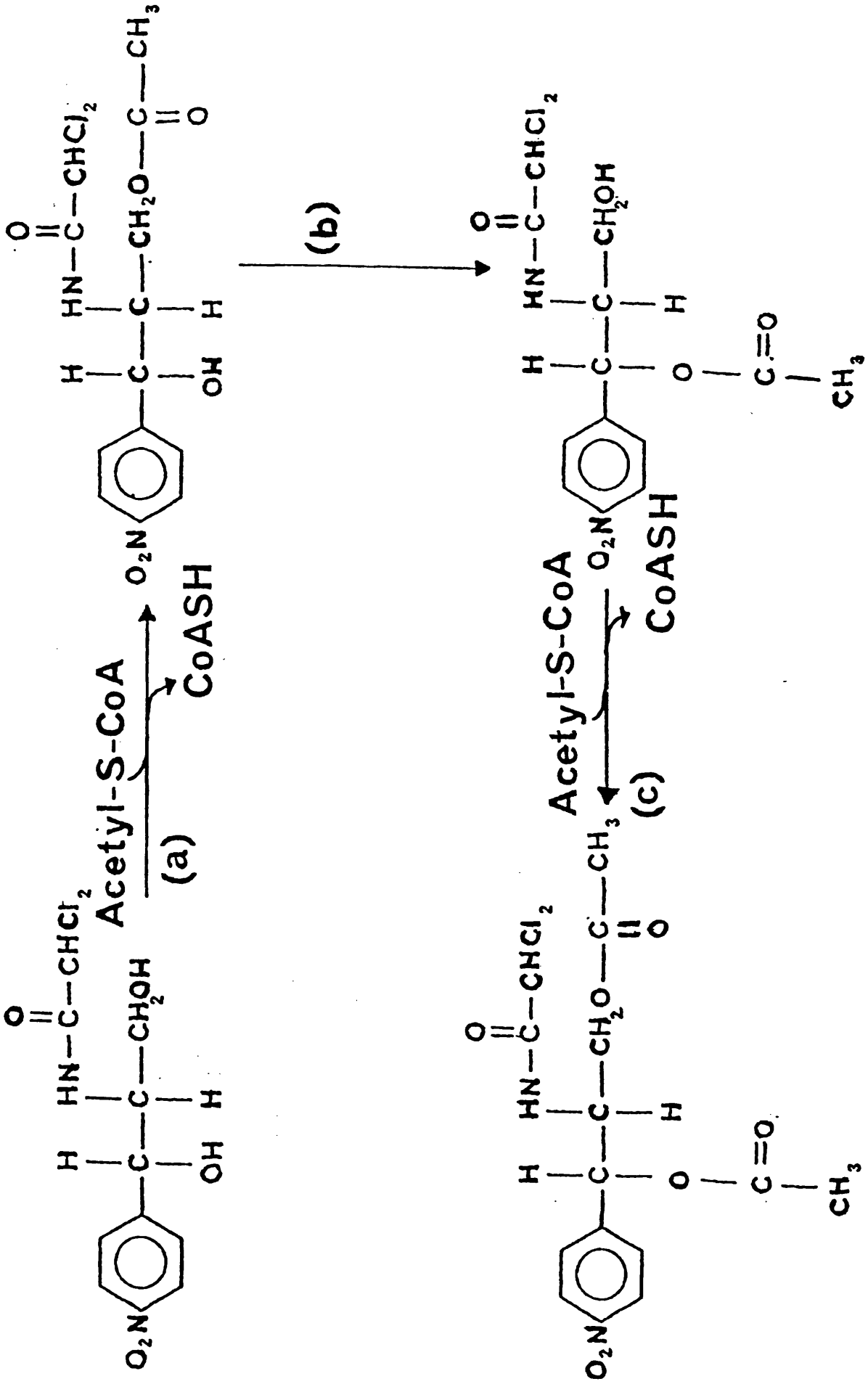
The persistance of chloramphenicol resistance and its appearance amongst many bacterial genera owes much to the linkage of the genetic determinant to other selectable markers and the frequency with which it is found to be part of the transmissable and transposable genetic elements, plasmids and transposons respectively.

In 1964 Miyamura (26) showed that, not only were E.coli and other Gram-negative bacteria with R factors for the drug able to inactivate chloramphenicol, but also some naturally occuring pathogens such as Staphylococcal aureus which were Gram-positive. The proteins from the E.coli

Fig.2.

The enzymic acetylation of Chloramphenicol by CAT.

The primary product of the reaction is 3-acetyl chloramphenicol (a). The 1,3-diacetyl chloramphenicol is found only after prolonged incubation (30,31) (c). The conversion of 3-acetyl chloramphenicol to 1-acetyl chloramphenicol is nonenzymic (32) (b). As the conversion of 1-acetyl chloramphenicol to 1,3-diacetyl chloramphenicol proceeds at a rate which is two orders of magnitude slower than the initial reaction, it doesnot interfere significantly with the assay of CAT.


Acetyl-S-CoA
CoASH
(a)
(b)
(c)

and related genera were found to be large (>20kb) and analogous in some respects to the F-like factor of E.coli (27). The plasmids frequently carried other drug resistances such as resistance to ampicillin. The genes for the inducible staphylococcal family of CAT variants are almost invariably found on small (<5kb) plasmids which carried no other drug resistances (28).

Only a few CAT variants have been studied in detail as catalytic proteins or multimeric polypeptide assemblies. In Gram-negative species of bacteria the enzyme is synthesized constitutively from the R plasmid cat gene and can constitute 1 percent of the soluble intracellular cell protein (29). When the genes are present on high copy number plasmids and cells grown on glycerol rather than glucose the enzyme yield may approach or exceed 10 percent (33). The staphylococcal CAT variants, however, require induction and are not available in such large quantities. Pure enzyme can be obtained in high yield by affinity chromatography on Sepharose columns substituted with chloramphenicol base as the ligand (34,35).

Initial studies of the enzyme variants compared and contrasted properties of the protein with a view to investigating the relationship of different CAT variants. Table 1 summarizes the data available from several naturally occuring CAT variants with respect to chemical, kinetic and immunological studies. From this data it is apparent that the variants constitute a superficially heterogenous group of proteins. There is also evidence of some heterogeneity among examples of the most common (Type I) variant of CAT (37). The limitations of a classification based on such properties will probably become more apparent as the primary structures of more of the variants become known.

Amino acid sequence data are available now on three variants. Initially these data were generated by protein sequencing methods but such studies have now been superceded by direct determinations of DNA sequences which have the added advantage of providing information about the regulatory

SELECTED PROPERTIES OF CHLORAMPHENICOL ACETYLTRANSFERASE VARIANTS

ENZYME TYPES	BINDING TO CHLORAMPHENICOL-SUBSTITUTED ACAROSE [1]	K_a^{app} (μM) CHLORAMPHENICOL	K_a^{app} (μM) ACETYL-CoA	RELATIVE V_{MAX} (per cent)	DTNB SENSITIVITY [1]	REACTION WITH ANTI-SERUM CAT_I	REACTION WITH ANTI-SERUM CAT_{III}	REACTION WITH ANTI-SERUM CAT_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	+	-	-
Haemophilus 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	-	-	(+)

Table 1

The data has been tabulated from Zaidenzaig et al.(36), Gaffney et al, (37), Fitton and Shaw (38) and Britz and Wilkinson (39). The results of unpublished experiments in the laboratory of W.V. Shaw are marked .

1. Graded 0-5. 0= no binding or no inhibition by DTNB.

2. Relative Vmax is an estimate of maximum catalytic rate in presence of saturating concentrations of both substrates. The turnover number (k_{cat}) for the Type III enzyme specified by plasmid R387 is 1500sec^{-1} and is taken as 100.

3. The anti-CAT_C serum neutralised two streptococcal variants but didnot give precipitation reactions (marked+). All others marked + gave both precipitation and neutralization reactions.

regions of the cat genes. The known sequence data to date is summarized in Table 2.

The data from the Type I and Type III E.coli variants and the staphylococcal variant specified by plasmid pC194 have also been used for secondary structure predictions as seen in Fig.3. Unfortunately X-ray crystallography work that was started by the late Dr. I.Swan is not far enough advanced yet to add extra knowledge to these studies, (135).

Until recently little data have been available on the mechanism of the enzyme reaction. Much work has been done on the structural requirements of the substrates, especially on analogues of chloramphenicol (26,45,46, 47,48). These were synthesized in the hope of finding a compound which had good antibiotic activity and yet was not a substrate for CAT. Table 3 summarizes the data available for the specificity of the acyl acceptor. Also tabulated are estimates of the effectiveness of chloramphenicol analogues and isomers to inhibit polypeptide synthesis from synthetic polyribonucleotides in cell free systems (49,50). It can be seen that CAT and the ribosomal target of chloramphenicol (peptidyltransferase) have a nearly absolute requirement for the D,threo isomer, 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 the inhibition of peptidyltransferase are more stringent than for CAT. The nature of the electronegative haloacetyl substituents at the C_2 amino group influences the effectiveness of the inhibition of protein synthesis more than the acetyl acceptor in the CAT assay.

An analogue that has recently been synthesized is a fluoro compound in which fluorine replaces the 3-hydroxyl group (51). This is a very effective competitive inhibitor with respect to chloramphenicol in the CAT system, yet also is a good antibiotic. This situation probably reflects the fact that fluorine has a similar van der Waals radius as the hydroxyl group and could form a hydrogen bond with the hydroxyl at C_1, hence

Table 2

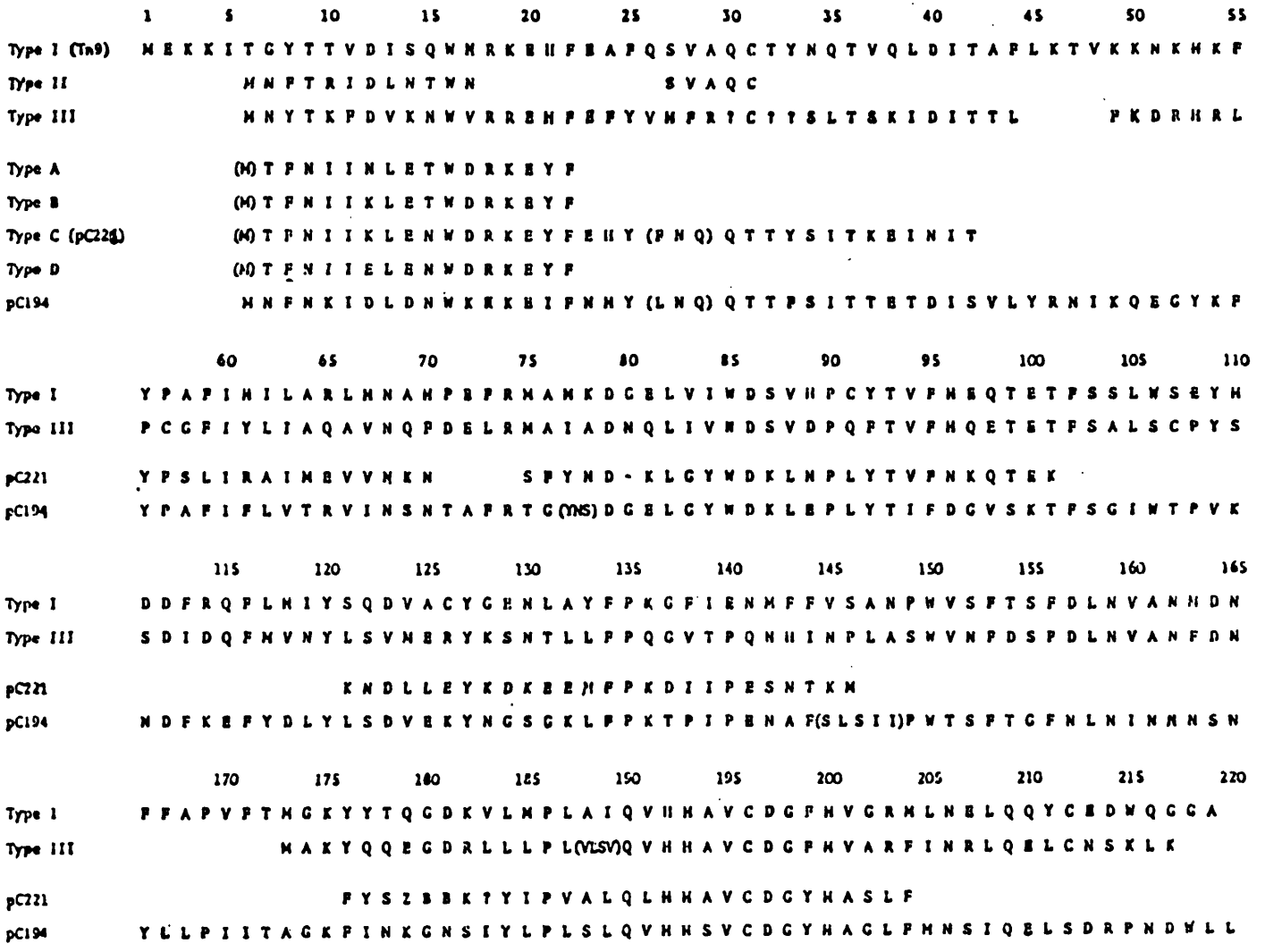
Primary structures of Chloramphenicol Acetyltransferases.

Type I structure is from Shaw et al (40), Alton and Vapnek (41) and Marcoli et al (42).

The Type II and Type III partial sequences have been determined(36,43) and the amino terminal sequences of the staphylococcal variants (38).

Some of the sequence displayed for the Type III and Type C proteins is from unpublished studies from the work of L.C. Packman, N.M.C. Kaye, J.E. Fitton and W.V. Shaw.

The amino terminal methionine of Types A,B,C and D has been inferred from the nucleotide sequence of pC194 (63). Each of the four staphylococcal variants has threonine as the first residue of the protein purified from induced bacteria (38).


1 5 10 15 20 25 30 35 40 45 50 55
Type I (Tn9) MEKKITGYTTVDISQWMRKEIFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKF
Type II MNFTRIDLNTWN SVAQC
Type III MNYTKPDVKNWVRREHFEFYVMFRTCTTSLTSKIDITTL PKDRHRL
Type A (M)TFNIINLETWDRKEYF
Type B (M)TFNIIKLETWDRKEYF
Type C (pC221) (M)TFNIIKLENWDRKEYFEHY(FNQ)QTTYSITKEINIT
Type D (M)TFNIIELENWDRKEYF
pC194 MNFNKIDLDNWKRKEIFNMY(LNQ)QTTPSITTETDISVLYRNIKQEGYKF
60 65 70 75 80 85 90 95 100 105 110
Type I YPAFIHILARLMNAHPEFRMAMKDGELVIWDSVHPCYTVFHEQTETFSSLWSEYH
Type III PCGFIYLIAQAVNQPDELRMAIADNQLIVWDSVDPQFTVFHQETETFSALSCPYS
pC221 YPSLIRAIMEVVNKN SFYND-KLGYWDKLNPLYTVFNKQTEK
pC194 YPAFIFLVTRVINSNTAFRTG(YNS)DGELGYWDKLEPLYTIFDGVSKTFSGIWTPVK
115 120 125 130 135 140 145 150 155 160 165
Type I DDFRQFLHIYSQDVACYGENLAYFPKGFIENMFFVSANPWVSFTSFDLNVANNDN
Type III SDIDQFMVNYLSVNERYKSNTLLFPQGVTPQNHINPLASWVNFDSPDLNVANFDN
pC221 KNDLLEYKDKEEIFPKDIIPESNTKM
pC194 NDFKEFYDLYLSDVEKYNGSGKLFPKTPIPENAF(SLSII)PWTSFTGFNLNINNNSN
170 175 180 185 190 195 200 205 210 215 220
Type I FFAPVFTMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCEDWQGGA
Type III MAKYQQEGDRLLLPL(VLSV)QVHHAVCDGFHVARFINRLQELCNSKLK
pC221 FYSZBBKTYIPVALQLHHAVCDGYHASLF
pC194 YLLPIITAGKFINKGNSIYLPLSLQVHHSVCDGYHAGLFMNSIQELSDRPNDWLL

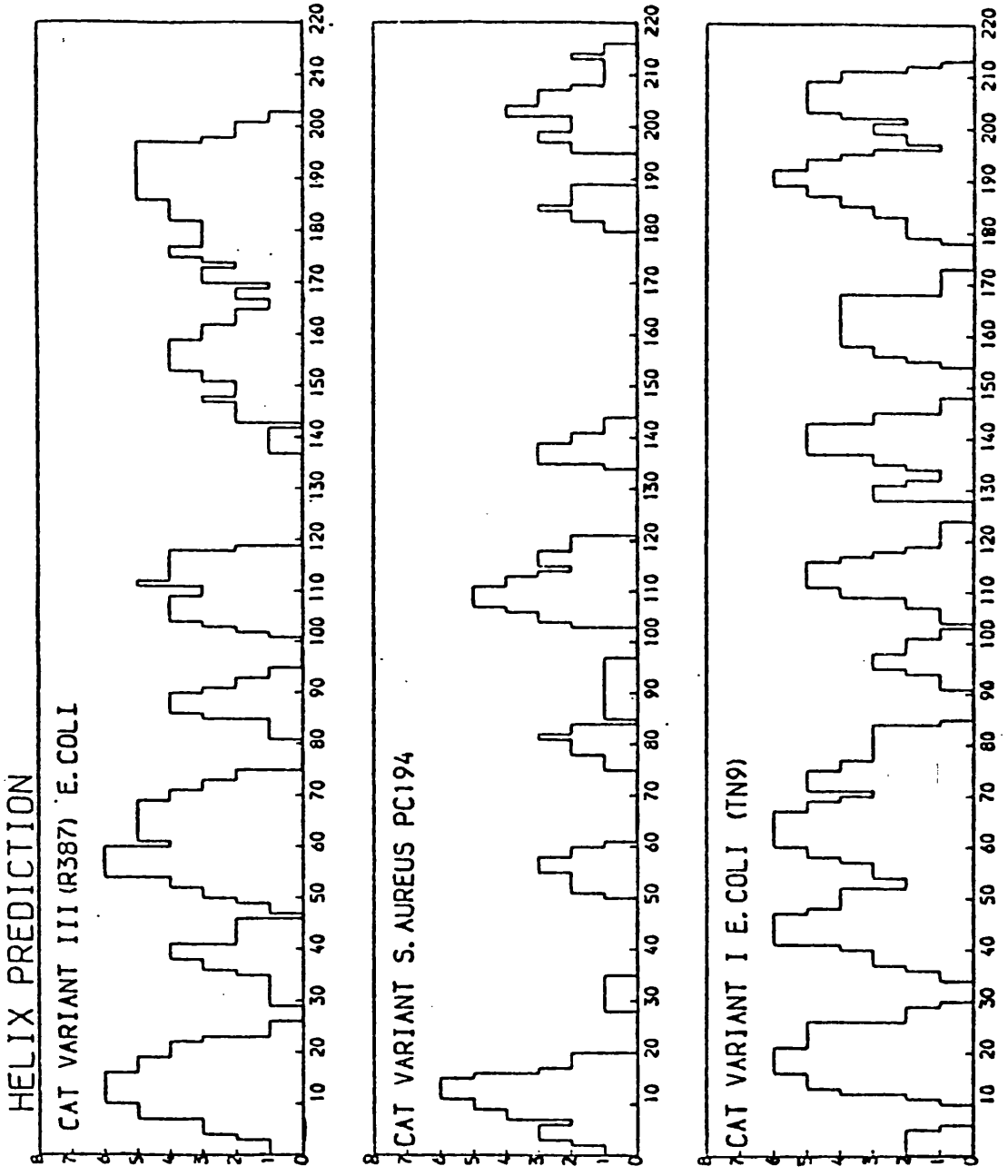


Fig.3.

Six methods were used by Eliopoulos (44) to predict the most likely structure of the three CAT variants. For predictive purposes the missing residues of the CAT_{III} were replaced by the corresponding residues in CAT_{I}. Residue 1 in each case is the amino terminal methionine of the protein.

EFFECTS OF CHLORAMPHENICOL ANALOGUES AND ISOMERS

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

Table 3

Analogues and isomers of chloramphenicol are compared with respect to their ability to inhibit a cell free E.coli protein synthesis system, their effectiveness as acetyl acceptors for two CAT variants (CAT_I and CAT_C) and their ability to induce the CAT_C protein encoded by pC221 in vivo.

allowing the formation of the five membered ring that is thought to be important for recognition by the ribosome and enzyme (52). Bustard et al. (53) have stated however, following theoretical conformational predictions, that the hydrogen bonding probably does not exist since H-bonds would form only in very low dielectric media or in a region of very low dielectric constant created by aggregation of non polar moieties. Minimal non-bonded interactions and solvent stabilizations are, in their opinions, responsible for the stabilizations of chloramphenicol rather than intramolecular hydrogen bonding.

Less work has been done on the specificity of the acyl donor (26). The complete and unmodified coenzyme structure is required since acetyl-S-dephospho CoA, acetyl-S-panthetheine and the S-acetyl derivative of the E.coli acyl carrier protein are not acetyl donors (29). The size of the acyl group transferred is also critical since transfer drops for substituents longer than the propionyl moiety (54). Some adenine nucleotides are effective as inhibitors of CAT_I and are competitive with respect to acetyl-S-CoA (K_m 0.1mM for acetyl-S-CoA, K_i for ATP and ADP, 2.2mM and 3mM) (55). Adenosine, pyrophosphate and AMP are not inhibitory. These results suggest that both adenine and the pyrophosphate diester portions of the CoA structure are important as determinants of coenzyme binding.

As stated previously, little work has been done on the catalytic mechanism of the enzyme. Results of studies by Tanaka et al (56), Zaidenzaig and Shaw (57) and Fitton and Shaw (38) are compatible with a ternary complex mechanism for Type I and Type C variants. A detailed kinetic analysis of the forward and reverse reactions with a Type III variant is presently being undertaken by Kleanthous. It has not been possible to demonstrate enzyme-bound radioactivity after incubating CAT with a stoichiometric amount of [^{14}C]acetyl-labeled substrate or product, confirming the kinetic analysis (58,59) and in contrast to several other acetyl-S-CoA dependent acetyltransferase systems in which an acetyl-enzyme intermediate appears

to be implicated in the mechanism (homoserine-O-acetyltransferase (60) and arylamine N-acetyltransferase (61)).

Chemical modification studies have been carried out on the CAT system with two ultimate goals. One has been to investigate the quaternary structure. Since CAT is a tetramer it would be interesting to know how the subunits associate to form functional tetramers. Results from amidination experiments with methylacetimidate indicate a unique unreactive residue to be present in each variant studied. Lysine 136 of CAT_I and Lysine 38 of CAT_{III} behave as inaccessible or 'buried' residues (62). It remains to be shown unambiguously, however, that the lysine residues participate in salt bridge formation with counteranions on contiguous subunits.

The second goal has been to identify reactive residues at or near the active site and to locate residues in the substrate binding sites. Initial results from experiments with the Type I variant (58), Type II variant (43) and Type C variant (38) have indicated that one or possibly two cysteine residues and one histidine residue are at or near the active site. Since much of this information is relevant to the theme of this thesis it will be presented and discussed in following chapters. The present study was carried out in order to investigate the active site and binding sites of a CAT variant in detail and to compare and contrast the data obtained with that from previous work.

Chemical modification is one method of gaining information about protein structure and the mechanism of action of enzymes. Reagents for chemical modification fall broadly into two catagories, site specific and group specific reagents. Although group reagents can be selective, they generally discriminate on the basis of accessibility of side chains - a parameter not always related to function. Careful control experiments examining stoichiometry, sites of modification and protection studies in the presence of substrate can determine whether the reagent is active site

directed. Characteristics of an enzyme can be taken into account, however, in designing a more specific reagent for chemical modification. For example, a reagent with substrate-like structural features to favour complex formation with the enzyme should be a more specific reagent. The selectivity is still determined, however, by the availability of amino acid side chains. Whether any amino acid ,in either case, is modified also depends on steric factors, catalytic factors, the polarity of the local enviroment and the nucleophilicity of the protein functional groups.

Although it is possible to modify in theory serine, methionine, tyrosine, tryptophan, threonine, glutamic and aspartic acid, lysine,arginine, cysteine and histidine residues, the present studies have centred on the modification of the latter three. Some reagents used are group specific whilst others have been chosen because of their structural similarity to the substrate chloramphenicol.

Finally, it should be noted that it is unlikely that the chemical modification of enzymes will ever be able to provide more than a fragmentary description of a mechanism of an enzyme, since it is always difficult to define the role of a particular residue with certainty. Information from several techniques (physical, chemical and biochemical) must be considered in order to define a mechanism more completely.

Chapter Two

Materials and Methods

Materials

Sigma London Chemical Co. Ltd.

Chloramphenicol; Chloramphenicol base; Cyanogen Bromide; Dansyl chloride; Diethylpyrocarbonate; 5,5 Dithiobis(2-nitrobenzoic acid); Dithiothreitol; Guanidine Hydrochloride; Elastase; Iodoacetic acid; Iodoacetamide; 2-Mercaptoethanol; Sodium dodecyl sulphate; Trizma base.

BDH Chemicals Ltd.

Acrylamine; 6-Aminohexanoic acid; Dansyl amino acids; Naphthalene Black 12 B; Polyamide thin layer sheets; Coomassie PG83, PG90; Urea; N,N,N,N-Tetramethylenediamine.

Pierce and Warriner (U.K.) Ltd.

Acetonitrile; Dimethylformamide; Triethylamine.

Rathburn Chemicals (Walkerburn) Ltd.

Butylacetate; Methanol; Pyridine.

The Radiochemical Centre, Amersham.

$[^{14}C]$Iodoacetamide; $[^{14}C]$Iodoacetic acid; $[^{14}C]$Methanol; $[^{14}C]$Chloramphenicol.

Whatman Biochemicals Ltd.

1 and 3 MM Chromatography paper.

Boehringer Mannheim.

Coenzyme A

Fluka Chemical Co.

CDC coupler; Monobromoacetone.

Roche Diagnostics.

Fluorescamine.

Pharmacia Ltd.

Sephadex; Sepharose 4B.

Eastman Kodak Co.

N,N Methylenebisacrylamide; p-Nitrobenzene sulphonyl chloride; Methyl p-nitrobenzene sulphonate; Cellulose plates.

Aldrich Chemical Co.

Iodomethane ; Butanedione.

Merck.

Polygram SilG (silica) plates.

Corning.

Agarose film; Amido Black.

The following compounds were gifts;

3-Fluorochloramphenicol,Schering Chemical Co.

5,5 Dithiobis(2-nitromethylbenzoate); 5,5 Dithiobis(2-nitrotoluene), M. Webb,Chemistry Department, University of Leicester.

G7063-2, Glaxo.

Methods

2.1 Preparation of cell free extracts.

(a) Growth of cells.

The pilot plant at the Microbiological Research Establishment (Porton Down) was supplied with 3 stab cultures of the mutant organism E.coli J53 (R387;Cm700), a high expression mutant selected by N. Kaye by growth on 700 µg per ml of chloramphenicol. A seed culture of 20l was innoculated and grown at 37°C for 24 hr. This seed culture was then added to 400l of culture medium and grown for 15 hr. at 37°C. This work was performed by Dr. A. Atkinson and J. Stretton. The cells were frozen at -20°C for storage.

(b) Preparation of cell paste.

The frozen cell paste was suspended by the use of a Waring Blender in a buffer containing TrisHCl (pH7.8) 50mM; chloramphenicol, 0.2mM; and 2-mercaptoethanol, 0.1mM (TCM). The frozen cells were then broken using a Dyno Mill, flow rate 1500ml per hr. The cell extract was centrifuged in a Beckman JA10 rotor at 10,000 rpm. The supernatant was heated slowly in a waterbath. When the temperature reached 60°C, the waterbath was switched off and the extract was stirred for a further 5 minutes. The extract was allowed to cool to room temperature and then placed in an ice waterbath and stirred until the temperature was 2°C or 3°C, then centrifuged at 18,000 rpm for 60 minutes to yield the crude extract.

2.2 Preparation of CAT-specific affinity resin.

Sepharose 4B (300g) was suspended in 600ml of water. NaOH (5M) was added to bring the pH to 11.0. Small aliquots of ground CNBr (55g) was then added (in a fume cupboard) to the slowly stirring suspension over a 30 minute period. The pH was maintained at pH 10.5-11.0 by addition of 5M NaOH and the temperature at 20°C by addition of ice. After 45 minutes the temperature was adjusted to 4°C by addition of excess ice, and the activated Sepharose was washed with 3.5l of ice cold carbonate/bicarbonate buffer pH 10. The moist gel was added to a solution of 6-aminohexanoic acid (164g) in 200ml of ice cold buffer and the pH adjusted to 10. The solution was

stirred slowly overnight. The hexanoyl-Sepharose was filtered and washed thoroughly with 1 litre of 0.1M $NaHCO_3$, pH 10; 4 litre of water; 1 litre 4M NaCl; 4 litre of water; 1 litre 0.1M acetic acid and 4 litre of water. Titration of the resin to determine the degree of substitution was carried out by the method of Yon and Simmonds (64) using 0.1M KCl as an electrolyte. The washed hexanoyl-Sepharose was mixed with 600ml of water containing 43g of N-cyclohexyl-N-(2-(4-morpholinyl)-ethyl)carbodiimide-methyl-p-toluene sulphonate (CDC) coupler. The pH of the mixture was adjusted to pH4.5 with HCl and a solution of 4.22g of chloramphenicol base in 100ml of water was added. When the pH required no further adjustment (30-60minutes), the mixture was left to stir slowly overnight at 30°C. The resin was washed and titrated as above and stored at 4°C.

2.3 Purification of CAT.

CAT was purified by the method of Zaidenzaig and Shaw (35). The crude extract was dialysed overnight against TM buffer (TrisHCl, pH7.8 and 50mM containing 0.1mM 2-mercaptoethanol) at 4°C in order to remove excess salt and chloramphenicol which is known to interfere with the binding of the enzyme. The resin was added in TM buffer to the stirring crude extract at 4°C. The binding of the enzyme to the resin was monitored by removing 100µl of the mixture, sedimenting the resin by centrifugation and assaying the supernatant for activity. The resin was poured into a column at 4°C and washed with three column volumes of TM buffer to remove any unbound protein. the resin was then washed with TM buffer containing 0.3M NaCl. The column effluent was monitored for protein at 280nm against an appropriate NaCl blank. When the absorbance at 280nm had dropped to 0.02, elution of CAT was achieved by addition of TM buffer containing 0.3M NaCl and 5mM chloramphenicol. 10ml fractions were collected. These were tested for CAT activity using the standard assay. Peak tubes contained more than 1mg/ml enzyme. The CAT was dialysed against TCM buffer and stored at -20°C. The CAT was assayed for purity by SDS-PAGE electrophoresis. The resin was washed with with TM buffer containing 1.0M NaCl; TM buffer and then stored

in TM buffer containing 0.02% sodium azide.

2.4 Assay of CAT.

The acetylation of chloramphenicol was determined by the spectrophotometric assay. This uses the reduction of 5,5 dithiobis(2-nitrobenzoic acid) (DTNB) by CoA to produce the thionitrobenzoate anion $\epsilon_{412} = 13.6 \times 10^3 M^{-1}cm^{-1}$ at pH 7.8 (29). The reaction was followed at 412nm using a Pye-Unicam SP1800 dual beam spectrophotometer. In a standard assay the chloramphenicol and acetyl-S-CoA concentrations were both set at 100μM.

2.5 Protein Determination.

The determination of the protein content of crude extracts was made by the method of Lowry (65). Pure preparations of CAT were determined by amino acid analysis.

2.6 Electrophoresis of proteins.

(a) Gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate (SDS).

SDS-PAGE was carried out using the discontinuous method of Laemmli (66). Electrophoresis in slab gels was performed at room temperature using a potential difference of 60V for the stacking gel and 120V for the running gel. The percentage of acrylamide in the running gel was varied according to needs, whilst the stacking gel was set at 3% acrylamide. Gels were stained in a solution of 50% methanol, 10% acetic acid containing 100mg Coomassie Blue R250 (BDH PG83) for an hour, followed by an overnight destain in 10% acetic acid, 5% methanol. Where necessary marker proteins were applied to the gel.

(b) Native gels on agarose strips.

1-2μl of protein solution was applied to the preformed well of an agarose gel, and the gel electrophoresed for 1hr. at 90V at room temperature in sodium barbitol buffer pH 8.0, using a Shandon thin layer electrophoresis apparatus. The gel was stained with a solution containing

.123% (w/v) napthalene black in 0.1M acetic acid for 30 minutes. The gel was then fixed onto its support using a stream of hot air. The gel was then destained in 5% (v/v) acetic acid.

(c) Histochemical stain.

Detection of the CAT activity in native electrophoresis of protein was performed by the method of Shaw and Brodsky (46) which utilizes the coupled reduction of nitrobluetetrazolium by free CoA, via a phenazine methosulphate carrier, to insoluble formazan.

2.7 Determination of K_m and K_i values.

K_m determinations were carried out at 37°C using a modification of the standard assay procedure (Methods 2.4). For the determination of the K_m of chloramphenicol, the acetyl-S-CoA concentration was set at the saturating concentration of 400µM and the chloramphenicol concentration was varied. The chloramphenicol concentration was set at 200µM for the determination of the K_m of acetyl-S-CoA. The data were plotted using the iterative least squares programme of Cleland (67). For the determination of a K_i value the assays were performed as above except that they were carried out in the absence and presence of the competing ligand.

2.8 Peptide mapping of CAT protein.

Peptide mapping of purified protein was carried out using the method of Cleveland et al (68). Column purified proteins were run on a 12.5% (w/v) polyacrylamide slab gel containing 0.1% SDS (Methods2.6a). Thin spacers (1.2mm) were used for this gel. Protein bands were located by staining with Coomassie Blue R250. The bands were cut out with a scapel blade and placed in 10ml of a solution containing 0.125M TrisHCl pH6.8, 0.1mM EDTA, 1%(w/v) SDS (buffer A), gently mixed and soaked for 10 minutes at room temperature. After this time the buffer was replaced with fresh buffer and soaked for a further 10 minutes. The pH of the solution was monitored over this period. If the pH was stable at pH 6.8 over this period then the gel slices were ready for digestion. If not, further buffer changes were carried out until

the pH stabilized.For digestion a 17.5% (w/v) polyacrylamide gel containing 0.1mM EDTA and 0.1% (w/v) SDS was used. 1.5mm spacers were used for this gel to facilitate the loading of the gel slice. The gel slice was pushed gently to the bottom of a well, which had previously been filled with buffer A, using a spatula. The gel piece was then covered with 10µl of buffer A containing 20% glycerol, 0.1% bromophenol blue and either 1µg of papain or 2µg of Staphylococcus aureus (S. aureus) V8 protease. The top reservoir was filled with running buffer containing 1mM EDTA. The gels were electrophoresed into the stacking gel until the dye was 4mm from the bottom of the stacking gel. The current was turned off for 30 minutes to allow the digestion to take place and then turned on again. The gel was stained with either Coomassie Blue R250 (Methods 2.6a) or by the silver stain method (Methods 2.9) to visulize the bands.

2.9 Silver stain. (69)

Gels were soaked for 1 hr. in 50% methanol, 10% acetic acid, then overnight in 5% methanol, 10% acetic acid. The gels were then soaked for 30 minutes in 10% unbuffered gluteraldehyde, rinsed and soaked for 2.5 hr. in distilled water with gentle agitation. The gels were stained in 200ml of a freshly made solution containing .075% NaOH, 7.8% $AgNO_3$, 2% NH_4OH for 10 minutes, rinsed and placed in 200ml of fresh 0.005% sodium citrate, .019% formaldehyde to develope. Development was stopped by washing extensively with water.

2.10 Amino acid analysis.

(a) Proteins

Salt free protein samples were hydrolysed for 24, 48 and 72 hr. in the presence of 6M HCl containing 10µm phenol at 105°C. Tubes containing the sample and acid were constricted in an oxygen gas flame and sealed under vacuum after degassing and flushing the sample twice with nitrogen After removal of the acid under vacuum, hydrolysates were resuspended in 0.02M HCl and aliquots were analysed on a single column Locarte

analyser using sodium citrate buffers (70). Values of serine and threonine were extrapolated to zero time of hydrolysis, whilst those obtained for isoleucine, leucine, and valine were extrapolated to 96 hr. hydrolysis time.

(b) Peptides.

Hydrolysis of peptides was carried out as in Methods 2.10a except that the hydrolysis was carried out for 24 hr. only.

2.11 Immunochemical techniques.

Crude antisera raised against purified CAT variants were used in these experiments. The CAT_I directed reagent was made from a goat immunized with CAT_I specified by plasmid JR66. CAT_{II} specified by plasmid Sa and CAT_{III} specified by plasmid R387 were injected into rabbits to raise the CAT_{II} and CAT_{III} directed reagents.

Ouchterlony diffusion tests (71) were performed in 0.75% agarose prepared in 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na_2HPO_4, 0.2% (w/v) KH_2PO_4 on plates. Plates were incubated for upto 72 hr. at 4^oC. Gels were stained with 0.123% (w/v) amido black in 5% acetic acid and destained with 7% acetic acid.

2.12 Equilibrium dialysis.

Equlibrium dialysis experiments were carried out using a multichambered apparatus. Each chamber was divided into two by the dialysis membrane which was inserted between the two halves of the apparatus. Each half chamber had a total volume of 500µl. Samples, CAT_{III} or $[^{14}C]$ chloramphenicol in 50mM TrisHCl pH 7.8 (standard buffer) were loaded into the chamber using a Hamilton syringe. An equal volume of standard buffer was placed in the chamber on the other side of the dialysis membrane. Initial experiments were carried out to find how long $[^{14}C]$ chloramphenicol took to equilibrate across the membrane. For this, samples (50µl) were taken with a Hamilton syringe from both sides of the membrane and counted for radioactivity. When protein and ligand were used in the experiment the

samples were mixed prior to loading in the chamber. The apparatus was gently rotated for 24hr. at 4°C. Samples were then removed (3x100µl) from each side of the membrane and counted for radioactivity. The activity of the enzyme was monitored throughout the experiment.

In experiments where methyl-S-CoA was included, a saturating amount of methyl-S-CoA (based on the K_i value determined) was placed on each side of the membrane and the experiments carried out as above. The results were plotted according to the Scatchard equation.

2.13 Inactivation of CAT_{III} with Iodoacetamide and Iodoacetic acid in the presence and absence of substrates.

CAT_{III} (0.2mg/ml) in standard buffer (100µl) was incubated in a silver foil covered tube at 37°C. 10µl was taken, diluted into 1ml of standard buffer and stored on ice in the dark for later measurement of activities using the standard assay. Inhibition was initiated by the addition of either iodoacetamide or iodoacetic acid. Samples (10µl) were taken at time intervals and treated as above. When chloramphenicol or acetyl-S-CoA (1mM) were included they were preincubated with the enzyme for 5 minutes prior to the addition of the inhibitor. The data were plotted according to the equation $\ln E/E_o = -kt$ where E_o is the activity at time zero, (100%) and E is the activity at anytime t, k is the pseudo first order rate constant for the inactivation (72).

2.14 Incorporation of ^{14}C Iodoacetamide in the presence and absence of substrates.

1mg/ml CAT_{III} was incubated in 500µl of standard buffer in the dark at 37°C. Inactivation was initiated by addition of $[^{14}C]$iodoacetamide (S.A 0.25 µCi/umole). Samples were taken (10µl) at time intervals and treated as described in Methods 2.13. When the inactivation had proceeded to 90% the reaction was stopped by addition of a 10 fold molar excess over sulphydryl groups of 2-mercaptoethanol. After exhaustive dialysis against 10mM ammonium bicarbonate pH 8.0, samples were lyophilized, taken up in

a small amount of standard buffer and counted for incorporation of label. When a time course was being constructed, samples for each time point were set up and treated as above. Protection experiments were carried out as above except acetyl-S-CoA or chloramphenicol (1mM) were added prior to the addition of $[^{14}C]$ iodoacetamide.

2.15 Identification of residues modified by $[^{14}C]$ Iodoacetamide.

(a) Inactivation.

1μmole of CAT_{III} (25mg in 2ml) was incubated in standard buffer at 37°C in a silver foil covered tube. Aliquots were taken at intervals over a 2hr. period, after the reaction had been initiated by addition of 10mM $[^{14}C]$ iodoacetamide, and treated as described in Methods 2.13. The reaction was terminated by addition of a 10 fold molar excess over sulphydryl groups of 2-mercaptoethanol and then the sample exhaustively dialysed against 10mM ammonium bicarbonate pH 8.0.

(b) Reduction and carboxymethylation.

Guanidine hydrochloride was added to a final concentration of 6M and a 4 fold molar excess of dithiothreitol (DTT) over total sulphydryl groups was added. The reaction vessel was flushed with nitrogen, sealed and stirred slowly in the dark for 2hr. After this time a 4 fold molar excess over sulphydryl groups (including DTT) of solid iodoacetic acid was added. After incubation for a further 2hr. to ensure alkylation of all unmodified cysteine residues, the reaction mix was quenched with excess 2-mercaptoethanol, exhaustively dialysed against ammonium bicarbonate (1.0M, 0.5M, 0.05M) and finally lyophilized.

(c) Digestion with protease of the modified protein.

Prior to the large scale digestion a trial digest with elastase was performed. 10nmole of protein was digestion with 1% w/w elastase for 4hr. in 50mM ammonium bicarbonate pH 8.0 at 37°C. Peptides were separated by electrophoresis in one direction followed by chromatography in a second direction (73).

Salt free samples were lyophilized and taken up in 1-2µl of standard HVPE (Methods 2.15e) electrophoresis buffer which contained fluorescent marker. The sample was applied to a 10cm x 10cm POLYGRAM SIL G silica thin layer plate, under a stream of warm air using a drawn out capillary.

Electrophoresis was carried out at 350V for 50 minutes, using a Shandon (Kohn U77) flat bed electrophoresis apparatus. The edges of the thin layer plate was connected to the electrode solutions by wicks of Whatman 3MM filter paper.

After drying ascending chromatography was carried out at right angles in butanol: acetic acid: water: pyridine (BAWP) 15:3:12:10 by volume. Peptides were located by autoradiography on Kodirex (Kodak) film, KDT2. Digestion of the protein sample was carried out as described above. Digestion was terminated by freezing the sample followed by lyophilization.

(d) Chromatography of peptides.

Peptides were separated on a Dowex 50 (sulphonated polystyrene) ion-exchange resin at 50°C. The column was preequilibrated in the pyridinium form with 5mM pyridine/acetate buffer pH 2.1. Peptides were fractionated using a pH and salt gradient (5mM pyridine/acetate pH 2.1 - 2.0M pyridine /acetate pH 4.9). The column was finally washed with 6.0M pyridine.

The sample was taken up in 5ml of formicacid with heating prior to loading. Peptides were identified by 280nm absorbance and by counting a 10µl sample of the 1ml sample collected. Peaks were pooled for HVPE.

(e) High voltage paper electrophoresis (HVPE).

Electrophoresis of peptides on Whatman 1 and 3MM chromatography paper was carried out in water cooled vertical tanks containing either toluene (pH 6.5) or white spirit (pH 2.1) as coolant. The electrode and wetting buffers were pyridine/acetic acid/water, 0.5:10:89.5 by volume (pH 6.5); acetic acid/formic acid, 8:2:90 by volume (pH 2.1) (74).

Salt free samples were lyophilized and taken up in a small amount of the appropriate buffer with warming. The solution was applied to a 1cm

square at the origin of the paper under a stream of warm air. Fluorescent markers (75) and amino acids (76) were applied as side markers. Buffer was carefully applied on either side of the origin to focus the sample. Excess buffer was removed with blotting paper. Electrophoresis at pH 6.5 proceeded at 3000V (60V/cm) until a marker dye had migrated 8.5cm. Chromatograms were dried in a stream of warm air. Radioactive peptides were located by autoradiography on Kodirex KDT2 film. Peptide containing areas were cut out and restitched onto fresh paper and electrophoresis at pH2.1 carried out. Radioactive peptides were again located by autoradiography.

(f) Elution of peptides.

Peptides were eluted from the paper with dilute acetic acid (5%) or dilute ammonia solution (5%) and collected in acid washes tubes. The peptides were stored at -20°C in a lyophilized state or in dilute acetic acid.

(g) Sequence analysis by the dansyl-Edman method.

Peptides were sequenced by the dansyl-Edman procedure as described by Hartley (77) and dansyl-amino acids identified by the method of Woods and Wang. (78).

2.16 Radioactive counting.

Radioactive counting was performed in a Packard scintillation counter Model 3385 using 'Brays Fluid' as the scintillation fluid. Counting efficiency was typically 80%.

2.17 Determination of λ max and extinction coefficient of 5,5 dithiobis (2-nitromethylbenzoate) (DTNMB) and 5,5 dithiobis(2-nitrotoluene) (DTNT).

1mM solutions of DTNMB and DTNT were made in dimethylformamide. Aliquots of these solutions were then diluted into 1ml of standard buffer, to give a solution of final concentration 50µM. These solutions were scanned in a Pye-Unicam SP1800 recording spectrophotometer against a standard buffer blank. An aliquot of dimethylformamide alone was added

to the blank. From these readings a λ max for each solution was established. 2-mercaptoethanol (final concentration 0.5mM) was then added to the sample and the blank tubes and the new λ max determined.

Samples of DTNMB and DTNT (10-50µM) in the presence and absence of 2-mercaptoethanol (0.5mM) were then made up and their absorbance at the appropriate λ max determined against a suitable buffer. The **extinction coefficient was determined from these experiments.**

2.18 Inactivation of CAT_{III} with DTNMB and DTNT.

100µl of CAT_{III}(0.2mg/ml) in standard buffer was incubated at 37°C. A 10µl sample was removed and diluted into 1ml of standard buffer for assaying by the standard method (Methods 2.4). DTNMB and DTNT (final concentration 0.05mM) were added from stock solutions in dimethylformamide and 10µl samples taken at intervals and treated as above.
A control sample of enzyme and dimethylformamide alone in standard buffer was also assayed as described above.

Similarily, a sample of enzyme and 5,5 dithiobis(2-nitrobenzoic acid) (DTNB) was also assayed for loss of activity except that the final concentration of DTNB was 0.5mM. Protection experiments were carried out as above except that chloramphenicol or acetyl-S-CoA was added to each sample tube.

2.19 Measurement of the incorporation of DTNMB into CAT_{III}.

1ml of CAT_{III} (0.5mg/ml) was incubated at 37°C in standard buffer. DTNMB (final concentration 5-50µM) was added from a stock solution of DTNMB in dimethylformamide and the absorbance at 420nm measured against a standard buffer blank. A 10µl sample was removed, diluted into 1ml of standard buffer and assayed by the standard method (Methods 2.4).

Samples were then applied to a Sephadex G10 desalting column (1x25cm) to remove excess reagent. The column had been preequilibrated with standard buffer. 1ml samples were collected and protein located by measuring 280nm absorbance. Tubes containing protein were pooled and concentrated to 1ml

using an Amicon B15 concentrator. An identical sample of unmodified enzyme was treated in the same manner.

2.20 Inactivation of CAT_{III} with Diethylpyrocarbonate (DEP).

DEP concentrations were measured by diluting samples of DEP into ethanol and reacting aliquots of this solution with 0.5mM imidazole in 50mM sodium phosphate pH 7.8 buffer. The N-carbethoxyimidazole formation was monitored at 240nm and the concentration of the stock solutions calculated using $\epsilon = 3.2 \times 10^3 M^{-1} cm^{-1}$ (79). All inactivation experiments were carried out at 25°C in 50mM sodium phosphate pH 6.0. CAT_{III} (0.8mg/ml) was incubated under these conditions, a 10µl sample was removed, diluted into 1ml of standard buffer and assayed by the standard method (Methods 2.4). DEP was added and further aliquots removed for assaying as described above. A control tube consisted of enzyme plus an aliquot of ethanol equal to that added to the sample tube. Spectral changes at 245nm of samples were measured against 50mM sodium phosphate buffer and enzyme blank.

After completion of the reaction 50µl of 1M hydroxylamine was added to the sample and control tubes and further measurements taken. Stock solutions of 1M hydroxylamine were made in 50mM sodium phosphate (pH 7.8) and the pH adjusted to pH 7.0 prior to use.

Protection experiments were carried out in the presence of chloramphenicol, acetyl-S-CoA and methyl-S-CoA as described for the inactivation studies, except that the enzyme samples were preequilibrated with substrates or substrate analogues prior to the addition of DEP.

2.21 Inhibition of CAT_{III} with Methyl p-Nitrobenzene Sulphonate and p-Nitrobenzene Sulphonyl Chloride.

Commercially purchased methyl p-nitrobenzene sulphonate (MNBS) was recrystallized from petroleum ether (100°-200°C). The concentration of stock solution of the compound in acetonitrile was determined by dissolving a sample of the solution in standard buffer and measuring the ultraviolet absorption. A λmax of 255nm and ϵ_{255} of 13.4×10^3 $M^{-1} cm^{-1}$ were used to

determined the stock solution's concentration.

100µl of CAT_{III} (0.1mg/ml) was incubated at 37°C in standard buffer. A 10µl sample was removedand diluted into 1ml of standard buffer and assayed by the standard method (Methods 2.4). MNBS was added and further 10µl samples removed at intervals and treated as described above. Control tubes consisted of enzyme plus acetonitrile.

Protection experiments in the presence of chloramphenicol, acetyl-S-CoA, acetonyl-S-CoA, methyl-S-CoA and 3-fluorochloramphenicol or combinations of these substrates and analogues were carried out as described above, except that the enzyme was preincubated with the substrate or substrate analogue.

Inhibition studies in the presence of p-nitrobenzene sulphonyl chloride were carried out as described for MNBS.

2.22 Photooxidation of CAT_{III} with Rose Bengal.

Photooxidation experiments were carried out in a watercooled Compton tube at 25°C. The tube containing 100µl of CAT_{III} (0.2mg/ml) in standard buffer was placed 10-14cm from a 100W light source. A 10µl sample was removed and diluted into 1ml of standard buffer in a silver foil covered tube and later assayed by the standard method (Methods 2.4). Rose bengal (final concentration 15µM) was added, samples taken at intervals and treated as described above.

Control experiments were carried out as above in the absence of light and the absence of dye.

Protection experiments in the presence of chloramphenicol and acetyl-S-CoA were carried out as described previously except that the enzyme was preincubated with substrate before addition of the rose bengal.

2.23 Inhibition of CAT_{III} with Butanedione.

100µl CAT_{III}(0.2mg/ml) was incubated at 37°C, either in 50mM borate pH 7.8 or 50mM sodium phosphate pH7.8. A 10µl sample was removed, diluted into 1ml of standard buffer and assayed by the standard method (Methods

2.4).Butanedione (concentration as described in text) was added and further samples removed and treated as described previously.

After inactivation the sample was desalted on a Sephadex G10 (1x25cm) which had been preequilibrated in 50mM sodium phosphate buffer pH 7.8. 1ml fractions were collected and protein located by measuring 280nm absorbance. Tubes containing protein were pooled and concentrated using an Amicon B15 concentrator.

Protection studies were carried out as described above except that the enzyme was preincubated with either chloramphenicol or acetyl-S-CoA prior to the addition of butanedione.

2.24 Inhibition of CAT_{III} with 4-amino-7-oxa-bicyclo(4,10)hept-3-ene-2, 5-dione-3-ene-2,5-dione-3-carboxamide (G7063-2).

Stock solutions of G7063-2 were made in standard buffer and stored at $4^{o}C$. Initially, solutions were pale yellow in colour but after storage for 1 week the solution had turned dark brown in colour.
100µl CAT_{III} (0.2mg/ml) in standard buffer was incubated at $37^{o}C$. A 10µl sample was removed, diluted into 1ml of standard buffer and assayed by the standard method (Methods 2.4). G7063-2 was added from the stock solution and further samples taken at intervals and treated as above.

Protection ezperiments were carried out as above except that chloramphenicol or acetyl-S-CoA was preincubated with the enzyme prior to addition of G7063-2.

Chapter Three

Purification and Characterization of CAT_{III}

3.1 Purification of protein.

Crude extracts were prepared according to Methods 2.1b. Initial studies on the enzyme indicated that the enzyme was stable to heating at 70°C for 10 minutes in a buffer containing 0.2mM chloramphenicol. This step was thus used as an initial step prior to the purification of the enzyme by affinity chromatography.

The technique of affinity chromatography has been successfully used to purify several of the CAT variants. Preliminary experiments performed using 1ml bed volume affinity columns containing resins of increasing substitutions indicated that a resin substitution of at least 9μeq of chloramphenicol base per ml of settled resin was necessary to bind the enzyme to the column and a salt concentration of 0.3M NaCl in the presence of 5mM chloramphenicol was needed to elute the enzyme from the column.

Large scale purifications were carried out according to Methods 2.2, 2.3. Yields varied between 65-80% of pure enzyme. A higher yield was usually obtained if the column was freshly prepared. Usually 95-100% of the total activity could be recovered. The CAT eluted from the resin in the buffer and salt washes could be recycled after dialysis to remove excess salt and chloramphenicol if required.

A typical purification profile is shown in Table 4. Concentrations of protein upto 5 mg/ml could be bound to the affinity resin. A maximum of 1 mg/ml was obtained for the CAT_I variant in previously described work (L.C. Packman, Ph.D Thesis, Univ. of Leicester 1978). Column fractions were tested for activity, pooled and dialysed against TCM buffer at 4°C. Samples were then frozen at -20°C for storage.

3.2 Electrophoresis of CAT_{III} variant.

(a) Electrophoresis in 0.1% SDS.

All purification steps were monitored by SDS gel electrophoresis as described in Methods 2.6a (Fig.4). As can be seen from this gel CAT represents a large percentage of the total cell protein (~5%).

Table 4

Purification of a CAT variant specified by E.coli J53 (R387;Cm700).

Purification Step	Volume (ml)	CAT (U/ml)	Protein (mg/ml)	CAT S.A (U/mg)	Purification Factor	Yield %
Crude extract	1000	1905	48	39.7	1.0	100
Heat step	780	2423	24	100.9	2.54	99
Affinity resin						
1. Unbound	1400	328	n.d			24
2. TM wash	450	95	n.d			2.2
3. TM + 0.3M NaCl wash	4500	40	n.d			9.4
4. TM + 0.3M + 5mM Cm wash	500	2465	1.3	1896	47.8	64.6

n.d not determined
One Unit is defined as the number of μmoles of product produced per minute in the standard assay.

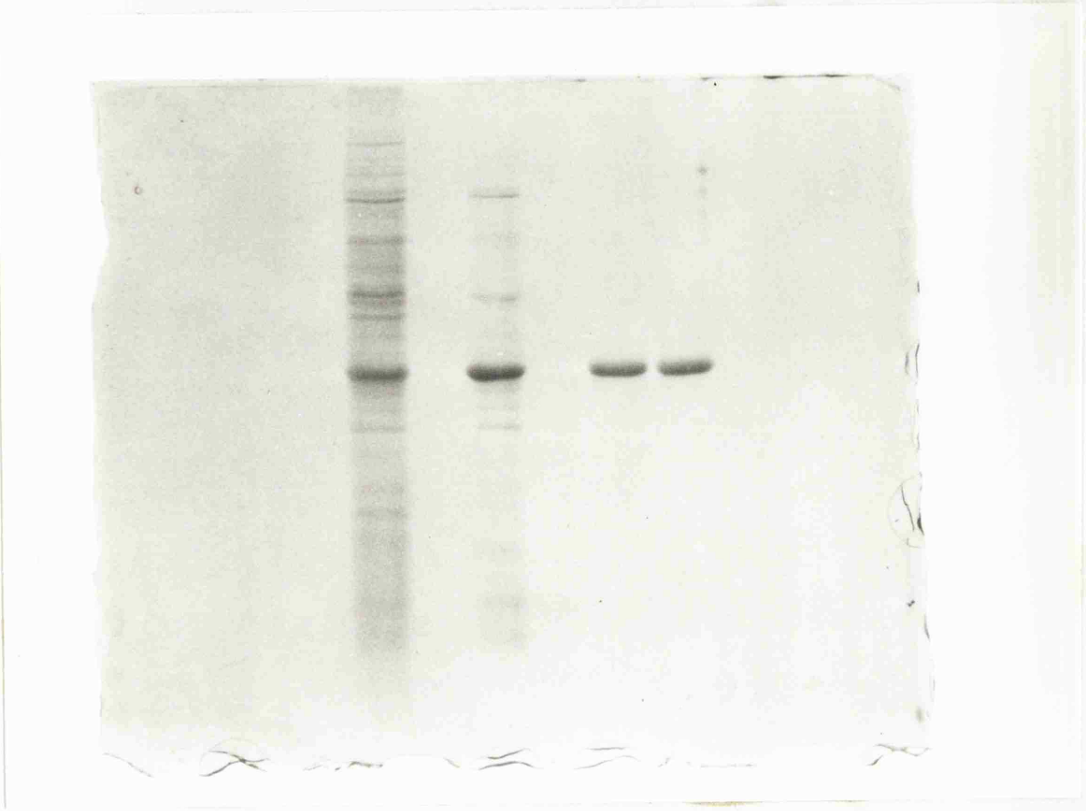


Fig.4.

Electrophoresis of samples from the purification of CAT_{III} under denaturing conditions in a 12.5% polyacrylamide gel containing 0.1%(w/v) SDS.

1. Crude extract 100µg
2. Heat treated protein 50µg
3. CAT_{III} 5µg
4. CAT_{III} 5µg

The protein was adjudged to be free of significant contamination by the Coomassie staining method.

Gel electrophoresis of pure CAT_{III} versus molecular weight markers was used to determine an apparent molecular weight for the protein (Fig.5).

Monomer molecular weights of CAT proteins determined by gel electrophoresis have tended to be an underestimate when compared to 'true' molecular weights determined from amino acid sequences. For example, CAT_I has a true molecular weight of 25,668, whereas it has an apparent molecular weight of 24,000 as determined by gel electrophoresis. It is thought that this may reflect difficulties in unfolding the protein properly making it run at a lower apparent molecular weight. Indeed, it has been previously reported that CAT_I specified by plasmid R429 requires the addition of 6M urea to the resolving and stacking gels to get reproducible tight bands on gel electrophoresis (L.C. Packman, Ph.D Thesis, Univ. of Leicester 1978). This procedure was not found to be necessary for CAT_{III} or CAT_I, although the protein was always boiled for at least 10 minutes in a well sealed tube prior to loading on the gel. Under these conditions reproducible molecular weights of 25,000 for CAT_{III} and 24,000 for CAT_I were observed. Whether the molecular weight determined for CAT_{III} is 'true' or apparent will not become clear until sequence studies are complete.

(b) Electrophoresis of native protein.

Electrophoresis of CAT_{III} under native conditions on an agarose strip was carried out according to Methods 2.6b. 1-2µl of a 40mg/ml protein solution was loaded in each well. After the gel had been run (90V for 50 minutes) the strip was divided into two and stained for protein (Methods 2.6b) and for CAT activity using the histochemical stain (Methods 2.6c). No higher molecular weight bands were seen either with the protein or activity stain (Fig.6). This result contrasts with that obtained with CAT_I in which multimers of native tetramer (hexamer and octamer) were seen, and makes CAT_{III} a more suitable candidate for NMR analysis than CAT_I.

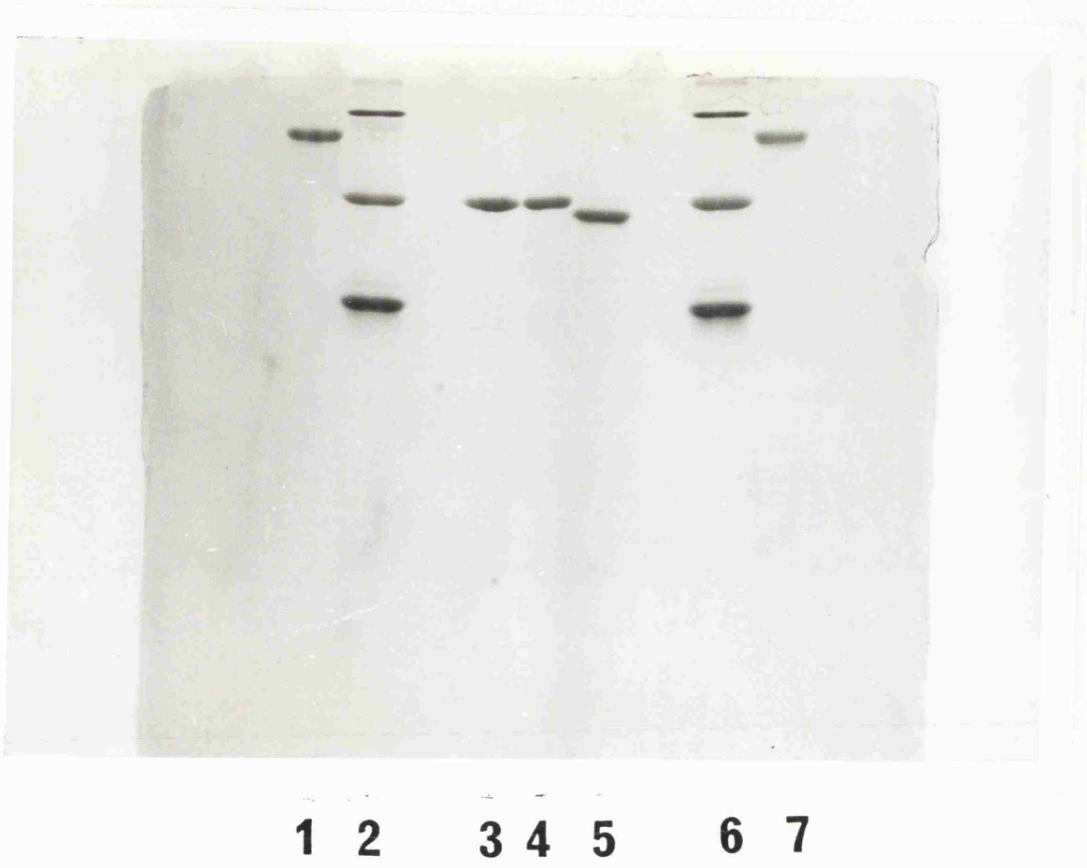


Fig.5.

Determination of monomer molecular weight of CAT_{III} in a 15% polyacrylamide gel containing 0.1% (w/v) SDS.

5µg of each protein was loaded on the gel.

1,7 Ovalbumin (45K)

2,6 BSA (67K), Chymotrypsinogen (25K), Cytochrome C (13.4K)

3,4 CAT_{III}

5 CAT_{I}

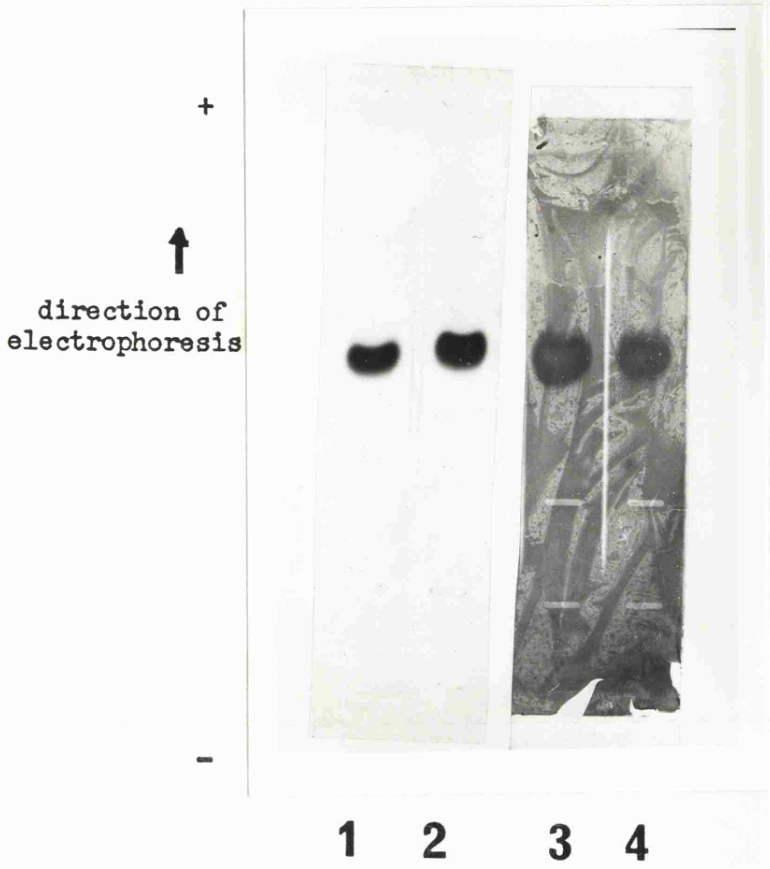


Fig.6.

Electrophoresis of CAT_{III} under native conditions on an agarose strip.

1-2µl of protein solution was applied to each track, and electrophoresed according to Methods 2.6b. The gel was then cut into two sections and one section stained for protein (Methods 2.6b) and the other section stained for activity using the histochemical stain (Methods 2.6c).

1,2 CAT_{III} stained for protein

3,4 CAT_{III} stained for activity

3.3 Amino acid analysis of CAT_{III}.

Acid hydrolysis and amino acid analysis as described in Methods 2.10 was carried out on salt free samples of CAT_{III}. The cysteine content of the variant was determined by analysis of performic acid oxidised protein.

The results were calculated using a monomer molecular weight of 25,000. A typical analysis is shown in Table 5.

3.4 Specific activity of CAT_{III}.

The specific activity of the enzyme was determined using amino acid analysis to determine the protein concentration. The value obtained of 1800-1900 μmole/minute/mg of monomer measured under standard assay conditions is the highest recorded specific activity of the CAT family.

3.5 Comparison of the peptide maps of CAT_I, CAT_{II} and CAT_{III}.

Previous studies have compared peptide maps of the CAT proteins using 2-D paper electrophoresis at pH 6.5 and pH 3.5 after digestion with a suitable enzyme (L.C. Packman, Ph.D Thesis, Univ. of Leicester).

In these studies CAT_{III} was compared to CAT_I and CAT_{II} by the peptide mapping method of Cleveland (68) (Methods 2.8).

Pure CAT bands were excised from a 12% polyacrylamide gel (0.1% w/v SDS) and after equilibration of the gel piece in 0.125mM TrisHCl pH 6.8, 0.1mM EDTA, 1% SDS loaded onto a second gel and digested in the stacking gel with either S. aureus V8 protease (Fig.7) or papain (Fig.8). The peptides generated were located by staining either with Coomassie Blue R250, or in the case of papain, since less protein had been loaded on the first gel, with the more sensitive silver stain (Methods 2.9). The results indicate that although the proteins are not identical, CAT_I, CAT_{II} and CAT_{III} are related since they show common peptides. Cleveland has demonstrated that unrelated proteins of the same size do not show common peptides (68).

Whilst the results obtained with the E.coli variants only confirmed previous results the technique was also used to examine the relatedness of Haemophilus influenzae variants of CAT. Two H. influenzae were shown

Table 5

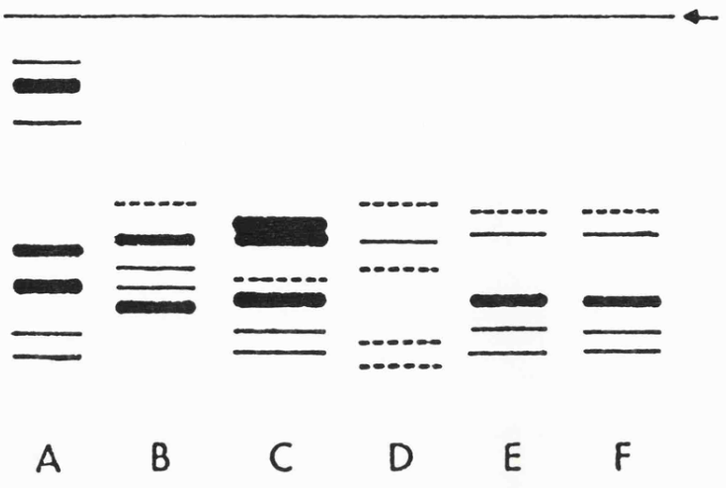
Amino Acid Analysis of CAT_{III}.

Amino Acid	Nanomoles recovered 24hr.	48hr.	72hr.	Best value	Residues per monomer
Cysteic Acid		3.7	4.1	3.9	4
Aspartic Acid	26.5	26.3	26.1	26.3	26
Threonine	11.5	11.6	10.5	11.8	12
Serine	18.3	19.3	17.5	18.8	19
Glutamic Acid	23.5	22.8	22.5	22.9	23
Proline	8.5	8.7	8.3	8.5	8/9
Glycine	8.9	10.2	9.8	9.7	9/10
Alanine	12.5	11.9	11.9	12.1	12
Valine	14.9	15.4	15.6	15.9	16
Methionine	4.9	4.9	4.6	4.8	5
Isoleucine	9.9	10.2	10.3	10.5	10/11
Leucine	19.0	17.9	19.2	19.4	19
Tyrosine	8.6	8.2	8.6	8.5	8/9
Phenylalanine	14.8	13.0	15.1	14.3	14
Histidine	6.6	6.7	6.6	6.6	7
Lysine	11.2	10.9	11.4	11.2	11
(Tryptophan)	not determined				
Arginine	9.1	10.2	8.8	9.3	9

Fig.7.

S. aureus V8 digestion patterns.

CAT variants were digested with S. aureus V8 protease according to Methods 2.8. The peptides generated were located by staining with Coomassie Blue R250. Tracks A,B and C are CAT_I, CAT_{II} and CAT_{III} respectively. Tracks D,E and F were Haemophilus influenzae variants of CAT which were purified in connection with studies carried out with Dr. M. Roberts. The arrow indicates the position of V8 on the gel. The line drawings were done prior to the gel being photographed.


A
B
C
D
E
F

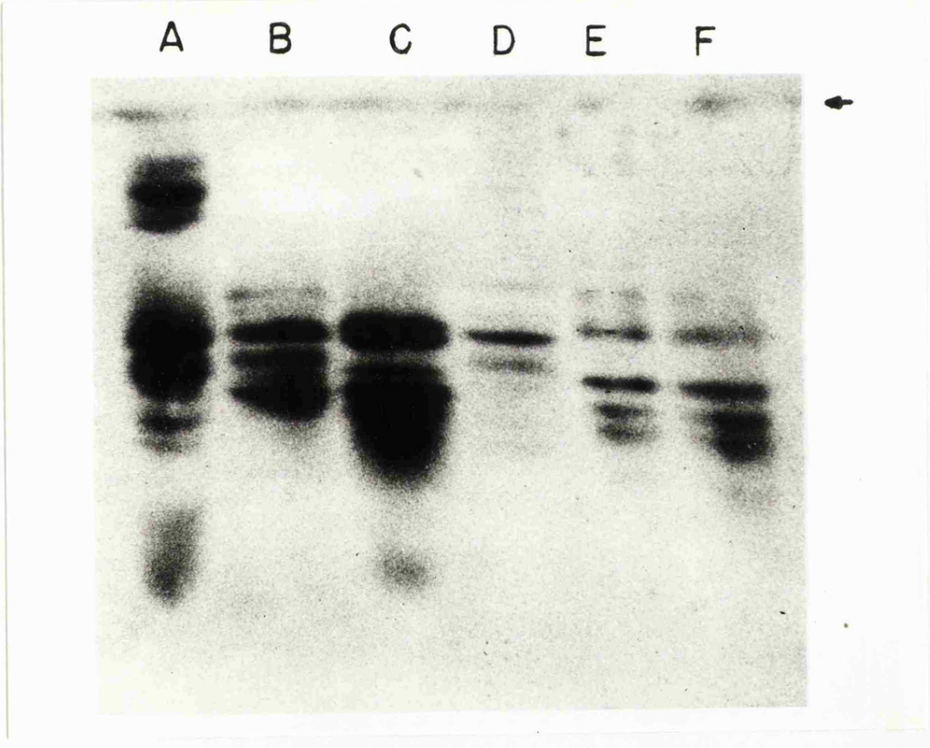
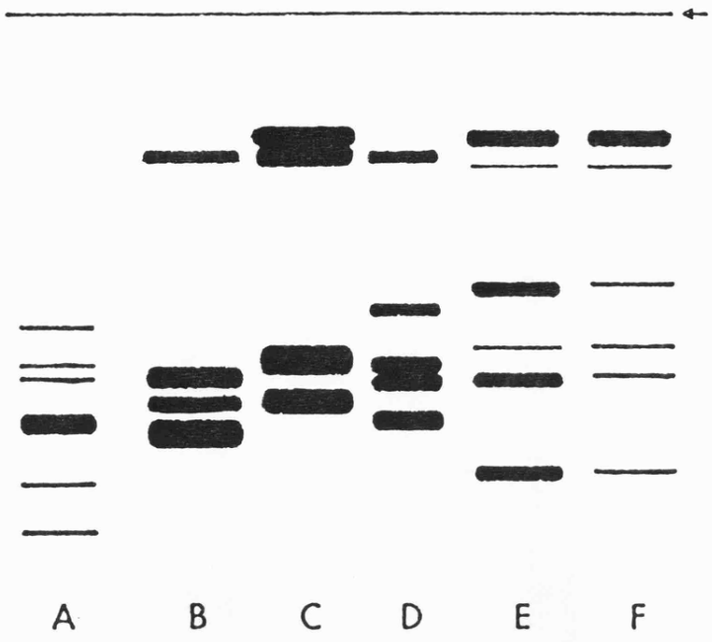
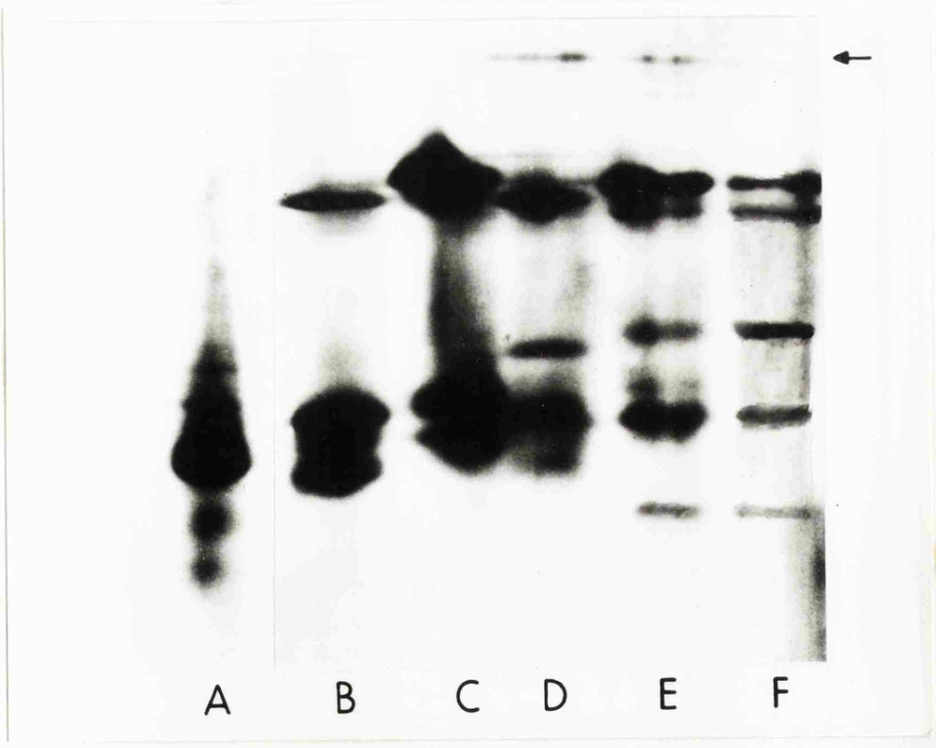

A
B
C
D
E
F

Fig.8.

Papain digestion patterns.

CAT variants were digested with papain according to Methods 2.8. The peptides were located using the silver stain (Methods 2.9). Tracks A, B and C are CAT_I, CAT_{II} and CAT_{III} respectively. Tracks D, E and F are Haemophilus influenzae variants of CAT which were purified in connection with studies carried out with Dr. M. Roberts. The arrow indicates the position of papain on the gel. The line drawings were done prior to the gel being photographed.


A
B
C
D
E
F


A
B
C
D
E
F

to have near identical peptide maps and all three variants examined had common peptides with CAT_I, CAT_{II} and CAT_{III}. This technique may therefore be useful in classifying CAT variants used in conjunction with chemical, kinetic and immunological studies.

3.6 Reaction of antibody raised against Type I, Type II and Type III protein with CAT_I, CAT_{II} and CAT_{III}.

Fig.9 shows the result of antibody precipitation experiments. The proteins show no cross reactivity despite the fact that the proteins are known to have homologous regions(Table 2). This may indicate that the homologous regions of the protein are buried .

3.7 Equilibrium dialysis of CAT_{III}.

Although it has always been presumed that CAT, which has four identical monomers, binds one mole of chloramphenicol per mole of monomer, this has never been established fully. Tanaka et al (56) determined a value of one molecule of chloramphenicol binding with one molecule of enzyme. They determined this using the equation $\log v/V_{max}-v = h \log(S)-\log K$ and interpreted h,the Hill constant,as the number of chloramphenicol binding sites. However, as discussed by Cornish-Bowden(80) h (sometimes referred to as n)doesnot have a physical meaning, although he also points out that many people have interpreted it spuriously as the number of ligand binding sites on each molecule of protein.

Equilibrium dialysis experiments were thus carried out on CAT_{III} in order to determine the number of ligand binding sites (Methods 2.12).

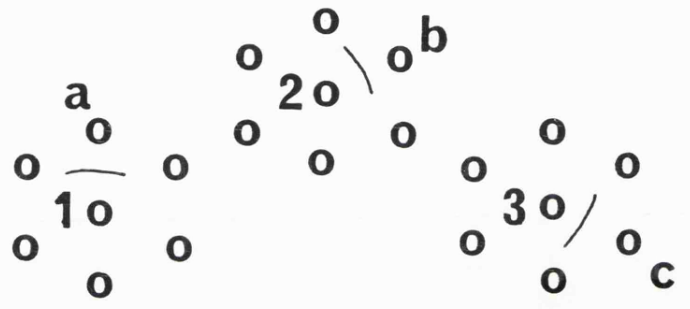
Initially $[^{14}C]$chloramphenicol alone (3.57uCi/μmole) was placed in one of the chambers in order to determine how long chloramphenicol took to equilibrate across the membrane. The time taken for equilibration was approximately 18 hr. as determined by these preliminary experiments (data not shown).

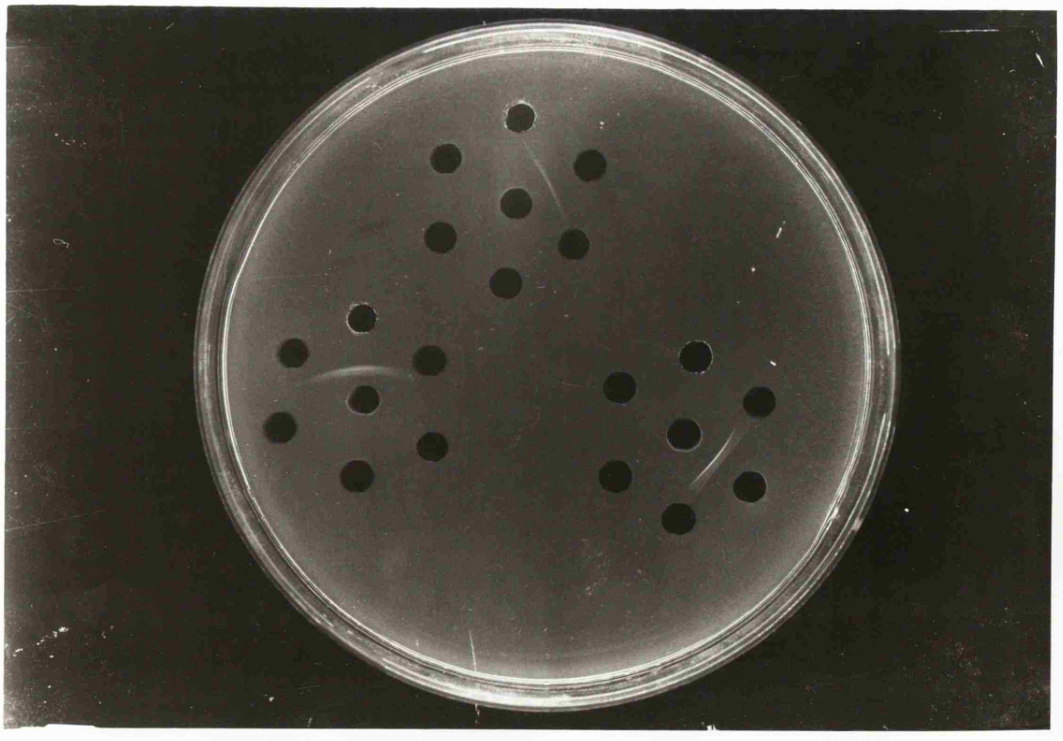
For the experiment an enzyme concentration of 0.05mM (monomer) was used and a range of $[^{14}C]$ chloramphenicol concentrations from 0.01-0.27mM

Fig.9.

Reaction of CAT variants I,II and III with CAT specific antisera .

Ouchterlony diffusion tests were carried out according to Methods 2.11. The CAT_I directed reagent was made from a goat immunized with CAT_I specified by plasmid JR66. CAT_{II} specified by plasmid Sa and CAT_{III} specified by plasmid R387 were injected into rabbits to raise the CAT_{II} and CAT_{III} directed reagents. 10µl of pure protein (1mg/ml) was used in each well; (a = CAT_I, b = CAT_{II}, c = CAT_{III}). 10µl of crude antiserum was placed in the appropriate centre well; (1 = CAT_I, 2 = CAT_{II}, 3 = CAT_{III}).





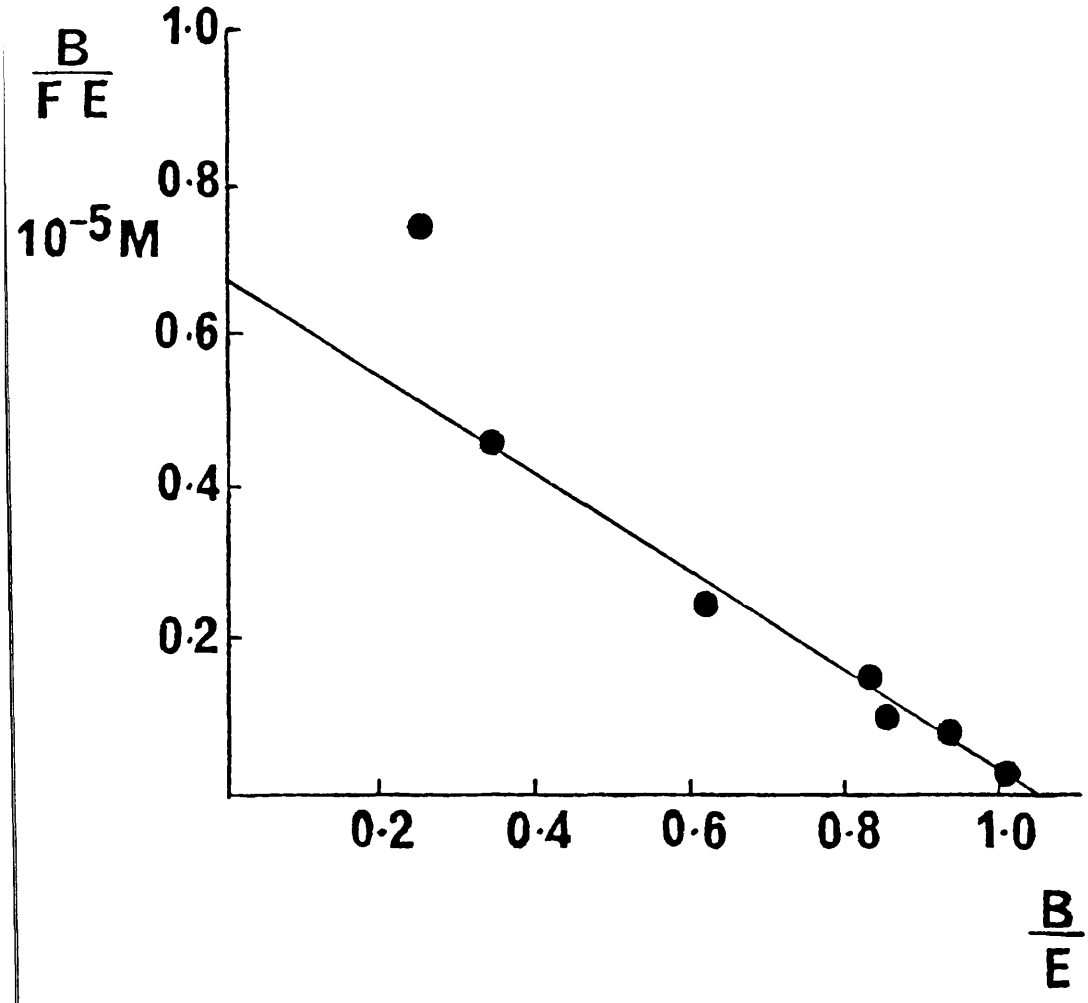


Fig.10.

Scatchard plot for equilibrium ligand binding.

Equilibrium binding studies were carried out according to Methods 2.12.

The enzyme concentration used was 0.05mM (monomer) and the chloramphenicol concentrations ranged from 0.01-0.27mM.

B = Bound ligand E = Enzyme concentration

F = Free ligand

(Fig.10).

The value determined from this data is 1.04 moles of chloramphenicol per mole of enzyme monomer (4 per tetramer). The dissociation constant was calculated at $15.5 \times 10^{-6} M^{-1}$ at $4^{\circ}C$ and pH 7.8. Since the bonding site ought to be the active site there are probably 4 active sites per tetramer. There is no indication from these results, however, whether each subunit has an active/binding site or whether residues from two or more subunits constitute a binding site. This has proved difficult to investigate because CAT is highly resistant to dissociation (L.C. Packman Ph.D Thesis, Univ. of Leicester).

3.8 Determination of K_m and V_{max} values for CAT_{III}.

Michaelis constants for the two substrates for CAT_{III} were determined using a modified spectrophotometric assay (Methods 2.7). The results are illustrated graphically in Fig.11,12. For each substrate a saturating concentration of the other substrate (400μM acetyl-S-CoA, 200μM chloramphenicol) was used in each assay.

The data was plotted using the iterative least squares programme of Cleland (67). K_m values of 17.6μM for chloramphenicol and 74μM for acetyl-S-CoA were determined.

Fig.11.

Lineweaver-Burk plot to determine the Michaelis constant for Chloramphenicol for the CAT_{III} variant.

Assays were carried out according to Methods 2.7. In each assay the amount of enzyme used was 3×10^{-4} nmoles, and the acetyl-S-CoA concentration was 400μM. The concentration of chloramphenicol was varied over the range 5-50μM. Values determined from the computer plot were: K_m = 17.6μM S.E 2.683, Vmax = 13.1 nmole/min/ml assay S.E 0.77.

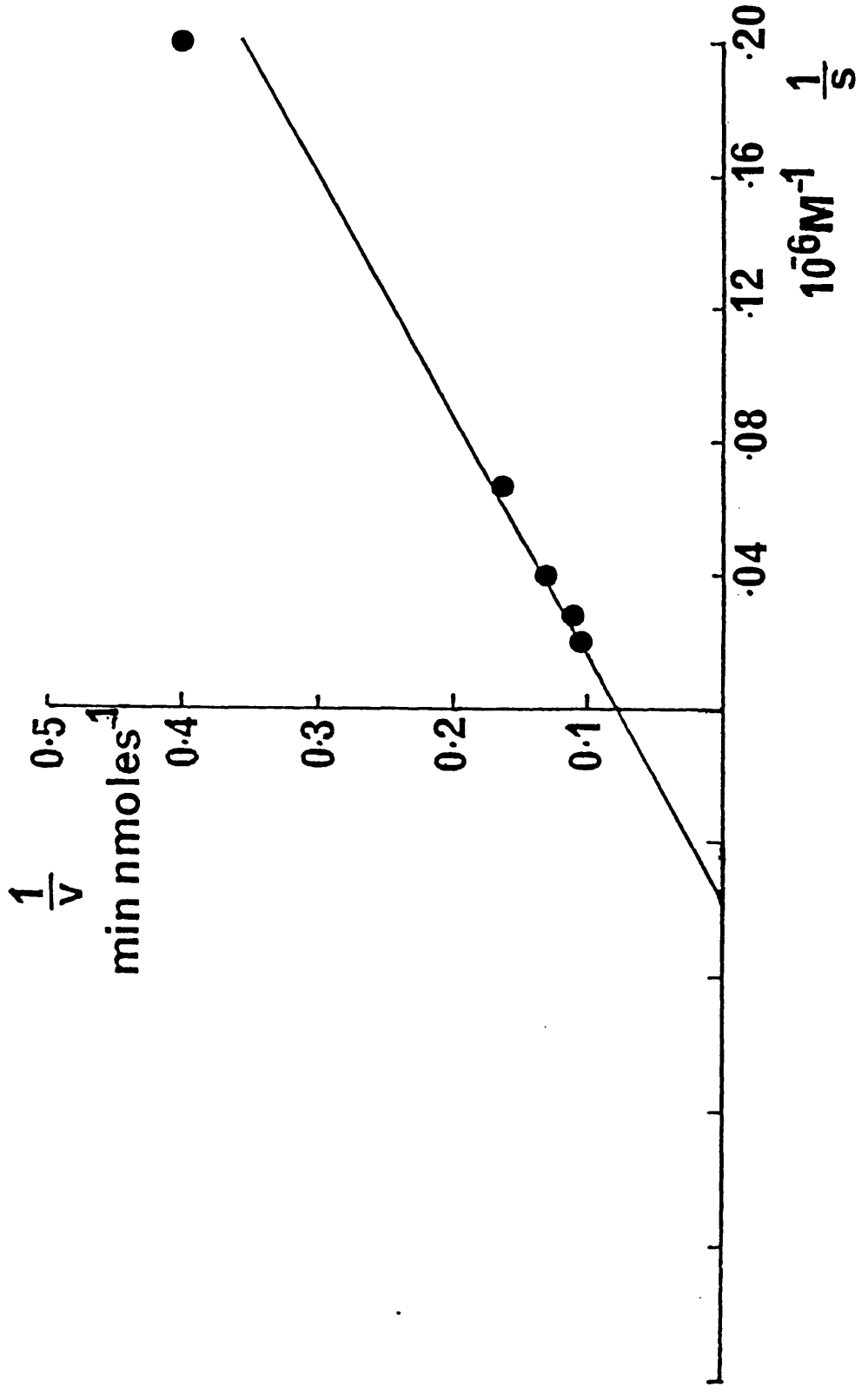
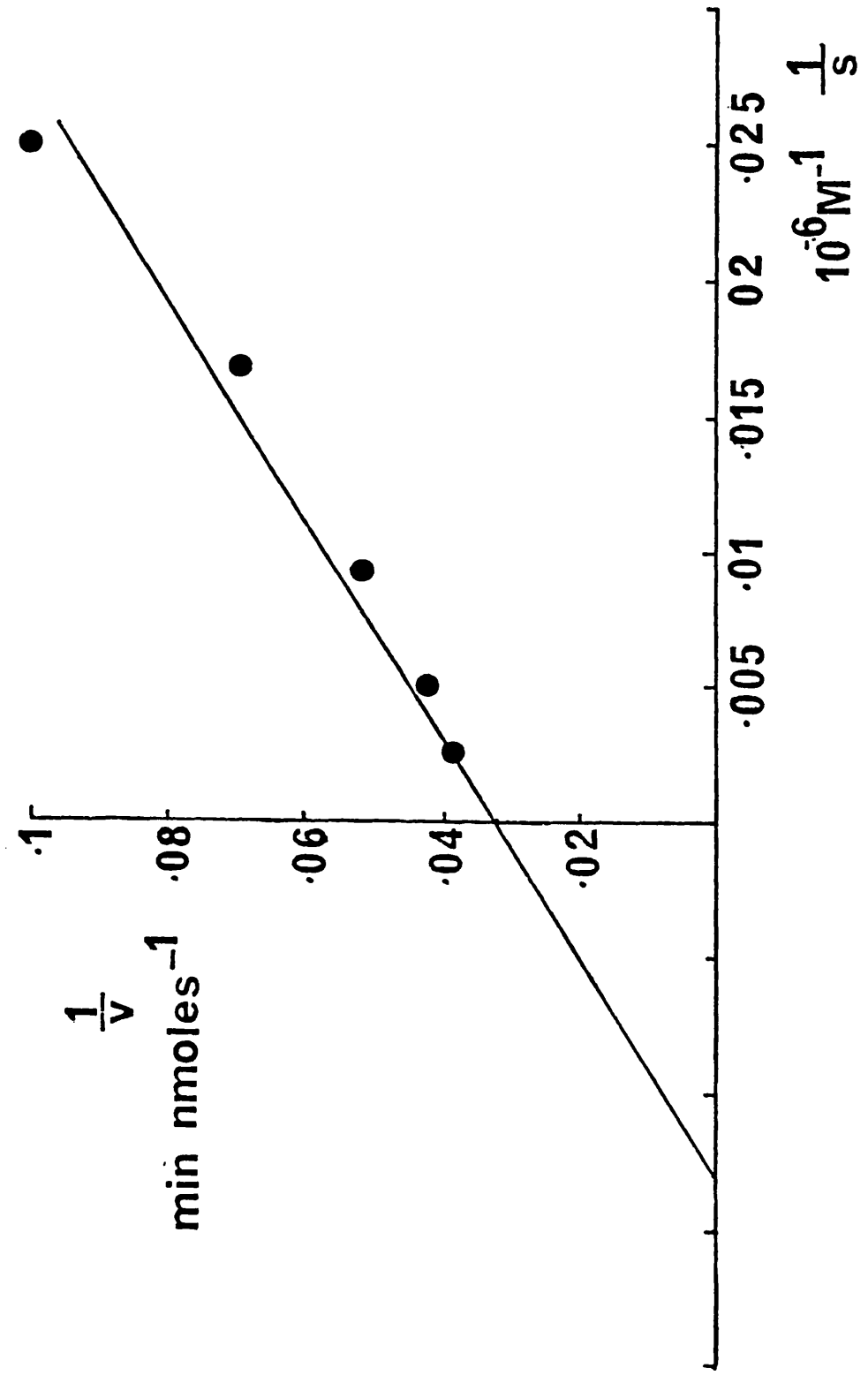

1/v
min nmoles⁻¹
0·5
0·4
0·3
0·2
0·1
·04
·08
·12
·16
·20
10⁻⁶M⁻¹ 1/s

Fig.12.

Lineweaver-Burk plot to determine the Michaelis constant for Acetyl-S-CoA for the CAT_{III} variant.

Assays were carried out according to Methods 2.7. In each assay the amount of enzyme used was 6×10^{-4}nmoles and the chloramphenicol concentration was 200µM. The concentration of acetyl-S-CoA was varied over the range 40-400µM. The K_m value determined was 74µM S.E 10.40, and the Vmax value determined was 30.9 nmole/min/ml assay S.E 1.46.


·1
·08
·06
·04
·02
1/v
min nmoles⁻¹
·005
·01
·015
02
·025
10⁻⁶M⁻¹ 1/s

Chapter Four

Chemical Modification Studies with Iodoacetamide and Iodoacetic Acid.

4.1 Introduction.

Early chemical modification experiments with CAT variants centred mainly on reagents which are known to modify sulphydryl residues. The reason for this arose mainly from the observation that the Type II variant was inhibited by DTNB under conditions obtaining in the standard assay (27,36). Table 6 compares inactivation data from experiments with CAT_I, CAT_{II} and CAT_C variants inhibited with iodoacetamide, iodoacetic acid, DTNB, dithiopyridine (DTP), fluorodinitrobenzoic acid (FDNB) and N-ethylmaleimide (NEM). The degree of protection seen with chloramphenicol and acetyl-S-CoA is also included.

Careful inspection of the table leads to two main conclusions. The CAT_C variant shows far less sensitivity to the reagents than CAT_I and CAT_{II} variants. Whereas the CAT_{II} variant is sensitive to all the reagents, CAT_I is not significantly inhibited by DTNB or iodoacetic acid although inhibition does occur with iodoacetamide and DTP.

In general chloramphenicol protects against inhibition by these reagents usually providing greater than 95% protection, whereas acetyl-S-CoA shows less than 5% protection under the same conditions. One exception is seen to this case. For the CAT_I variant 35% protection by acetyl-S-CoA is seen for inhibition with iodoacetamide.

Further experiments (43) with the CAT_{II} variant resulted in the incorporation of one mole of thiopyridine or thionitrobenzoate after addition of DTP or DTNB. A stochiometric amount of DTP was used. A second group was modified only after addition of eight moles of reagent per monomer. Titration with DTNB required three moles and a second group was modified only after addition of sixteen moles of DTNB. The data thus indicated that one cysteine residue was readily modified by the chromogenic disulphide reagents and also that CAT_{II} was modified faster by DTP than by the negatively charged DTNB. Treatment with DTP caused loss of 90–95% of the enzyme activity. If chloramphenicol was present the enzyme activity

Table 6

Summary of the pseudo first order rate constants of inactivation of CAT variants with Iodoacetamide, Iodoacetic Acid, DTNB, DTP, FDNB and N-ethylmaleimide (38,43,58).

Variant	mg/ml	Reagent	Rate of Inhibition 10^{-3} min^{-1}	%Protection Cm	%Protection Ac
I	2.5	Iodoacetamide 10mM	29	95	35
II	0.18	Iodoacetamide 5mM	65		
C	0.20	Iodoacetamide 5mM	7.6	95	5
I	2.5	Iodoacetic acid 10mM	0.4		
II	0.18	Iodoacetic acid 5mM	21.1		
C	0.20	Iodoacetic acid 10mM	0.1		
I	0.15	DTNB 0.5mM	1.2		
II	0.18	DTNB 0.01mM	34.5		
C	0.20	DTNB 1mM	0.1		
I	0.15	DTP 0.02mM	65	99	0
II	0.18	DTP 0.01mM	156		
C	0.20	DTP 1mM	0.01		
I	0.15	FDNB 1mM	9.9		
II	0.18	FDNB 0.04mM	32.2		
C	0.20	FDNB 1mM	11.7	95	5
I	0.15	NEM 1mM	72		
II					
C	0.20	NEM 1mM	2.1	95	5

Rates of inactivation were calculated from the equation $2.3 \log \frac{E}{E_o} = -kt$ (72)

E_o = initial enzyme activity, E = activity after time t.

was protected from inactivation(43).

The conclusions drawn from all these experiments are as follows:
(a) the E.coli variants CAT_I and CAT_{II} are sensitive to 'sulphydryl' reagents. CAT_I, however, is not sensitive to the negatively charged reagents DTNB and iodoacetic acid whilst the CAT_{II} variant is sensitive to all the reagents.

It has, therefore, been postulated that the CAT_I variant has a negatively charged residue(s) at or near the active site which hinders electrostatically inactivation of the enzyme by negatively charged inhibitors.
(b) The stapylococcal variant CAT_C is relatively insensitive to these sulphydryl reagents.
(c) Protection experiments have indicated that a cysteine residue modified in experiments with DTNB and DTP is located at or near the chloramphenicol binding site.

Identification of the 'sulphydryl' residues modified in these experiments was carried out using $[^{14}C]$ iodoacetamide and $[^{14}C]$ NTCB. The peptides isolated together with the position of the incorporated label are displayed in Table 7. In the case of CAT_C a sulphydryl residue was not modified as expected. Instead 3-carboxymethylhistidine was found. It should also be noted that the data presented in Table 7 for the CAT_I is subject to some debate since in a repeat of the inactivation experiment with $[^{14}C]$ iodoacetamide 1.97 moles of $[^{14}C]$ label were found to be incorporated. Amino acid analysis of the inactivated protein showed 0.7 moles of 3-carboxymethylhistidine and 1.3 moles of carboxymethylcysteine per mole of enzyme monomer. The position of the label in peptides was not established (J.E.Fitton, Ph.D Thesis, Univ. of Leicester, 1977).

Inactivation experiments have been carried out with the CAT_{III} variant, therefore, using iodoacetamide and iodoacetic acid in order to compare and contrast the resultsobtained with those from experiments with the CAT_I and CAT_C variant.

Table 7

Summary of peptides isolated in chemical modification studies using [^{14}C] Iodoacetamide and [^{14}C] NTCB.

[^{14}C] Iodoacetamide

CAT_I (58); 1.47 moles [^{14}C] label incorporated per mole of enzyme monomer.

	% of Total Radioactivity
Gln-Ser-Val-Ala-Gln-CmCys-Thr-Tyr	60
His-Ala-Val-CmCys-Asp-Gly-Phe	20
CmCys-Asp-Glu-Trp-Gly-Ala-Gly-Gln	12

position of label

CAT_C (38); 0.8 moles [^{14}C] label per mole of enzyme monomer.

His-CmHis-Ala-Val-Cys

[^{14}C] NTCB

CAT_{II} (43); 0.82 mole [^{14}C] label per mole of enzyme monomer.

Ser-Val-Ala-Gln-Cys

4.2 Inactivation of CAT_{III} by Iodoacetamide and Iodoacetic acid.

Inactivation experiments were carried out with iodoacetamide and iodoacetic acid according to Methods 2.13. The data were plotted on a semilogarithmic plot according to the equation $2.303\ E/E_o = -kt$ (72) (Fig.13). The k values obtained are shown in Table 8.

The CAT_{III} variant behaves very similarily to the CAT_I variant as regards the inhibition by iodoacetamide and iodoacetic acid. Iodoacetic acid inhibits CAT_{III} at less than 7% of the rate of that obtained with an equivalent concentration of iodoacetamide

Table 8

Rates of inhibition (k) of CAT_{III} by Iodoacetamide and Iodoacetic acid.

	$k\ min^{-1}$
control	4.1×10^{-4}
Iodoacetic acid (20mM)	1.7×10^{-3}
Iodoacetamide (5mM)	5.4×10^{-3}
Iodoacetamide (10mM)	11.4×10^{-3}
Iodoacetamide (20mM)	24.4×10^{-3}

4.3 Incorporation of $[^{14}C]$ Iodoacetamide in the presence and absence of substrates.

Initial incorporation experiments were carried out in which the enzyme samples and $[^{14}C]$ iodoacetamide were allowed to proceed to 90% inactivation, then quenched with 2-mercaptoethanol and exhaustively dialysed (Methods 2.14). A total of 0.96 moles of label were incorporated per mole of enzyme monomer.

Time course of incorporation.

Inhibition of the enzyme in the presence and absence of substrates was initiated by addition of $[^{14}C]$ iodoacetamide (S.A. 0.25μCi/umole). Samples were taken for assaying for residual activity just prior to

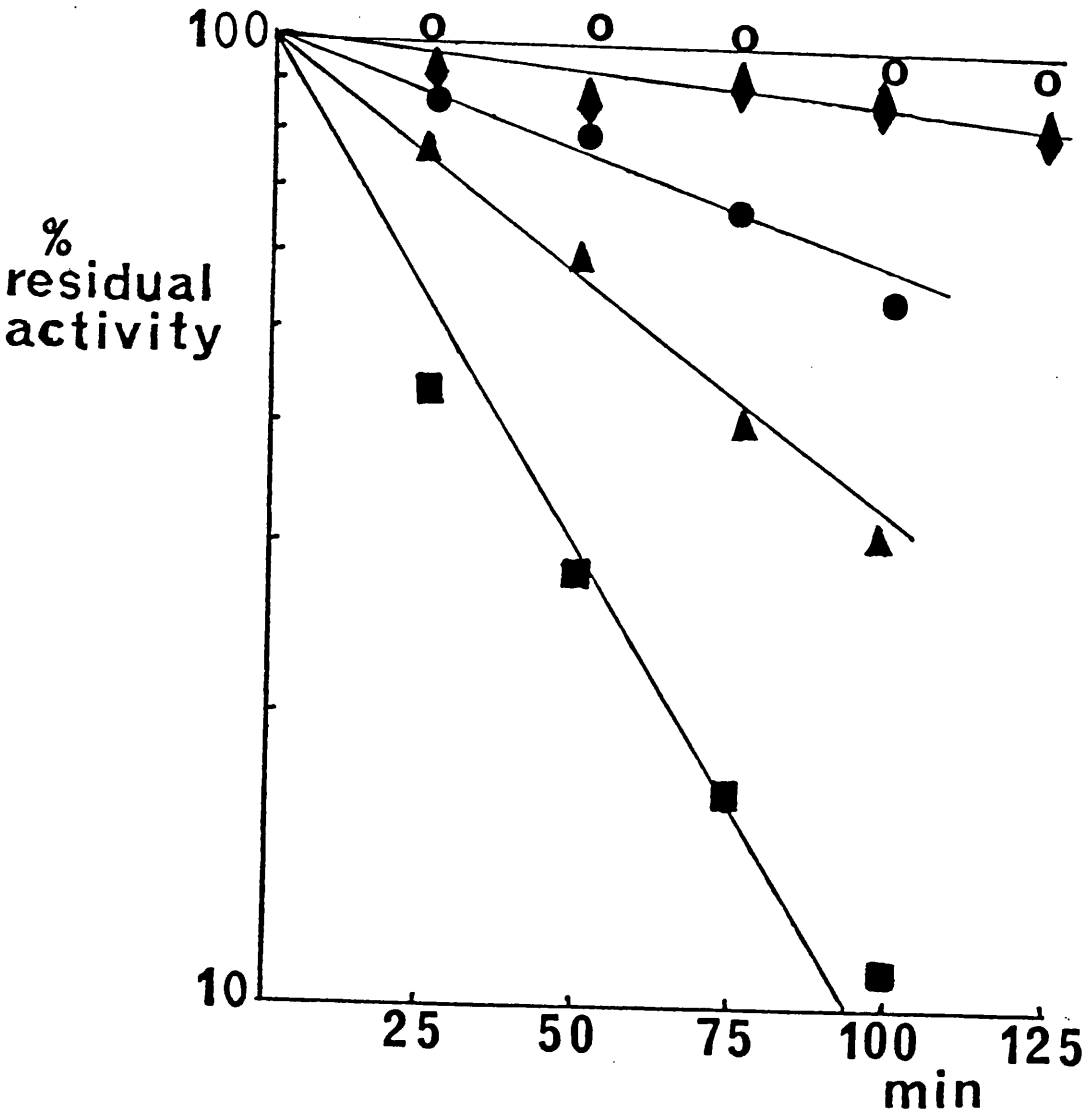
Fig.13.

Time course for the inhibition of CAT_{III} with Iodoacetamide and Iodoacetic Acid.

Enzyme (0.2mg/ml) in standard buffer was incubated at 37°C with 20mM iodoacetic acid ♦, 5mM, 10mM, 20mM iodoacetamide ● , ▲ , ■ . The control, ○ , consisted of enzyme alone.

Samples were taken, diluted into standard buffer and assayed by the standard method (Methods 2.13,2.4).

Rates of inactivation were calculated using the equation $2.3\log\frac{E}{E_o} = -kt$ are displayed in Table 8.


100
10
% residual activity
25
50
75
100
125
min

quenching and measured in the standard assay (Fig.14). After the appropriate time the reaction was quenched by the addition of a 10 fold molar excess (over sulphydryl groups) of 2-mercaptoethanol. After exhaustive dialysis against 10mM ammonium bicarbonate, samples were lyophilized, taken up in standard buffer and counted for incorporation of label (Fig.15). The degree of protection of the rate of loss of activity in the presence of chloramphenicol was 68%, whilst the degree of protection of the rate of incorporation of label seen was 69% (see legend Fig.14). No data is available for the rate of incorporation of label into the protein in the presence of acetyl-S-CoA since it was impossible to remove all the unbound label on dialysis. Since this was not observed with enzyme and iodoacetamide alone, or with enzyme, iodoacetamide and chloramphenicol, it would appear to be due to the consequence of having enzyme and acetyl-S-CoA together. Indeed, when 10μmoles of $[^{14}C]$ iodoacetamide were mixed with acetyl-S-CoA and dialysed in a spectropore membrane (cut off 3500) 25% of the counts remained in the bag after exhaustive dialysis. The reason for this is not known.

It should be noted that acetyl-S-CoA protects the inhibition of CAT_{III} by iodoacetamide as well as chloramphenicol does. This is in contrast to the data obtained with the CAT_C and CAT_I variants. The degree of protection seen with these was considerably lower (5% and 35% respectively).(See also Chapter 6 for discussion on this point.)

4.4 Identification of the residues modified by $[^{14}C]$ Iodoacetamide.

2umole of CAT_{III} was incubated in standard buffer with 10mM $[^{14}C]$ iodoacetamide. Aliquots were taken at intervals over a 2hr. period and assayed for residual activity. After this time the reaction was terminated by addition of 2-mercaptoethanol. The reaction mix was dialysed against 10mM ammonium bicarbonate and then denatured with guanidine hydrochloride. The protein was reduced with excess dithiothreitol and then incubated with cold iodoacetamide to ensure total alkylation of all cysteine residues. The reaction was quenched with excess 2-mercaptoethanol, then dialysed

Fig.14.

Time course for the inhibition of CAT_{III} by Iodoacetamide in the presence of Chloramphenicol and Acetyl-S-CoA.

CAT_{III} (1mg/ml) was incubated at 37^0C in standard buffer with 10mM [^{14}C] iodoacetamide in the presence of either acetyl-S-CoA (1mM) or chloramphenicol (1mM). Samples were taken over a 100 minute period, diluted into standard buffer and the samples assayed by the standard method (Methods 2.4, 2.14). Controlo , enzyme + iodoacetamide ▲, enzyme + iodoacetamide + acetyl-S-CoA ● , enzyme + iodoacetamide + chloramphenicol, ■ .

	-Cm	+Cm	%Protection
Rate of loss of activity	9.7×10^{-3}	3.3×10^{-3}	68
Rate of incorporation	9.9×10^{-3}	3.1×10^{-3}	69

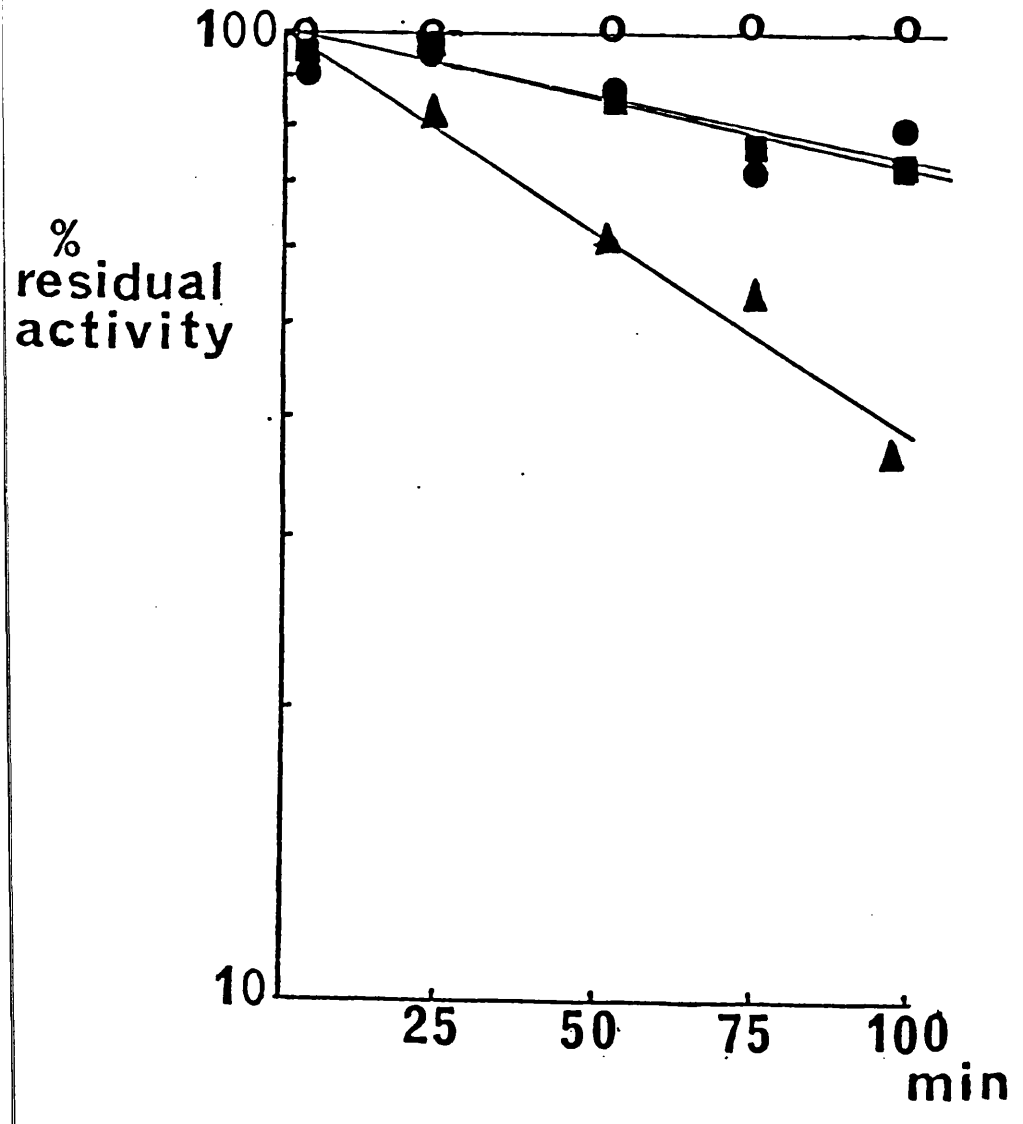
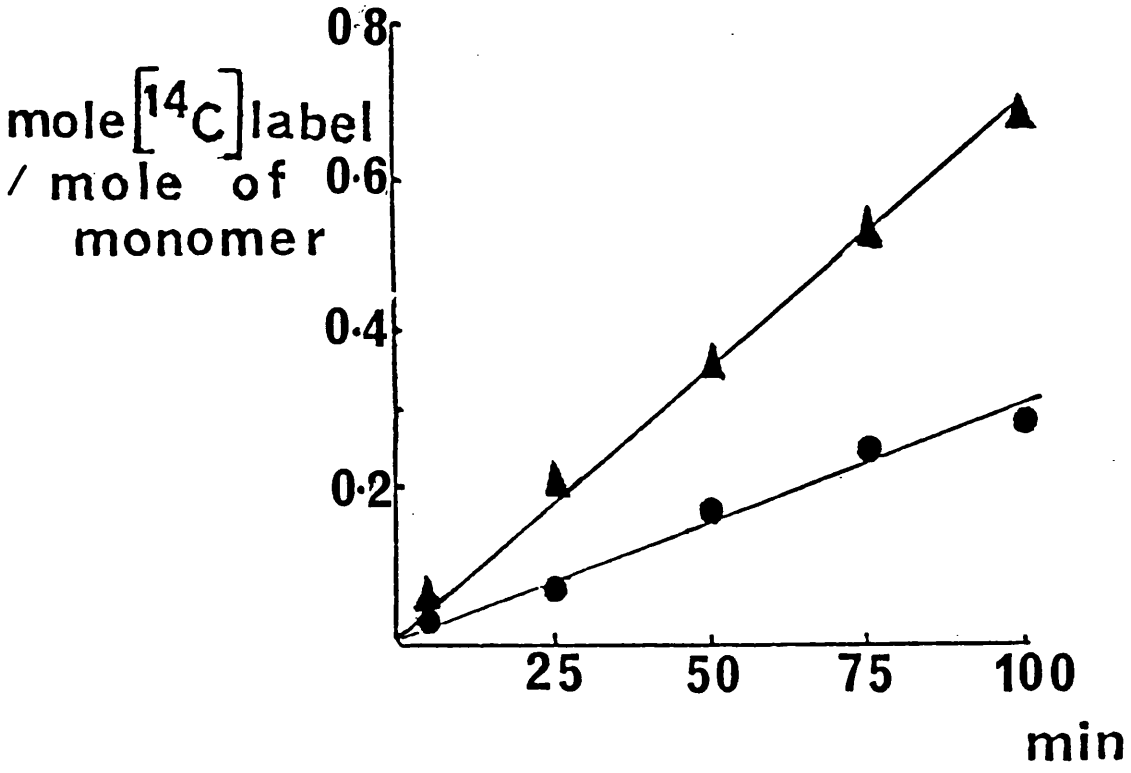

100
%
residual
activity
10
25
50
75
100
min

Fig.15.

Incorporation of [^{14}C] Iodoacetamide into CAT_{III}.

CAT_{III} (1 mg/ml) was incubated at 37°C in standard buffer with [^{14}C] iodoacetamide. Samples were taken for measurement of residual activity (Fig.14). After quenching with 2-mercaptoethanol to stop the reaction and exhaustive dialysis the samples were counted (Methods 2.14). CAT_{III} + iodoacetamide (10mM) ▲ , enzyme + iodoacetamide (10mM) + chloramphenicol (1mM) ● .


0·8
0·6
0·4
0·2
mole [14C] label / mole of monomer
25
50
75
100
min

exhaustively against ammonium bicarbonate and finally lyophilized (Methods 2.15a,b).

Prior to a large scale digestion a trial digest of 10nmoles of the total protein was performed with 1% w/w elastase (Methods 2.15c) and the peptides generated were separated by electrophoresis at pH 6.5 in one direction on a thin layer silica plate followed by chromatography in BAWP in a second direction (Methods 2.15c).

Inspection of the autoradiogram revealed six radioactive spots (data not shown). It was decided, therefore, that elastase would be a suitable enzyme with which to carry out the large scale digest. The protein was digested with 1% w/w elastase for 4hr. at 37°C in 50mM ammonium bicarbonate buffer pH 8.0. Digestion was terminated by freezing the sample, followed by lyophilization. Radioactive peptides were separated initially on a Dowex 50 ion exchange column using a pH and salt gradient at 50°C (Methods 2.15d). Peptides were identified by monitoring the 280nm absorbance and radioactive peptides by counting a 10µl sample of the 1ml sample collected. Pooled radioactive peptides were then separated by high voltage paper electrophoresis (Methods 2.15e). Radioactive peptides were located by autoradiography. These were eluted with 5% ammonia solution and 5% taken for counting and amino acid analysis.

The large scale digest revealed a large number of radioactive peptides (14 in total). The total amount of radioactivity incorporated was 0.98 moles $[^{14}C]$ label per mole of enzyme monomer. Three peptides which were adjudged pure on amino acid analysis were sequenced by the dansyl-Edman method (Methods 2.15g).

Carboxymethyl derivatives of histidine were prepared by reacting a 5 fold molar excess of iodoacetic acid with histidine at pH 8.0 for 8hr. The products were purified by high voltage paper electrophoresis at pH 6.5 for 30 minutes. After location of the products by light staining with fluoram, they were eluted with 5% ammonia solution. 3 peaks were found

on amino acid analysis and were identified according to the position they ran as described by Crestfield et al (81). 1,3-dicarboxymethylhistidine elutes prior to aspartic acid, 1-carboxymethylhistidine after proline and 3-carboxymethylhistidine after alanine. A ninhydrin colour factor equivalent to that of glycine was assumed for the analysis (J.E. Fitton, Ph.D Thesis, Univ. of Leicester 1977).

Peptide DX456

Analysis: CMCys (0.7), Pro (0.9), Gly (1.0), Leu (1.0), His (1.1), Arg (1.5).

Yield: 81 nmole

Radioactive yield: 27 nmole

Incorporation: 0.33

Sequence: Arg-Leu-Pro-CmCys-Gly

Peptide DX511

Analysis: CmCys (0.7), Glu (0.7), Pro (0.8), Gly (0.8), Leu (1.0), Phe (1.1), His (1.0), Arg (1.7).

Yield: 232 nmole

Radioactive yield: 90 nmole

Incorporation: 0.38

Sequence: Arg-Leu-Pro-CmCys-Gly-Phe

Peptide DX512

Analysis: Ala (0.7), CmCys (0.85), Val (1.1), His (0.9).

Yield: 64 nmole

Radioactive yield: 47 nmole

Incorporation: 0.73

Sequence: His-3CmHis-Ala

Peptides DX456 and DX511 would appear to have resulted from incomplete digestion of the protein. Of the remaining 11 peptides some were adjudged impure on analysis and most were found to have low levels of incorporation. These were not investigated further. Some peptides could be further

products of the three peptides sequenced.

4.5 Apparent pK_a of inactivation of CAT_{III} by Iodoacetamide.

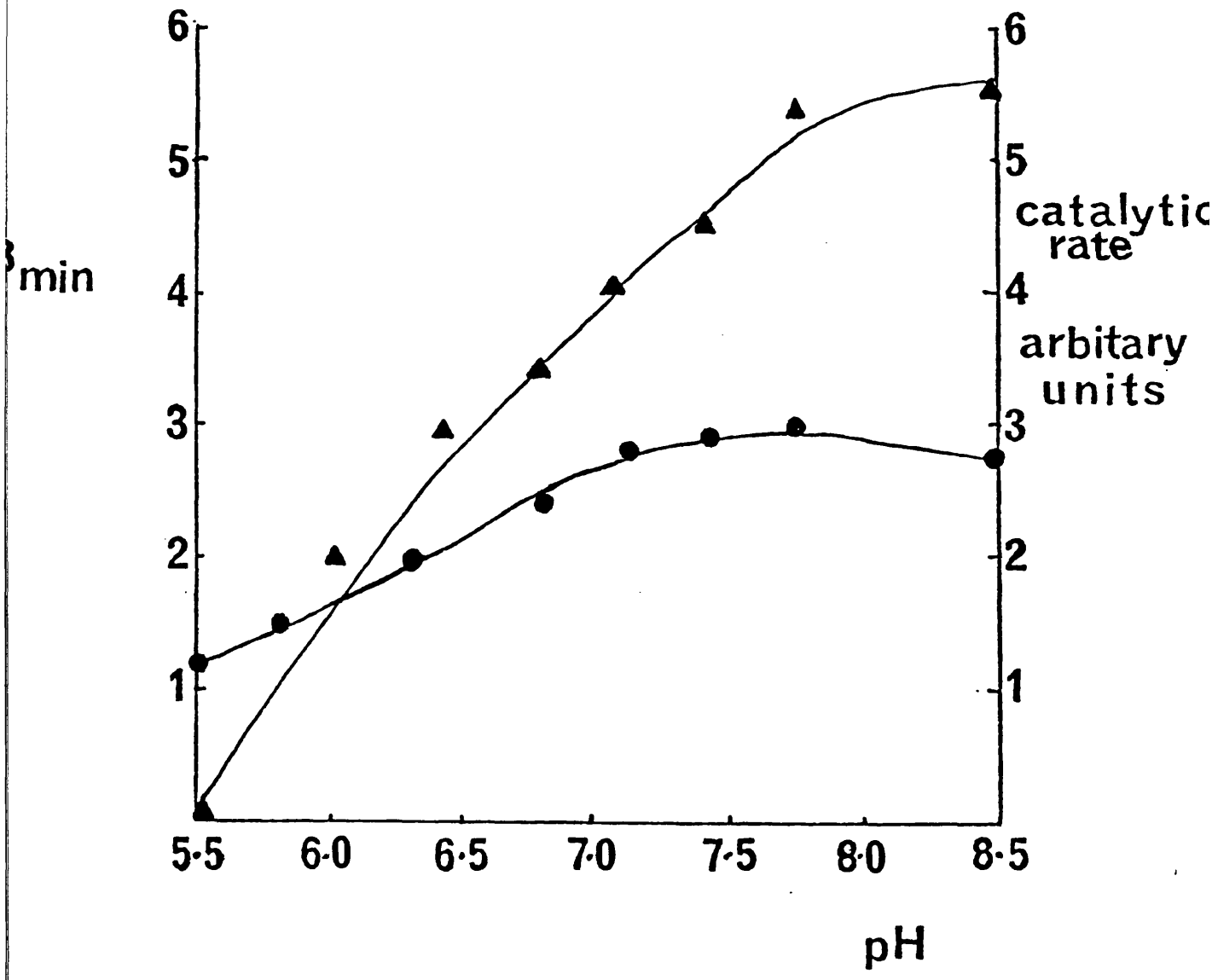
The pK_a of the inactivation of CAT_{III} by iodoacetamide was determined as described in Methods 2.16, using a range of Tris-Maleate buffers. Fig.16 shows the data obtained. For a comparison the catalytic rate versus pH curve (also determined in Tris-Maleate buffers) is also shown. Connolly and Trayer (82) have pointed out that it is important to test whether the enzyme is stable over the pH range tested when carrying out pK_a determination. Since in this case the catalytic values are not altered drastically over the pH range then there doesnot appear to be any large conformational changes that could result in modification of the catalytic mechanism and/or increased availability of an amino acid residue. The increased inactivation would appear, therefore, to be due to the ionization of a group to produce a reactive species.

A pK_a of 6.4 was determined for the CAT_{III} variant which contrasts with an approximate pK_a of 7.5 estimated for CAT_I under different conditions (58). A tentative assignment to a histidine residue could thus be made, although it is appreciated that anomalously high pK_a values or anomalously low pK_a values are frequently observed (83).

Fig.16.

PH dependence of the rate of inactivation of CAT_{III} by Iodoacetamide.

CAT_{III} (0.2mg/ml) was incubated in Tris-Maleate buffer at the appropriate pH. After taking an initial t =0 sample for assaying iodoacetamide (5mM) was added. Samples were taken at intervals and treated according to Methods 2.13, 2.4. Pseudo first order rate constants were calculated for each pH tested. To test the catalytic rate CAT_{III} was dialysed against the appropriate Tris-Maleate buffer and then assayed by the standard method (Methods 2.4).


min
6
5
4
3
2
1
catalytic rate
arbitary units
5·5
6·0
6·5
7·0
7·5
8·0
8·5
pH

Chapter Five

Chemical Modification Studies with 5,5 Dithiobis(2-nitrobenzoic acid) 5,5 Dithiobis(2-nitromethylbenzoate) and 5,5 Dithiobis(2-nitrotoluene).

5.1 Introduction.

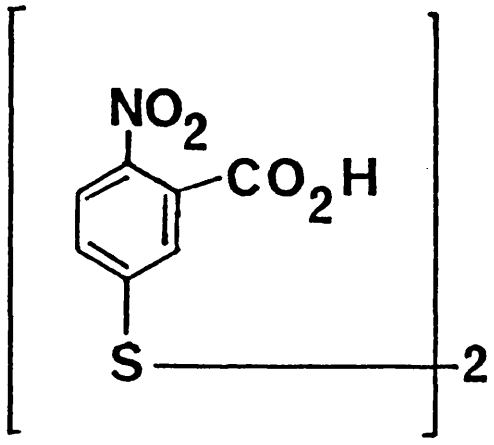
5,5 dithiobis(2-nitrobenzoic acid) (DTNB) has been widely used as a chemical modification reagent (eg. 72,84,85) and for quantitative determination of sulphydryl groups in proteins (86).

It has previously been noted (see Chapter 4) that whilst the CAT_{II} variant is inhibited both by DTNB and iodoacetic acid, CAT_I and CAT_C are not affected to any significant degree. Data is also presented in that chapter which shows that CAT_{III} is also not inhibited by iodoacetic acid. Such observations are compatible with the view that one or more negatively charged residues (an ionized aspartate or glutamate) may be at or near the active site in the CAT_I and CAT_C variants and, from the evidence presented in Chapter 4, in the CAT_{III} active site as well. A series of experiments were thus devised with Dr. D Smith and M. Webb (Dept. of Chemistry, Univ. of Leicester) to explore the hypothesis that charge repulsion was involved in the lack of inhibition by some reagents. Two analogues of DTNB were synthesized in which either the carboxyl groups were modified or replaced. The chemical structure of these compounds together with the parent compound DTNB are shown in Fig.17.

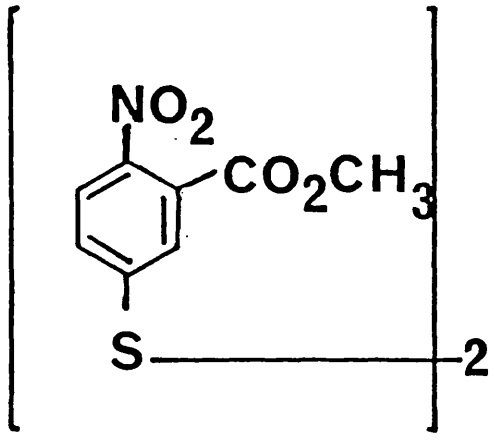
5.2 Determination of λmax and extinction coefficient of DTNMB and DTNT. Comparison with DTNB.

Stock solutions of DTNB, DTNMB and DTNT were made. DTNMB and DTNT were difficult to dissolve but all three compounds were soluble in varying degrees in dimethylformamide. Table 9 summarizes the λmax and extinction coefficient values for these compounds in the presence and absence of 0.5mM 2-mercaptoethanol (Methods 2.17). Fig.18 shows the shift in λmax and change in absorbance on addition of 0.5mM 2-mercaptoethanol to a 50µM solution of DTNMB. In a similar experiment with DTNT (Fig.19) a completely different trace was obtained. No sharp peaks were obtained as with DTNMB and DTNB (data not shown) solutions which made it very difficult to determine λ max and extinction coefficient values. Little change was seen on addition of

DTNB



DTNMB



DTNT

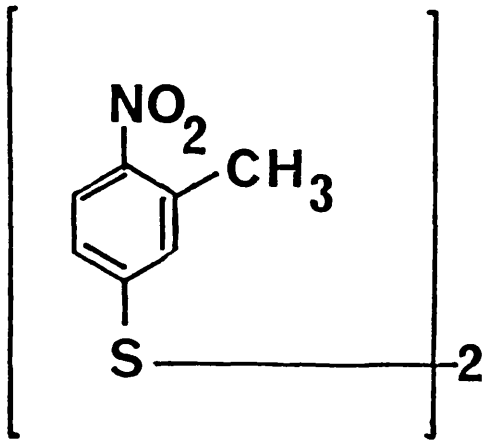


Fig.17.

Chemical structures of 5,5 dithiobis(2-nitrobenzoic acid) DTNB , 5,5 dithiobis(2-nitromethylbenzoate) DTNMB and 5,5 dithiobis(2-nitrotoluene) DTNT .

Table 9

Summary of λ max and extinction coefficients of DTNB, DTNMB and DTNT in the presence and absence of 2-Mercaptoethanol.

	- 2-Mercaptoethanol		+ 2-Mercaptoethanol	
Reagent	λ max (nm)	ε $M^{-1}cm^{-1}$	λ max (nm)	ε $M^{-1}cm^{-1}$
DTNB	324	16.6×10^3	412	13.1×10^3
DTNMB	340	16.2×10^3	420	16.5×10^3
DTNT	330	n.d	n.d	n.d

n.d = not determined

Fig.18. (page 62)

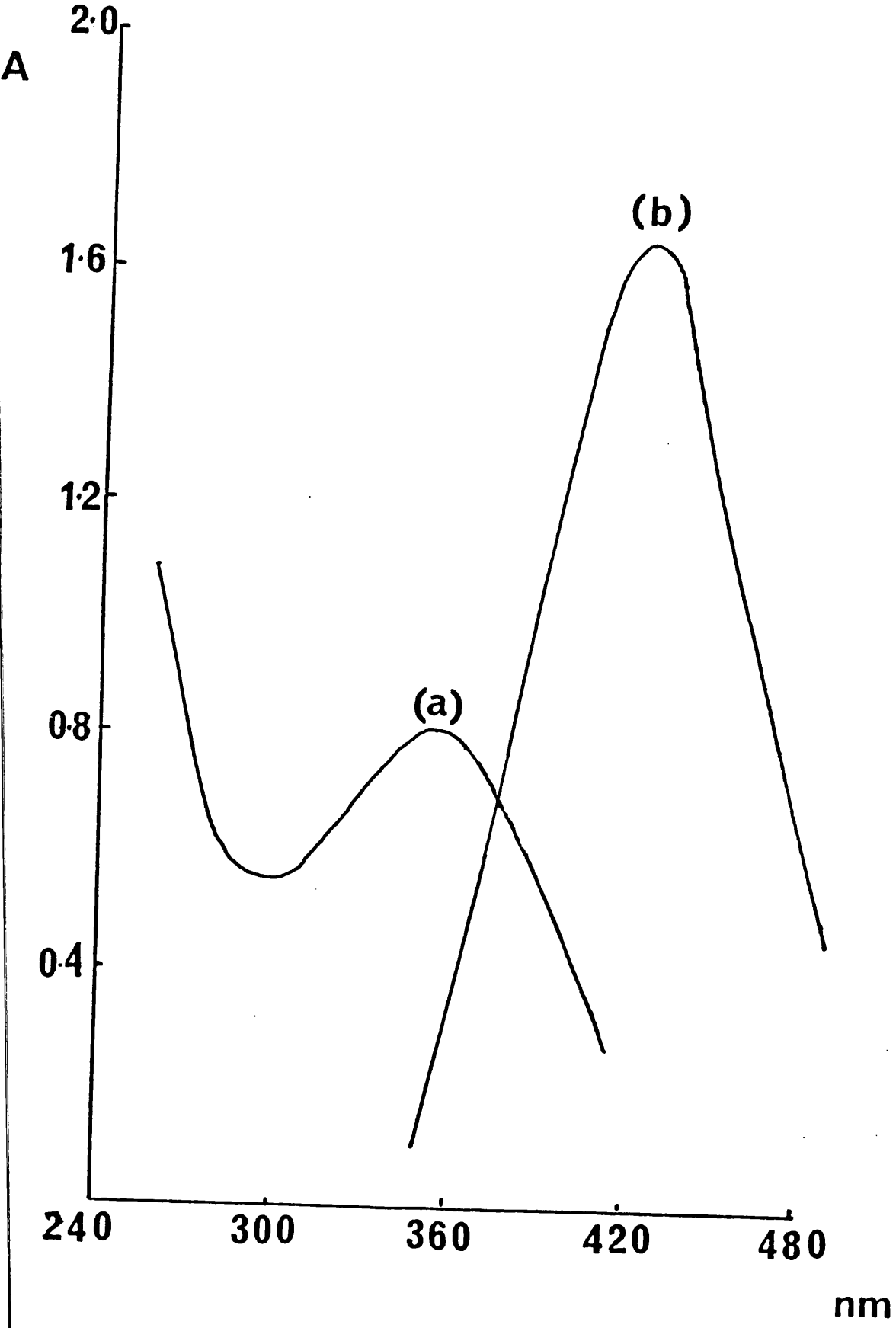
Effect of 2-Mercaptoethanol on the absorption spectrum of DTNMB.

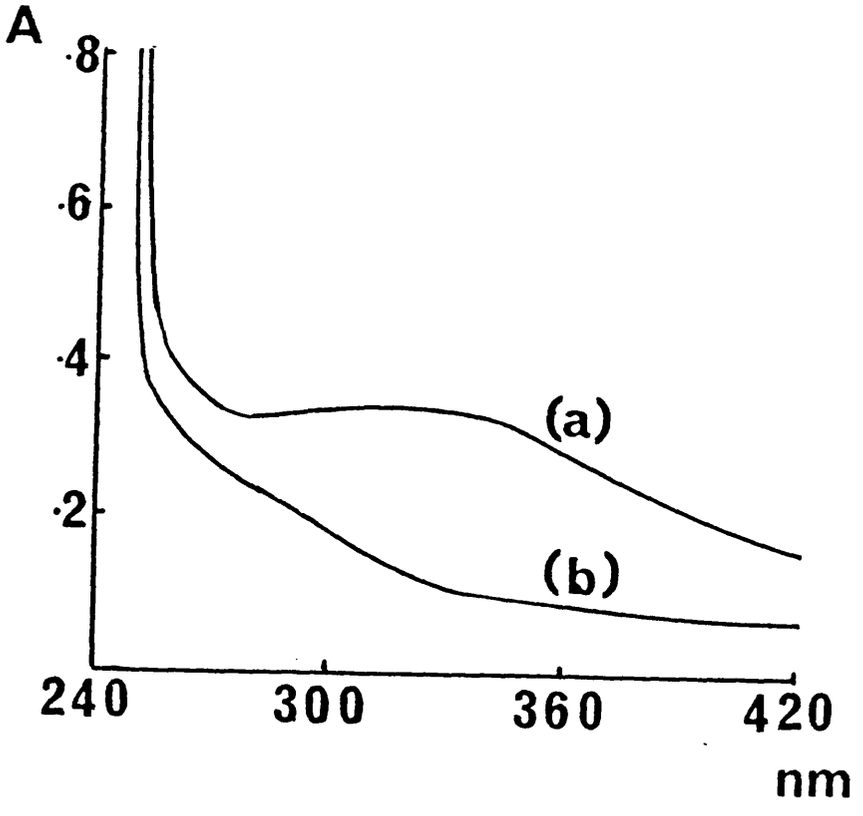
DTNMB (50μM) in standard buffer was used. The absorption of the solution was measured over a range of wavelengths (a), then 2-mercaptoethanol (0.5mM) added and the absorption spectrum remeasured (b).

Fig.19. (page 63)

Effect of 2-Mercaptoethanol on the absorption spectrum of DTNT.

DTNT (50μM) in standard buffer was used. The absorption spectrum of the solution was measured (a), then 2-mercaptoethanol (0.5mM) added and the spectrum remeasured.


A
2·0
1·6
1·2
0·8
0·4
(a)
(b)
240
300
360
420
480
nm


A
·8
·6
·4
·2
(a)
(b)
240
300
360
420
nm

2-mercaptoetanol. Higher concentrations of 2-mercaptoethanol were tried upto a 10,000 fold molar excess over sulphydryl groups but this had little effect on the absorption or λ max. A different buffering system was also tried (50mM glycine/NaOH pH 10.4) but again no significant change was seen. Addition of 2-mercaptoethanol to DTNB or DTNMB solutions produced the characteristic yellow colour of the anions as expected, but no yellow colour formation was observed with the DTNT solution.

5.3 Initial inactivation experiments with DTNB, DTNMB and DTNT.

CAT_{III} (0.2mg/ml) was incubated in standard buffer in the presence of 0.05mM DTNMB and DTNT or 0.5mM DTNB. Samples were taken at intervals, diluted into standard buffer and assayed for residual activity (Methods 2.18). There is a rapid loss of activity in the presence of DTNMB (90% in 4 minutes Fig.20) but at a concentration ten times higher DTNB causes virtually no loss of activity. DTNT also has no effect on the activity of the enzyme. From these initial experiments it would appear that removal of the negative charge from DTNB causes a dramatic increase in the rate of inhibition of the enzyme. It would have been nice, however, to have confirmed this result with the DTNT reagent.

5.4 Incorporation of DTNMB into the CAT_{III} variant.

In order to investigate the stoichiometry of incorporation into protein, the production of the free anion RS^- was followed spectrophotometrically using the previously established λ max (420nm) and the extinction coefficient of $16.5 \times 10^3 M^{-1} cm^{-1}$ (Table 9).

For the incorporation experiments a final concentration of DTNMB of 5-50μM was used. More concentrated solutions couldnot be used because of turbidity due to insolubility of the reagent. Fig.21 shows a plot of %residual activity and moles of RS^- incorporated per mole of enzyme monomer versus the number of moles of DTNMB added per mole of enzyme monomer. DTNMB (5-50μM) was added to CAT_{III} (0.5mg/ml) and the

Fig.20.

Time course for the inhibition of CAT_{III} with DTNB, DTNMB and DTNT.

Enzyme (0.2mg/ml) was incubated in standard buffer at 37°C with DTNB (0.5mM)● , DTNT (0.05mM)▲ , and DTNMB (0.05mM)■ . Samples were taken at intervals, diluted into standard buffer and assayed by the standard method (Methods 2.4). The control contained enzyme alone○ .

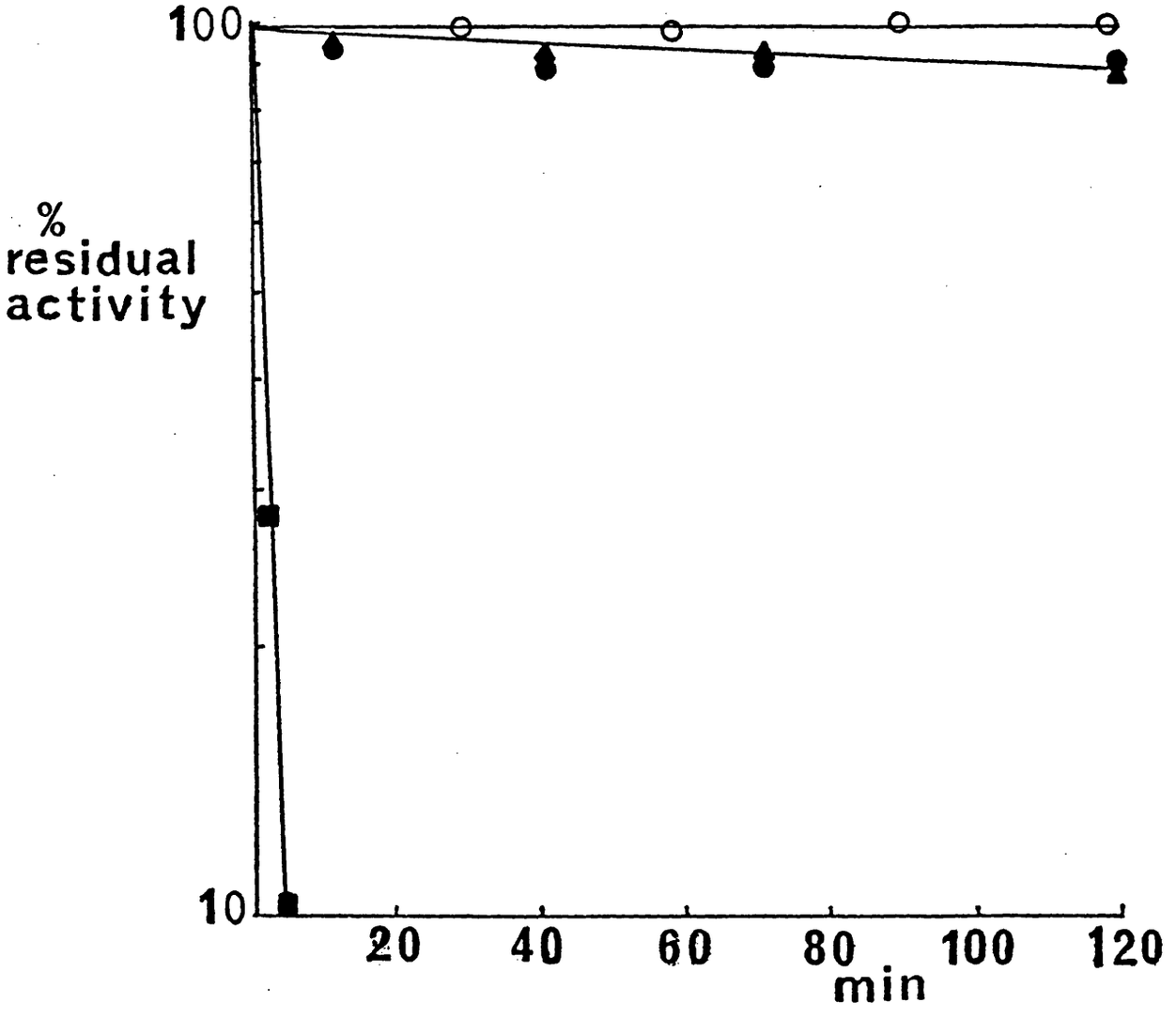
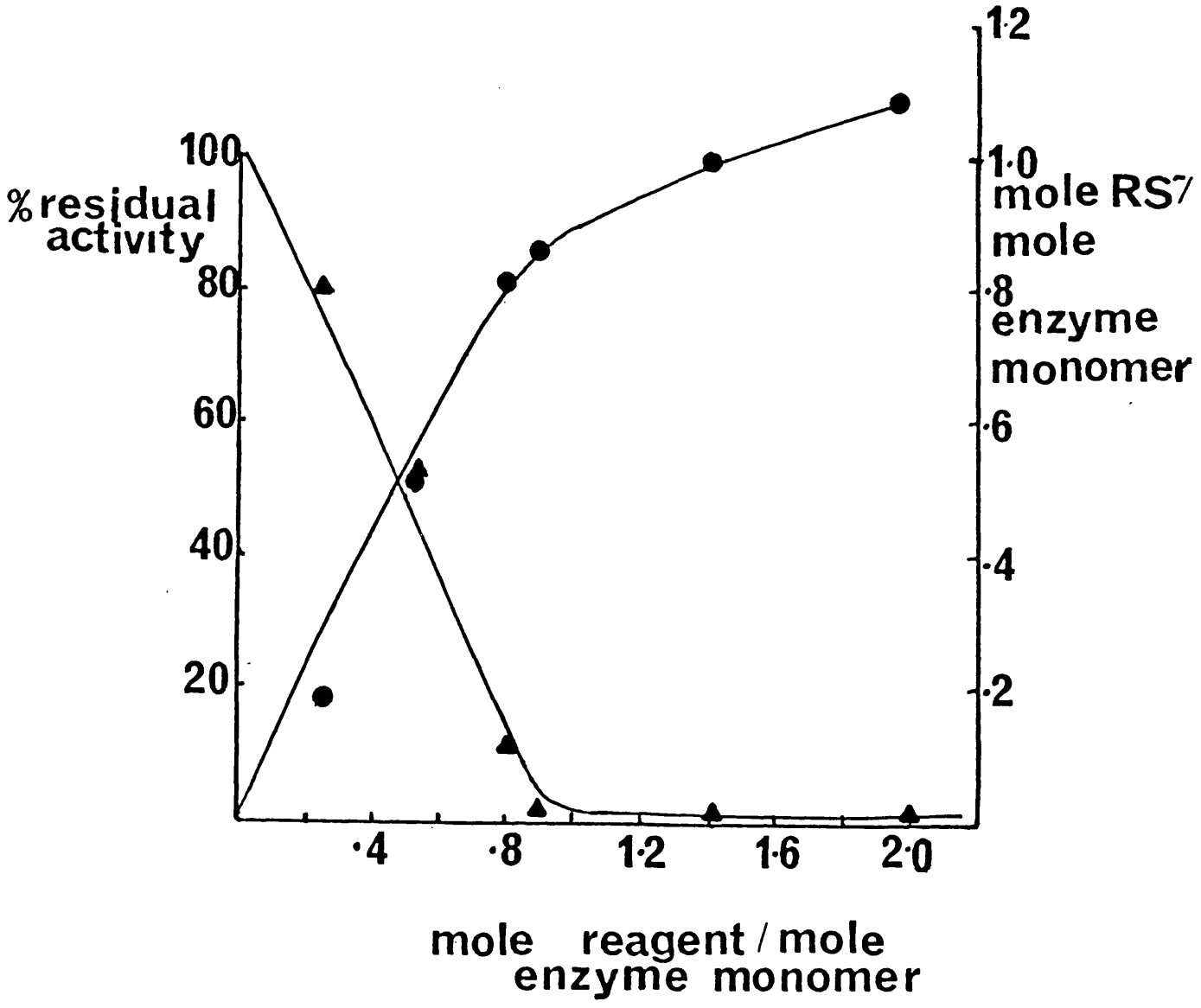

100
%
residual
activity
10
20
40
60
80
100
120
min

Fig.21.

Incorporation of Thionitromethylbenzoate (RS^-) into CAT_{III} as a function of activity and concentration of reagent (DTNMB) added.

CAT_{III} (0.5mg/ml) in standard buffer was used in each assay. DTNMB was added (5-50μM) and the absorbance at 420nm measured (Methods 2.19). Using $\varepsilon = 16.5 \times 10^3 M^{-1} cm^{-1}$ the number of moles of reagent incorporated per mole of enzyme monomer could be calculated. The residual activity of each sample was also measured by the standard method (Methods 2.4).


% residual activity
100
80
60
40
20
·4
·8
1·2
1·6
2·0
mole reagent / mole enzyme monomer
1·2
1·0
·8
·6
·4
·2
mole RS
mole
enzyme
monomer

production of free RS^- measured (Methods 2.19). The maximum absorbance was generally reached within two minutes, after which time an appropriate aliquot taken to assay for residual activity. Incorporation and inactivation matched the stoichiometry expected from the ratio of reagents to enzyme within experimental error.

5.5 Addition of 2-mercaptoethanol to CAT_{III} modified by DTNMB.

CAT_{III} variant was modified with DTNMB (0.81 moles/mole enzyme monomer) and excess reagent was removed by gel filtration using Sephadex G10 equilibrated in standard buffer (Methods 2.19). Spectra of the modified and unmodified enzyme (after desalting) are shown in Fig.22. An increase in absorbance between 320-360nm is observed in the modified enzyme, similar to that seen with DTNB-modified isocitrate dehydrogenase which has an increased absorbance between 310-325nm (72).

Attempts to reactivate the modified enzyme by addition of 2-mercaptoethanol (150mM) were only partly successful in that 89 percent removal of the modifying group (monitored spectrophotometrically by the absorbance of the anion RS^- released, over a 30 minute period) yielded a return to activity to only 36 percent of the pre-addition level (Table 10). Likewise, isocitrate dehydrogenase modified by DTNB could only be partially reactivated by treatment with 2-mercaptoethanol (72). Upon storage,however, a secondary change was found to take place releasing thionitrobenzoate and eliminating the ability to be reactivated. This secondary inactivation appears to result from disulphide interchange with unreacted sulphydryl groups in the partially modified enzyme. The possibility of disulphide exchange in the presence of DTNB was first postulated by Fernandez Diez et al.(87) and is illustrated in the following scheme:

$$\text{Protein}\begin{matrix}\text{SH}\\\text{SH}\end{matrix} + \text{RSSR} \longrightarrow \text{Protein}\begin{matrix}\text{SSR}\\\text{SH}\end{matrix} + RS^-$$

$$\text{Protein}\begin{matrix}\text{SSR}\\\text{SH}\end{matrix} \longrightarrow \text{Protein}\begin{matrix}\text{S}\\\text{S}\end{matrix} + RS^-$$

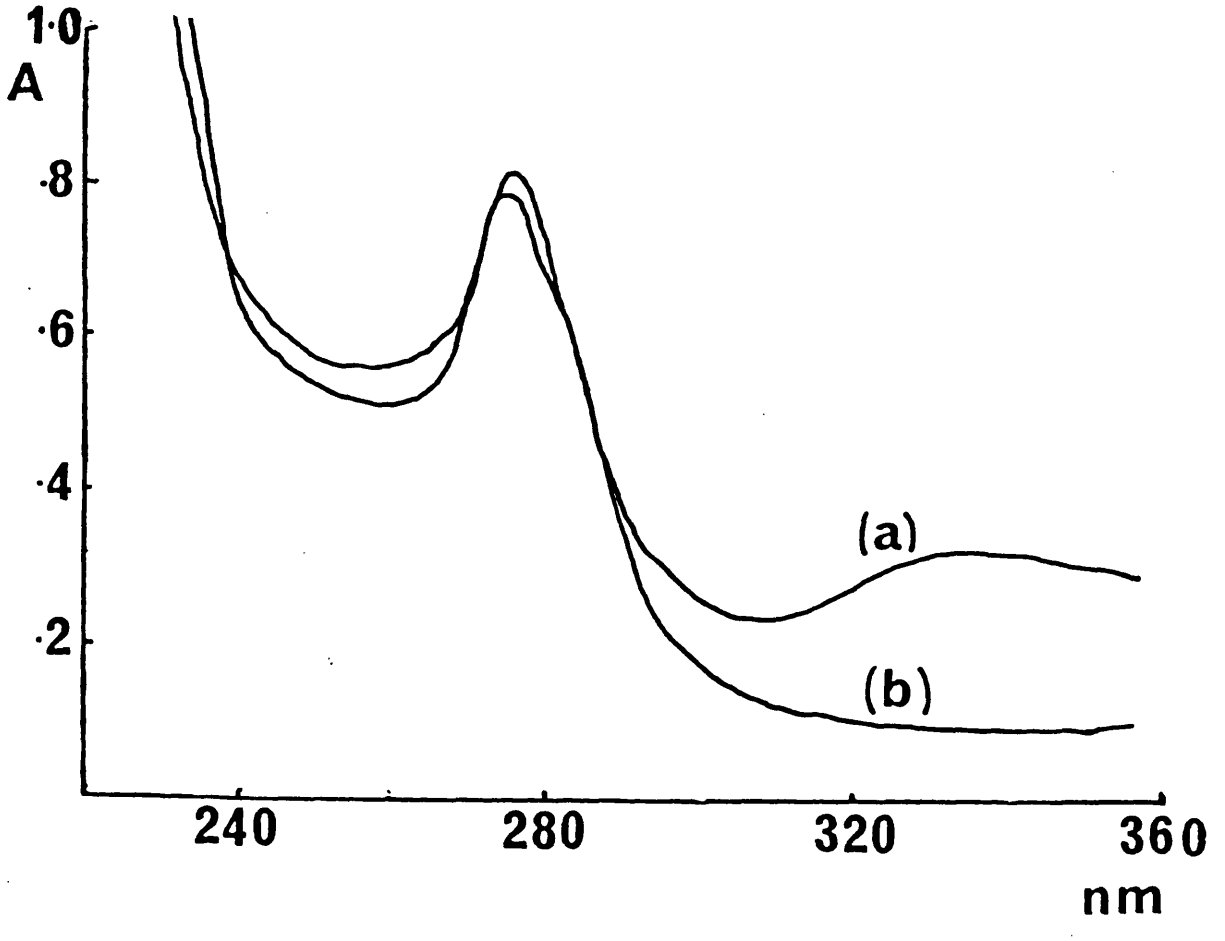


Fig.22.

Absorption spectrum of CAT_{III} modified with DTNMB.

The absorbance of a solution of CAT_{III} (0.5mg/ml) in standard buffer was measured before (b) and after modification with DTNMB (a), (Methods 2.19).

To test for disulphide exchange partially modified enzyme was monitored for secondary inactivation by following absorbance at 420nm as evidence for any increase in the RS^- species (Table 10). There was, however, no spectroscopic evidence for internal disulphide formation since after modification and removal of excess reagent there was no further decrease in activity or increase in 420nm absorbance. On addition of 2-mercaptoethanol, although 89 percent of the RS^- species was recovered only 36 percent of the activity was recovered initially. Almost full recovery was achieved, however, with respect to the control if the enzyme was left for a day (Table 10).

This observation suggests that the enzyme had undergone a conformational change when it was modified and was slowly regaining an active conformation upon storage.

5.6 Time dependence of inactivation and the effect of substrates.

Measurements of the rate of inactivation were carried out under the same conditions described for the spectrophotometric study of modification of CAT (equimolar reagent and enzyme monomer) but the concentrations of each were reduced to 1.25μM in a final volume of 1ml. The data (Fig.24,25) reveal non-linear kinectics of inactivation, which differ from the linear profiles observed with iodoacetamide (Chapter 4), a result most readily explained by the equimolar concentrations of reagent and enzyme monomers and the certain ty that the reaction must be bimolecular rather than pseudo first order. The latter situation usually is found when the inhibitor is in great excess or the process is observed at very early times before there has been a significant change in the concentration of inhibitor.

Initially it appeared as though both chloramphenicol and acetyl-S-CoA were able to protect the enzyme from modification. However, it was observed that the sample which contained acetyl-S-CoA turned yellow over the time course of the experiment. Since formation of the DTNMB

Table 10

Time course of absorbance and activity changes of DTNMB-modified CAT_{III}.

Time	Absorbance 420nm	Enzyme Activity relative to unmodified control
0	0	100%
2 min	0.26 (0.81mole/mole monomer)	12%
1 day (after desalting)	0.004	14%
Addition of 150mM 2-mercaptoethanol	0.24 (0.72mole/mole monomer)	36%
1 day after addition 2-mercaptoethanol		82%

Fig.23.

Time course for the inhibition of CAT_{III} by DTNMB in the presence of Acetyl-S-CoA.

CAT_{III} (0.03mg/ml) was incubated in standard buffer at 37°C with DTNMB (1.25uM) ■, in the presence of 1.25uM ●, and 6.25uM ▲ acetyl-S-CoA. The controlo consisted of enzyme alone. Samples were taken at intervals and assayed for residual activity by the standard method (Methods 2.4).

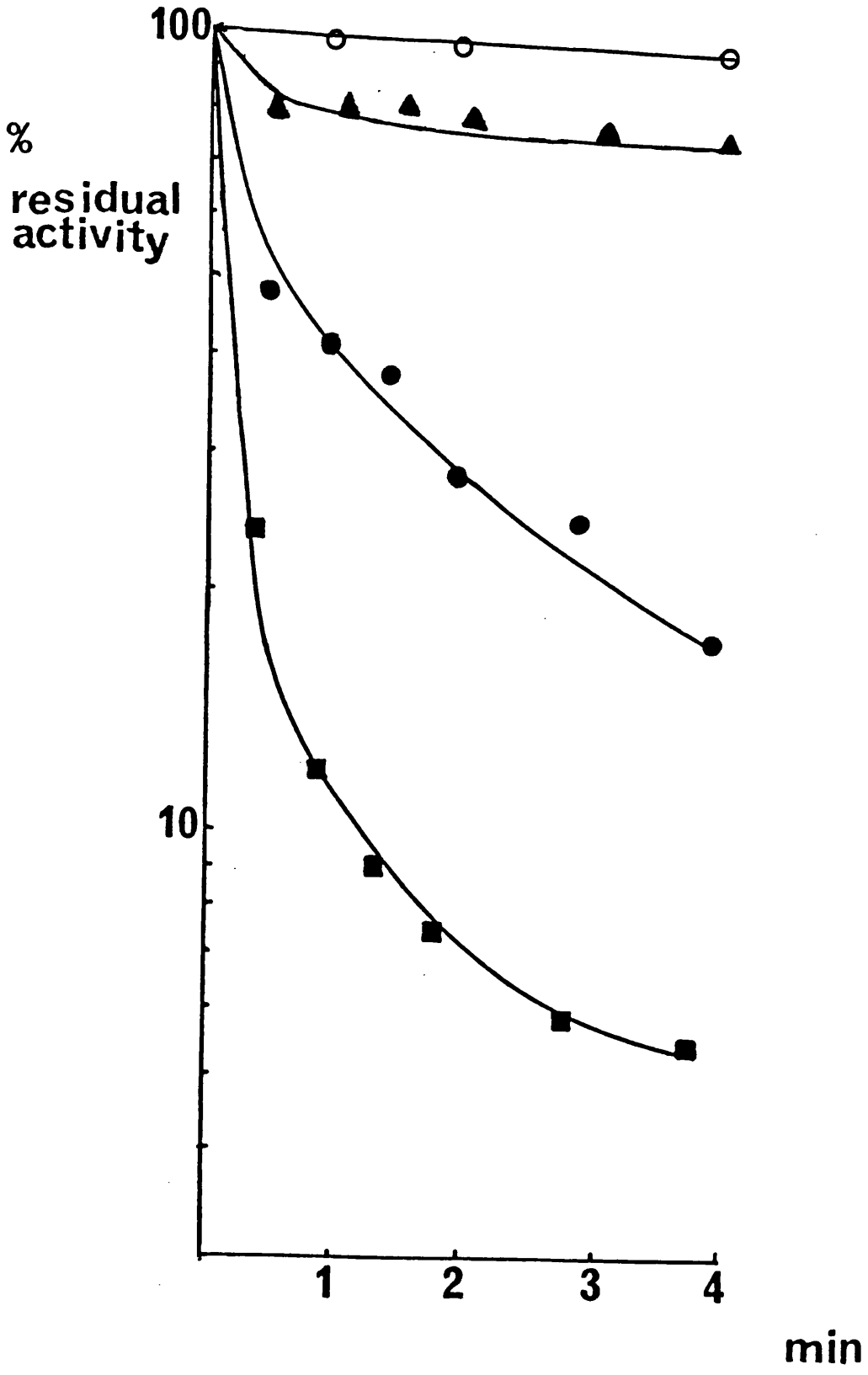
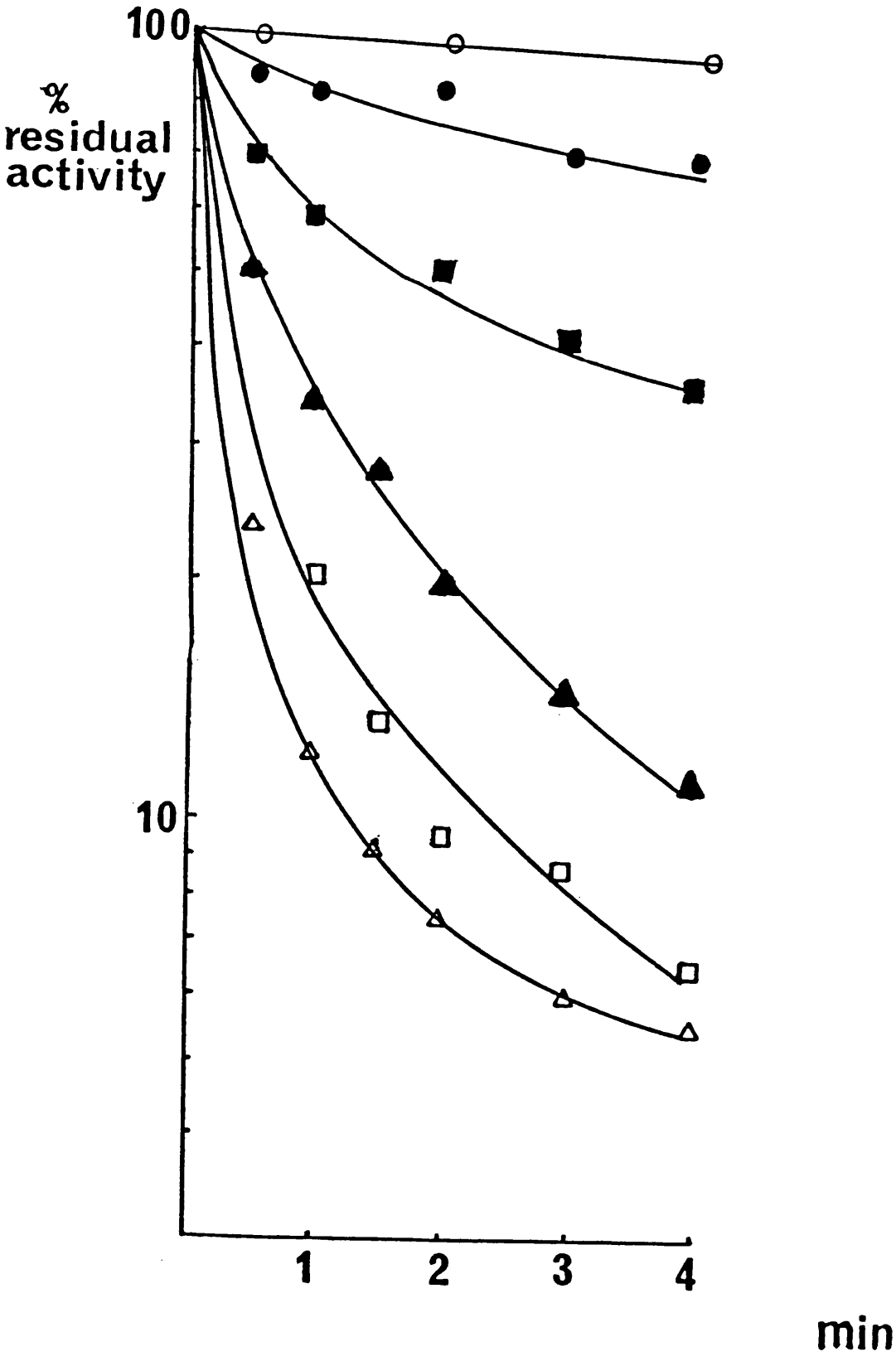

100
%
residual
activity
10
1
2
3
4
min

Fig.24.

Time course for the inhibition of CAT_{III} by DTNMB in the presence of Chloramphenicol.

CAT_{III} (0.03mg/ml) was incubated in standard buffer at 37°C with DTNMB (1.25uM) △, in the presence of 1.25uM □, 6.25uM ▲, 12.5uM ■ and 25uM ● chloramphenicol. The control consisted of enzyme alone. Samples were taken at intervals and assayed for residual activity by the standard method (Methods 2.4).


100
%
residual
activity
10
1
2
3
4
min

anion is normally associated with modification and loss of activity, the apparent and unexpected protection needed further investigation. DTNB was used in this case since it was more freely available, although the results were confirmed subsequently with the DTNMB analogue.

It has been reported previously (58) that if substrate levels of enzyme and acetyl-S-CoA are incubated together a slow but measurable hydrolysis of acetyl-S-CoA occurs. It has been postulated that water acts as the hydroxyl acceptor so acetate ion, reduced CoA is formed and the enzyme remains unchanged.

Table 11 shows the absorbance at 412nm or 420nm obtained after incubating enzyme and DTNB or DTNMB in the presence and absence of acetyl-S-CoA. In the presence of DTNB or DTNMB the free CoA formed in the hydrolysis reaction will react to form a mixed disulphide RSSCoA and the yellow coloured free anion RS^-. In fact, the presence of DTNB or DTNMB could enhance the hydrolysis of acetyl-S-CoA. From the data presented it would appear that acetyl-S-CoA is being hydrolysed, the free CoA reacting with the reagent, reducing its effective concentration and giving an apparent protection effect.

To overcome the problem observed with acetyl-S-CoA two non-reactive analogues were synthesized for use in protection experiments (see Chapter 6).

Table 11

Absorbance of a solution of CAT_{III} in the presence of acetyl-S-CoA and DTNB or DTNMB.

CAT_{III} (0.5mg/ml) was incubated in standard buffer with DTNB (20µM or 200µM) or DTNMB (20µM) in the presence of acetyl-S-CoA (20µM or 100µM). The reagents were mixed and the absorbance measured at either 412nm or 420nm after 5 minutes.

Enzyme (µM)	DTNB (µM)	Acetyl-S-CoA (µM)	Absorbance (412nm)
20	20		0.028
20	200		0.042
20	20	20	0.229
20	100	100	1.37
Enzyme (µM)	DTNMB (µM)	Acetyl-S-CoA (µM)	Absorbance (420nm)
20	20	20	0.325

Chapter Six

Synthesis and use of the Acetyl-S-CoA analogues Acetonyl-S-CoA and Methyl-S-CoA.

6.1 Introduction.

Acetonyl-S-CoA has been reported as a potent competitive inhibitor, with respect to acetyl-S-CoA, of citrate synthase, phosphotransacetylase and carnitine acetyltransferase (38). This analogue didnot, however, activate E.coli phosphoenolpyruvate carboxylase or rat liver pyruvate carboxylase, two enzymes which require acetyl-S-CoA as an obligate activator. Acetonyl-S-CoA will not compete with acetyl-S-CoA for binding to these enzymes showing in these cases the apparent absolute requirement of these two enzymes for a thioester group on the activating ligand. Since acetonyl-S-CoA is a thioether rather than a thioester it cannot be turned over in the CAT system and hence was synthesized for use as a non-reactive analogue of acetyl-S-CoA.

6.2 Synthesis of Acetonyl-S-CoA.

Acetonyl-S-CoA was synthesized from CoA and 1-bromoacetone (88). CoA (12.7μmole) was dissolved in degassed ice-cold water and dithiothreitol (1.36μmole) added to ensure complete reduction of the CoA. The pH was adjusted with sodium hydroxide to pH 8.0. Monobromoacetone, dissolved in 95% ethanol prior to use, was used for the alkylation. Disappearance of free sulphydryl was measured by adding an aliquot of the reaction mix to DTNB solution in standard buffer and reading the absorbance at 412nm. Fig.25a shows the loss of 412nm absorbance on addition of monobromoacetone. Solvent and any unreacted monobromoacetone were removed by lyophilization. The sample was then taken up in 250μl of water and applied to a Sephadex column (1.0x25cm) which had been equilibrated with water. Fractions containing the highest concentrations of 260nm absorbing material were pooled. Fig.25b shows that the Sephadex column used completely separated the acetonyl-S-CoA from diacetonyl dithiothreitol, which is a byproduct of the reaction.

Analysis of the product on cellulose thin layer plates developed in butanol:pyridine:acetic acid:water solvent (50:33:1:40 v/v) showed

Fig.25a.

Decrease in absorbance on addition of Monobromoacetone to CoA.

The sample consisted of CoA (12.7μmole) and DTT (1.36μmole) in 1ml of water. Monobromoacetone was added and then 5μl samples removed to test for free CoA using a DTNB solution, by monitoring the absorbance of the solution at 412nm.

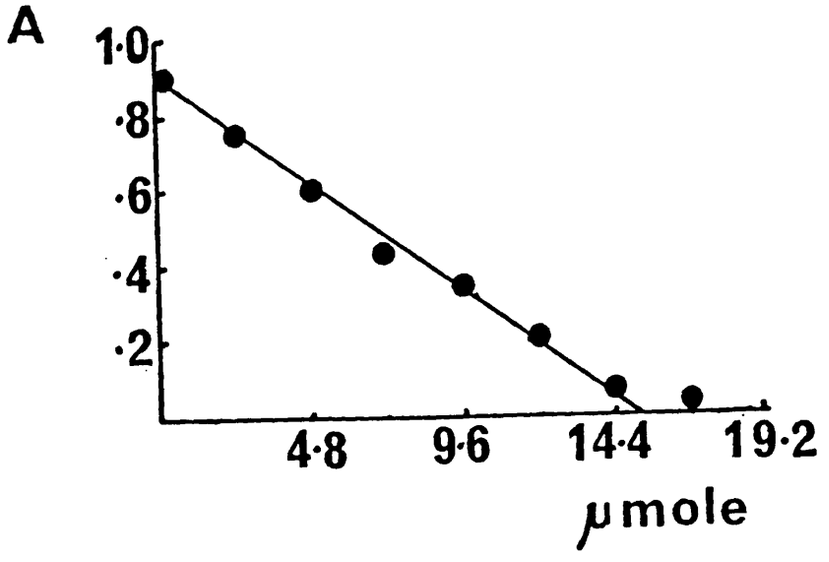
Fig.25b.

Absorbance at 260nm and change in absorbance at 576nm of fractions collected from a Sephadex G10 column.

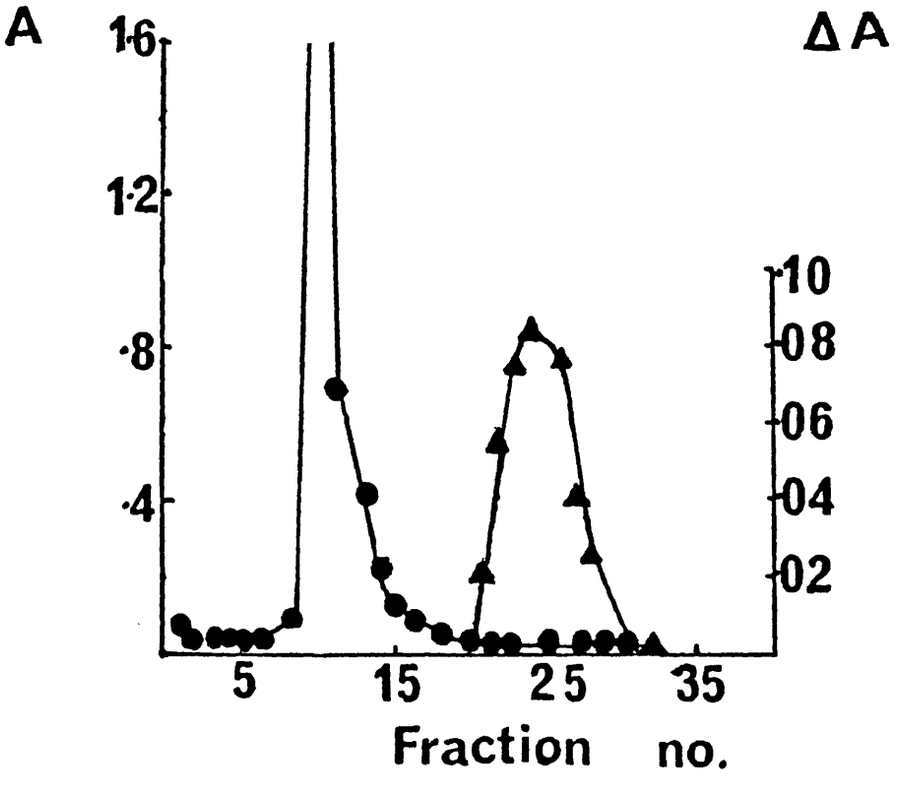
Acetonyl-S-CoA was prepared as described in the text. The lyophilized sample was dissolved in 250μl of water and applied to a Sephadex G10 column (1 x 25cm) which had been equilibrated with water. 1ml fractions were collected and the acetonyl-S-CoA located by 260nm absorbance (A ●).

Dithiothreitol was reacted with a two fold molar excess of monobromoacetone and after lyophilization this mixture was applied to the column. 1ml fractions were collected and the alkylated dithiothreitol was located by following its reaction with $KMnO_4$ at 576nm (Δ A ▲), (88).

a


A
1·0
·8
·6
·4
·2
4·8
9·6
14·4
19·2
μmole

b


A
ΔA
1·6
1·2
·8
·4
10
08
06
04
02
5
15
25
35
Fraction no.

a single spot R.F 0.18 (acetyl-S-CoA 0.16, CoA 0.40). These values donot agree with those quoted in reference 88 but were consistently found.

Acetonyl-S-CoA was also applied to a polyethyleneimine cellulose plate and developed in 0.3M LiCl. Acetonyl-S-CoA and CoA both had mobilities of 0.02 showing that acetonyl-S-CoA hadnot lost any of its phosphate groups during the synthesis.

The U.V spectra of CoA and acetonyl-S-CoA were identical. The concentration of acetonyl-S-CoA was calculated using an extinction coefficient $\epsilon = 15.4 \times 10^3 M^{-1} cm^{-1}$ at 260nm. An average yield of 90% was obtained. Free CoA was measured using DTNB. An average of 0.3% free CoA was found in the acetonyl-S-CoA preparations.

6.3 Determination of K_i of Acetonyl-S-CoA for binding to CAT_{III}.

Addition of acetonyl-S-CoA to the enzyme under standard assay conditions yielded no change in absorbance at 412nm as was expected for a non-reactive thioether.

A K_i for the binding of acetonyl-S-CoA to CAT_{III} with respect to acetyl-S-CoA was determined as described in Methods 2.7. In all experiments the chloramphenicol concentration was set at 200μM and a range of acetyl-S-CoA concentrations was used in the presence and absence of acetonyl-S-CoA. Acetonyl-S-CoA was found to be a competitive inhibitor (Fig.26) with respect to acetyl-S-CoA and have a K_i of 41 ± 7μM This low binding constant thus makes acetonyl-S-CoA a suitable non-reactive analogue for use in protection experiments.

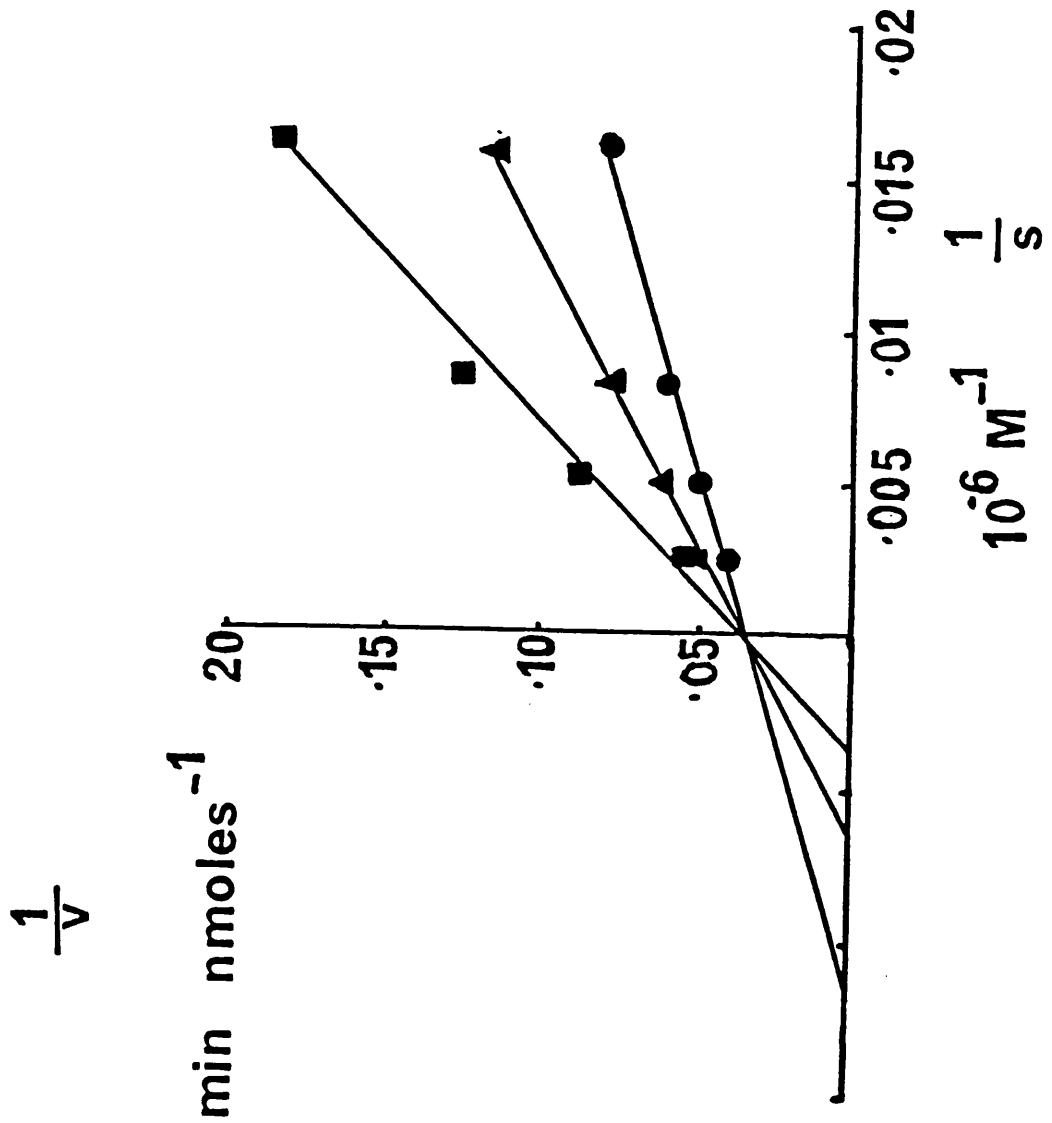
6.4 Synthesis of Methyl-S-CoA.

Methyl-S-CoA was made in a similar manner to acetonyl-S-CoA using iodomethane as the alkylating agent. A larger molar excess of iodomethane was required for the full alkylation of free sulphydryl groups. The average yield of methyl-S-CoA was 74% and the average free CoA 1%. A K_i for the binding of methyl-S-CoA to the enzyme was determined as dsecribed in 6.3 and Methods 2.7. A K_i of 305 ± 30μM was obtained and

Fig.26.

Determination of K_i for Acetonyl-S-CoA binding to CAT_{III}.

Assays were performed as described in Methods 2.7. The acetyl-S-CoA concentration was varied from 55μM to 400μM, whilst the chloramphenicol concentration was set at 200μM. 6×10^{-4} nmoles of enzyme were used in each assay. The assays were performed in the absence ● and presence of 40μM ▲, and 80μM ■ acetonyl-S-CoA.


1/V
min nmoles^{-1}
20
.15
.10
.05
.005
.01
.015
.02
10^{-6} M^{-1}
1/S

again the methyl-S-CoA analogue was found to be a competitive inhibitor with respect to acetyl-S-CoA.

6.5 Inhibition of CAT_{III} with DTNMB in the presence of Acetonyl-S-CoA.

In order to test whether the protection observed against inactivation by DTNMB with acetyl-S-CoA was due to the interference of the substrate with the inhibitor giving an apparent protection effect, the experiment was repeated using acetonyl-S-CoA in the place of acetyl-S-CoA. CAT_{III} (1.25μM) was incubated with DTNMB (1.25μM) in the presence and absence of acetonyl-S-CoA. No protection of the inhibition was seen when the acetonyl-S-CoA was used at concentration of 1.25 and 125μM (data not shown). This result would appear to confirm that the protection observed with acetyl-S-CoA (see Chapter 5) is artifactual and true protection is afforded by chloramphenicol only.

6.6 Inhibition of CAT_{III} with Iodoacetamide in the presence of Acetyl-S-CoA, Acetonyl-S-CoA and Methyl-S-CoA.

No protection against inactivation by iodoacetamide was seen with and *S. aureus* Type C enzyme (38), whereas 35% protection was observed for the Type I enzyme (58). However, in the case of the CAT_{III} variant 65-75% protection was seen routinely. Since it had been observed that acetyl-S-CoA could give an apparent protection effect, it seemed sensible to repeat the experiments with the inhibitor iodoacetamide but using the non productive analogues, acetonyl-S-CoA and methyl-S-CoA, as the protective agents.

CAT_{III} (2.5mg/ml) was incubated with iodoacetamide (10mM) in the presence and absence of acetyl-S-CoA, acetonyl-S-CoA and methyl-S-CoA (1mM). It has previously been reported (58) that incubation of CAT_I alone (0.15mg/ml) with acetyl-S-CoA (0.5mM) gave a measurable rate of inactivation ($k=11x10^{-3}min^{-1}$), therefore CAT_{III} was also incubated with the substrate and analogues in the absence of iodoacetamide. In all cases an appropriate aliquot was taken over a 2hr. period, diluted into standard buffer and assayed by the standard method.

Table 12 shows the k values determined from this data which indicate that in the absence of iodoacetamide little inhibition was observed. All the k values obtained were considerably lower than those of the CAT_I variant (Table 12). The protective effect of acetonyl-S-CoA against the rate of loss of activity was almost identical with that of acetyl-S-CoA and would seem to suggest that the protection observed with the latter was real in this case. The degree of protection seen with the substrate analogue methyl-S-CoA,whilst significant,was lower than that of the other two substrates.This could reflect the fact that the analogue binds less tightly to the enzyme.

Both analogues were used in further experiments, the results of which are discussed in the following chapters.

Table 12

Protective effect of Acetyl-S-CoA, Acetonyl-S-CoA and Methyl-S-CoA on the inhibition of CAT_{III} by Iodoacetamide.

Enzyme (2.5mg/ml) was incubated in standard buffer at 37°C with acetyl-S-CoA (1mM) or acetonyl-S-CoA (1mM) or methyl-S-CoA (1mM) in the presence and absence of iodoacetamide (10mM). Samples were taken over a 2hr. period and the residual activity measured in the standard assay (Methods 2.4). K values were calculated from the expression $2.3 \log \frac{E}{E_o} = -kt$ (72).

CAT_{III} +	k min^{-1} -Iodoacetamide	k min^{-1} +Iodoacetamide
-	1×10^{-3}	22×10^{-3}
Acetyl-S-CoA	0.5×10^{-3}	5.1×10^{-3} (77%)
Acetonyl-S-CoA	2.0×10^{-3}	6.0×10^{-3} (73%)
Methyl-S-CoA	0.07×10^{-3}	11.1×10^{-3} (50%)

The degree of prctection observed is shown in brackets.

Chapter Seven

Chemical Modification Studies with Diethylpyrocarbonate

7.1 Introduction.

Diethylpyrocarbonate (DEP) has been widely used as a reagent for the chemical modification of proteins. Rapid inactivation has been observed for, among others, enzymes including ribonuclease and trypsin (89), glyceraldehyde phosphate dehydrogenase (90), lactate dehydrogenase (91) and peptidyl transferase (92).

The attraction of DEP has been a direct consequence of the chemistry in that only the carbethoxylation of imidazole groups (Fig.27) produces a spectral change at 230-240nm (93). This can be used to quantitate the extent of reaction using a molar extinction coefficient of $\varepsilon=3.0\times10^{3}$ M^{-1} cm^{-1} at 230nm (94) or $\varepsilon=3.2\times10^{3}$ $M^{-1}cm^{-1}$ at 240nm (79).

DEP can also react with the side chains of lysine, tryptophane, tyrosine and cysteine (94,95,96) as well as causing some protein polymerization (97). At pH 6.0, however, DEP has considerable specificity for imidazole groups (79,93). In several cases other amino acids have been positively demonstrated to remain unchanged under conditions of complete reaction of accessible histidines, for example cysteine, tyrosine and tryptophane side chains of glutamate dehydrogenase (98).

Deacylation of the carbethoxy groups and recovery of activity can often be acheived in a few minutes with hydroxylamine (94).

Since a histidine residue was modified in CAT_{III} by the inhibitor iodoacetamide (Chapter 4) it was decided to investigate further the modification of histidine residues using DEP as an inhibitor.

7.2 Initial experiments with CAT_{III} and DEP.

Initial experiments with DEP indicated that histidine residues of CAT_{III} were modified (Methods2.20). A difference spectrum of treated and untreated enzyme produced a λmax of 245nm (Fig.28b). Since no difference in the spectra was seen between 260-290nm it would appear that tyrosine and tryptophan residues havenot been altered (Fig.28a).

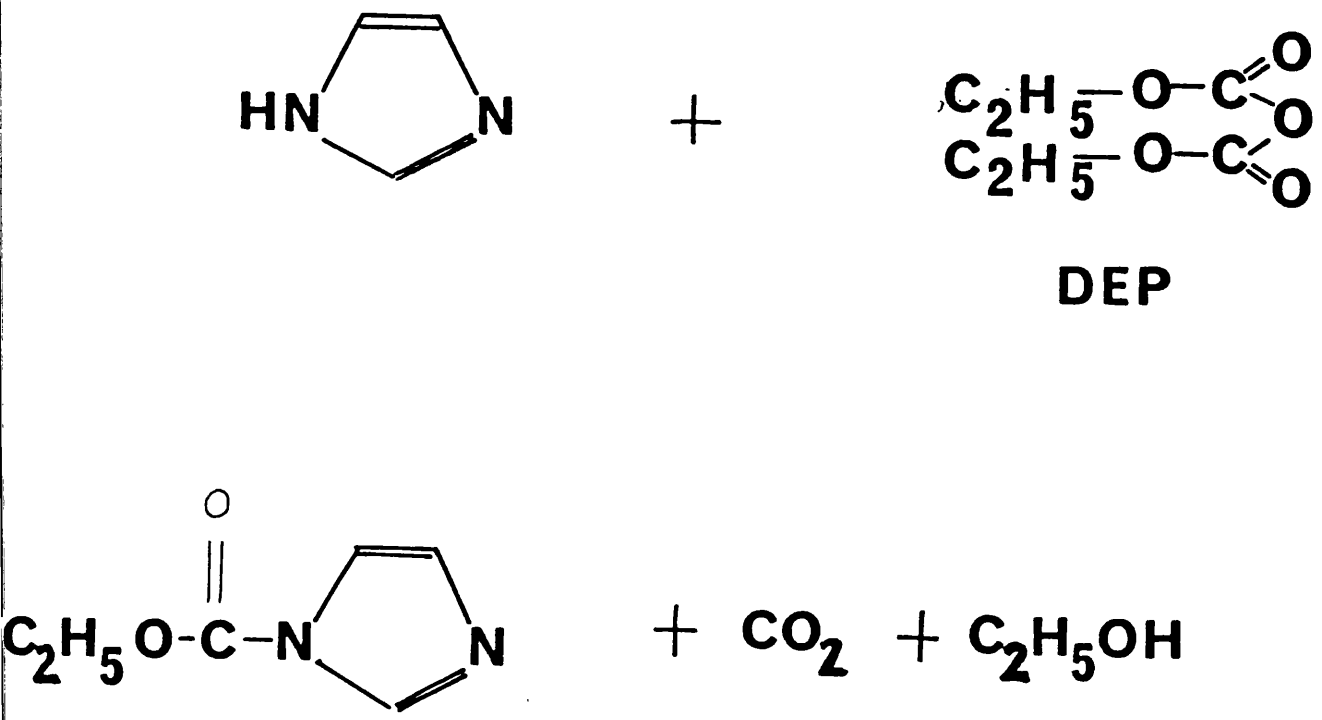


Fig.27.

Reaction of Imidazole with Diethylpyrocarbonate

Fig.28a.

Absorption spectrum of CAT_{III} modified with Diethylpyrocarbonate (DEP).

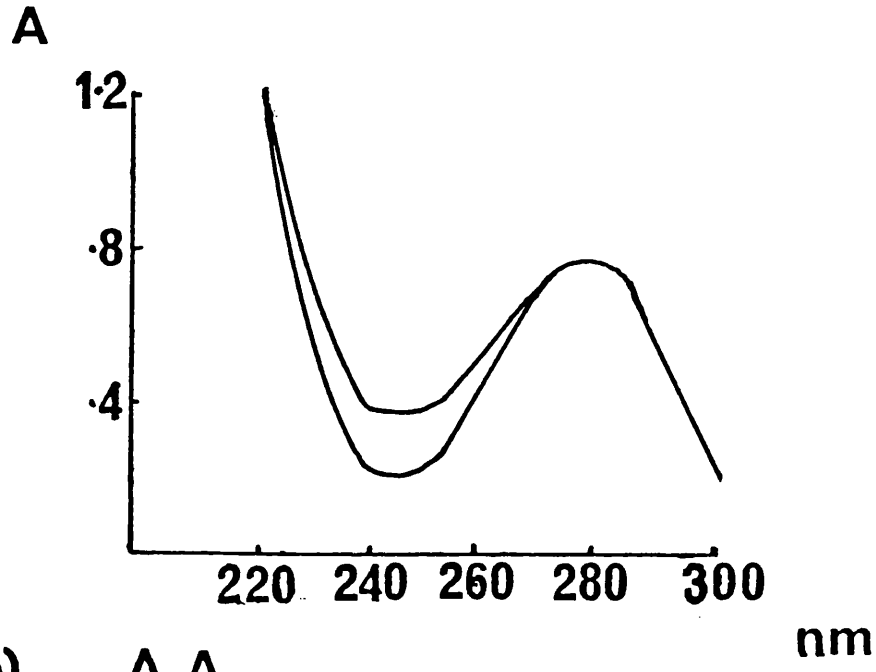
CAT_{III} (0.8mg/ml) was incubated at room temperature in sodium phosphate buffer (50mM, pH 6.0) in the presence and absence of DEP (0.25mM). The absorbance of each solution was measured against a buffer blank over a range of wavelengths.

Fig.28b.

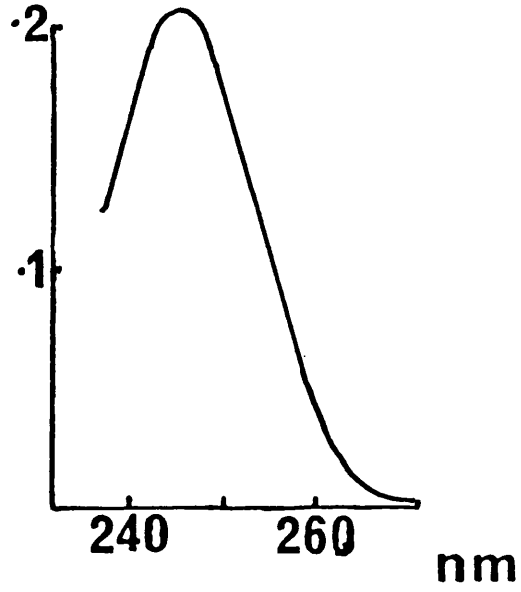
Difference spectrum of CAT_{III} modified with DEP.

CAT_{III} (0.8mg/ml) was incubated at room temperature in sodium phosphate buffer (50mM, pH 6.0) in the presence and absence of DEP. The absorbance of the treated enzyme was measured over a range of wavelengths against untreated enzyme.

(a)



(b)



7.3 Concentration dependence of the modification of CAT_{III} with DEP.

The modification of CAT_{III} was studied over a range of DEP concentrations, from 0.1mM to 5mM using the spectrophotometric assay (Methods 2.2). After the spectrophotometric change had reached a maximum, hydroxylamine was added (final concentration 0.05M) and the regeneration of histidine groups from carbethoxyhistidine measured. A plot of the number of moles of carbethoxyhistidine formed per mole of enzyme monomer (before and after addition of hydroxylamine) as a function of DEP concentration is shown in Fig.29.

It can be seen that at the highest concentration of inhibitor used 5-6 histidine residues have been modified. After addition of hydroxylamine incomplete reversal of the change in 245nm absorbance was observed, even at low concentrations of DEP. A single concentration of DEP (0.25mM) was taken for further studies.

7.4 Loss of activity of CAT_{III} with DEP.

CAT_{III} (0.8mg/ml) was incubated with DEP (0.25mM) at room temperature in 50mM sodium phosphate (pH 6.0). Samples were taken, diluted into standard buffer and assayed by the standard method (Methods 2.4). An identical sample of enzyme was monitored for change in 245nm absorbance.

After 15 minutes hydroxylamine was added and further measurements made. A biphasic loss of activity (Fig.30) was observed accompanied by a change in absorbance at 245nm. After 15 minutes 95% of the activity of the enzyme was abolished. Addition of hydroxylamine caused a decrease in the absorbance at 245nm of approximately 50% but only a 10% regain of activity. A total of 2 moles of carbethoxyhistidine were initially formed with one group being removed on addition of hydroxylamine.

Analysis of the loss of activity and rate of incorporation of carbethoxy groups into the protein on semilog plots reveals both 'fast' and 'slow' phases with pseudo first order rate constants of k_1 =2.7 min^{-1} and k_2 = 0.15 min^{-1} (loss of activity) and k_1 = 3.0 min^{-1} and k_2 = 0.16 min^{-1}

Fig.29.

Concentration dependence of the modification of CAT_{III} by DEP in the presence and absence of Hydroxylamine.

CAT_{III} (0.8mg/ml) was incubated at room temperature in sodium phosphate buffer (50mM, pH 6.0) with DEP (0.1mM-5mM), and the absorbance change at 245nm measured against untreated enzyme ● .After the reaction had gone to completion hydroxylamine (0.05M) was added and the absorbance remeasured, ▲ .The number of moles of carbethoxyhistidine groups present per mole of enzyme monomer was calculated in each case using $\varepsilon = 3.2 \times 10^3\ M^{-1}cm^{-1}$ (79).

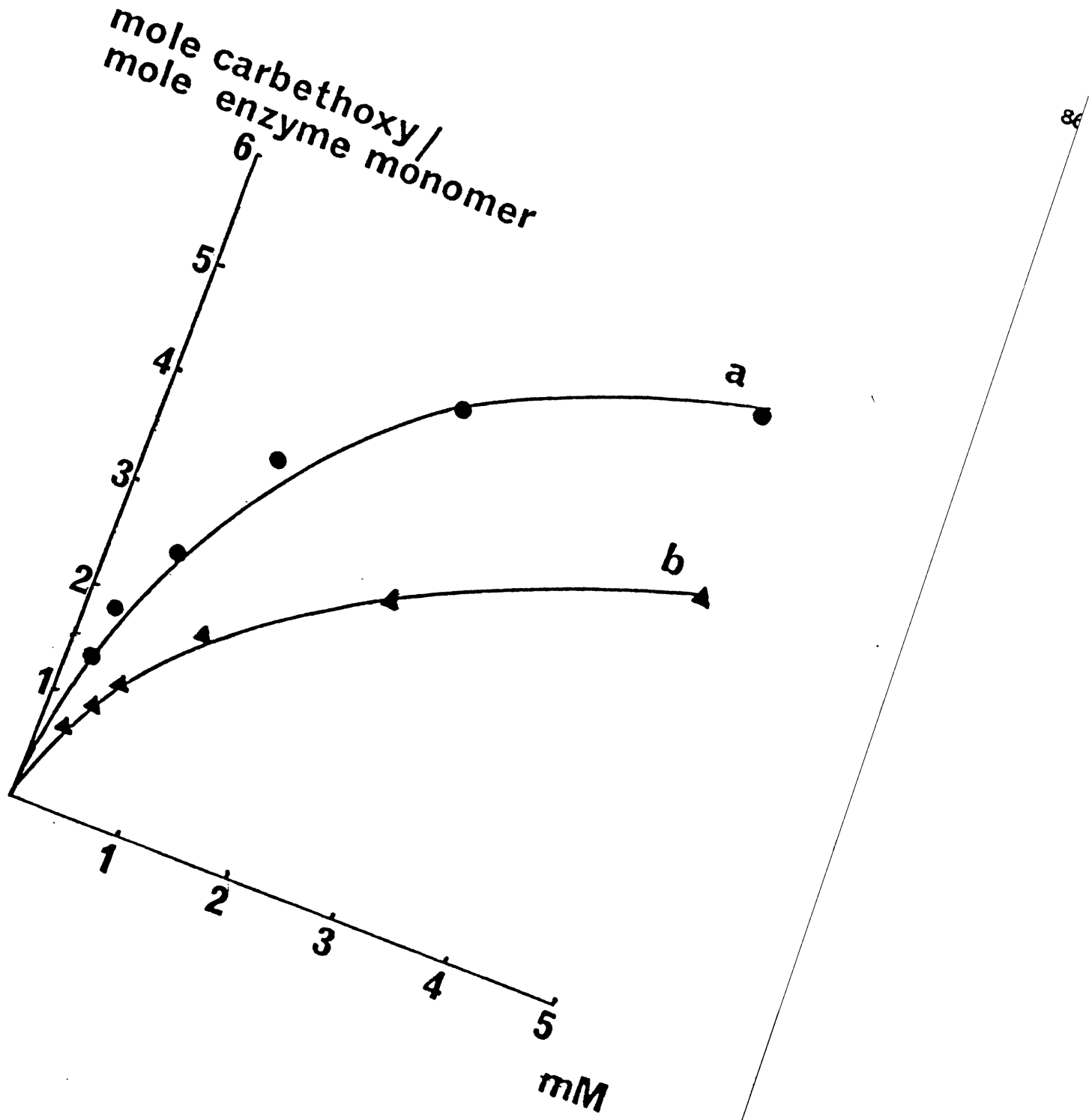
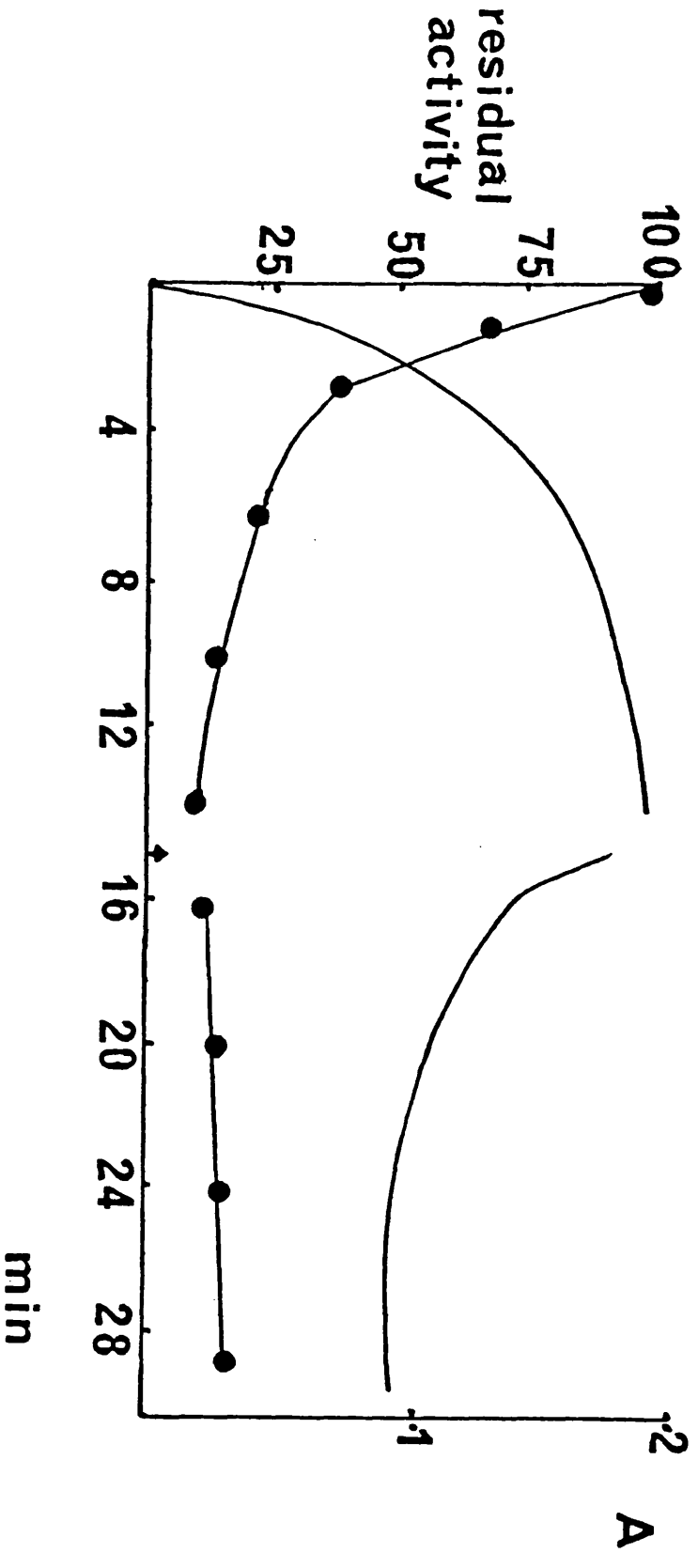

mole carbethoxy /
mole enzyme monomer
6
5
4
3
2
1
a
b
1
2
3
4
5
mM

Fig.30.

Time dependence of inhibition and change in absorbance of CAT_{III} modified by DEP before and after addition of Hydroxylamine.

CAT_{III} (0.8mg/ml) was incubated at room temperature in sodium phosphate buffer (50mM, pH 6.0) with DEP (0.25mM). Time samples were taken, diluted into standard buffer and assayed by the standard method (Methods 2.4) ● . Another identical sample was continuously monitored at 245nm using a Pye-Unicam SP1800 (Methods 2.20). After 15 minutes (↑) hydroxylamine (0.05M) was added and further measurements made. A control of enzyme alone was incubated with ethanol for 15 minutes before addition of hydroxylamine. No loss of activity was observed.


residual activity
100
75
50
25
4
8
12
16
20
24
28
min
2
1
A

(rate of incorporation) respectively. The loss of activity thus correlates well with the formation of carbethoxyhistidine, providing good evidence that the loss of activity is due to the modification of histidine residues.

Examples of non reversal by hydroxylamine of modification by DEP have been reported previously (99) and the phenomenon has been thoroughly investigated by Loosemore and Pratt (100). These authors found that even under mild conditions for modification (1-5mM) cleavage (Bamberger cleavage (101)) of the imidazole ring can occur. The proposed reaction scheme is indicated in Fig.31. If Bamberger cleavage occurs anomalous results can be obtained. Spectral changes at 240nm greater than allowed by the total histidine content of the protein and non reversal of the DEP induced spectral change in the presence of hydroxylamine are often seen. Should Bamberger cleavage be responsible for such effects (rather than protein conformational change or the modification of an amino group (the modification of which cannot be reversed by hydroxylamine)), its presence can be tested by amino acid analysis since it should involve the disappearance of histidine residues.

7.5 Amino acid analysis of CAT_{III} treated with DEP.

The amino acid analysis of modified and unmodified protein gave the following results;

	Histidine content
CAT_{III}	7.0 ± 0.4
CAT_{III} + 0.25mM DEP	6.3 ± 0.17
CAT_{III} + 0.05M hydroxylamine	7.2 ± 0.23
CAT_{III} + 0.25mM DEP + 0.05M hydroxylamine	6.1 ± 0.35

From this data it would appear at first glance that addition of DEP does result in the loss of a histidine residue, although the data was subject to some variability. The acidic conditions used in the amino acid analysis are sufficient to remove carbethoxy groups from histidine but not those which have gone through Bamberger cleavage (102).

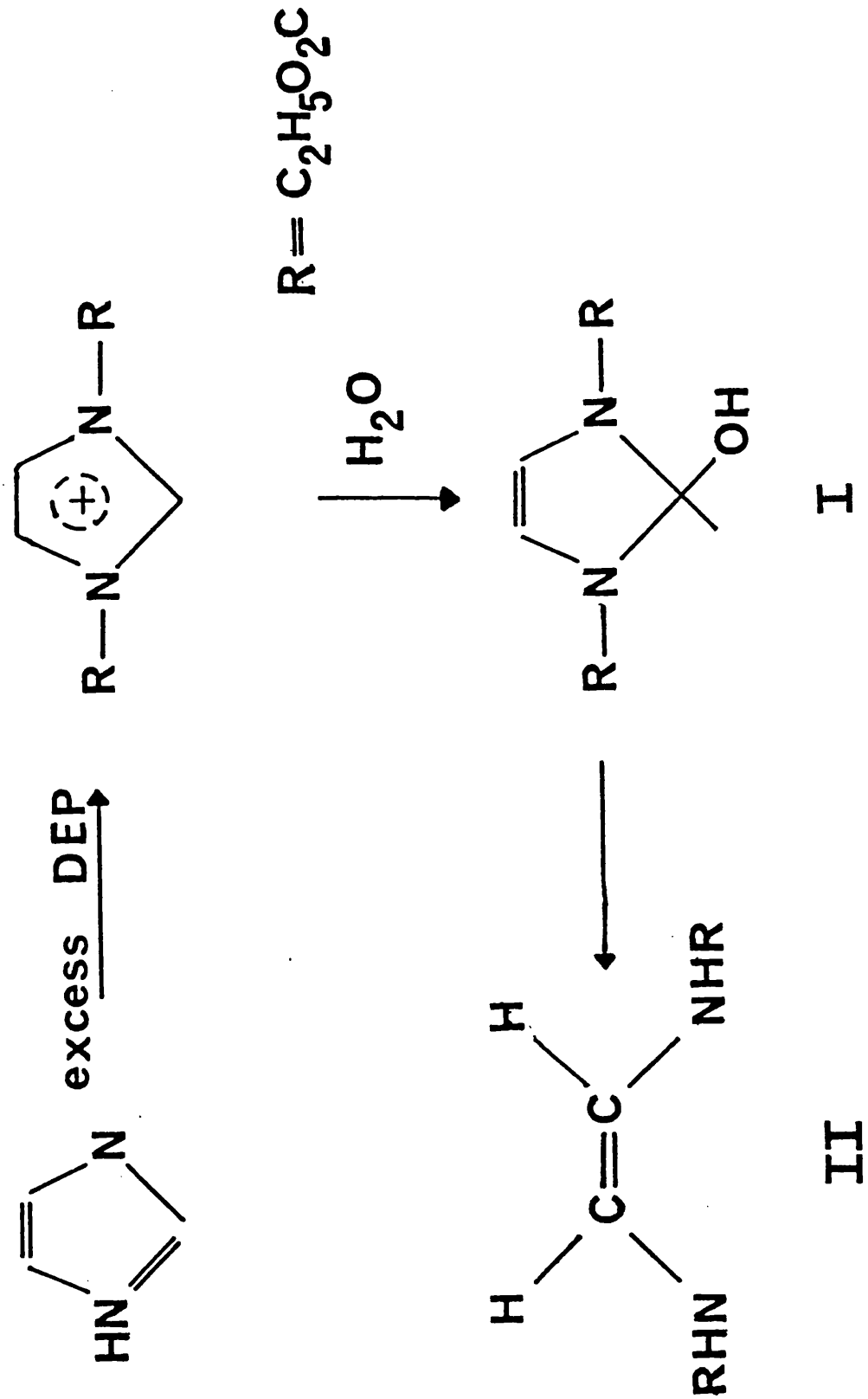


Fig.31.

Proposed reaction scheme for Bamberger cleavage.

Therefore the groups that are removed from the protein in the presence of hydroxylamine (see Fig.30) are also removed by the acidic conditions in the analysis. There should, therefore, be no difference in the histidine content of the modified protein in the presence and absence of hydroxylamine.

Although the amino acid analysis data does indicate that the lack of reversal of the loss of activity is due to the destruction of a histidine residue further attempts were made to increase the regain of activity on addition of hydroxylamine by altering the reaction conditions.

7.6 Reactivation with hydroxylamine of modified CAT_{III}.

Addition of 0.1M hydroxylamine to modified protein caused an increase in activity from 4% to 12% over a period of one hour. Addition of 1.0M hydroxylamine and incubation for a further hour, however, caused a 30% loss of activity in the control sample (unmodified protein) and a decrease in activity to 5% in the modified protein sample. Hence no significant change in activity was observed on addition of higher concentrations of hydroxylamine.

It has been reported previously that the degree of non reversal often depends on how far the inactivation has proceeded. When pyridoxamine phosphate activity had been reduced by 26% or 60% full activity could be restored by a twenty minute incubation with 0.9M hydroxylamine. When the activity was more markedly reduced by a higher concentration of modifier it was only partially restored by hydroxylamine. The authors suggested that dicarbethoxylation may have occured (103). Attempts to partially modify the protein using low concentrations of DEP were unsuccessful since irreproducible results were obtained. It has been reported that at low concentrations DEP rapidly disappears from solution (104). Not only does DEP rapidly hydrolyze in solution but even weak nucleophiles can attack DEP and accelerate its rate of decomposition.

When enzyme, however, was incubated with DEP (0.25mM) and samples diluted into either standard buffer or standard buffer plus hydroxylamine

Table 13

Reactivation with Hydroxylamine of CAT_{III} modified by DEP.

CAT_{IIII} (0.8mg/ml) was incubated at room temperature in sodium phosphate buffer (50mM, pH 6.0) with DEP (1mM). Samples were taken at intervals and diluted either into standard buffer or standard buffer plus hydroxylamine (0.05M) and then assayed by the standard method (Methods 2.4).

Time (min)	% Residual Activity -Hydroxylamine	+Hydroxylamine
0	100	100
0.5	7	91
1	5	83
2	6	77
4	3.5	63
10	2	27

then the data in Table 13 was obtained. Hydroxylamine clearly is able to restore enzyme activity provided it is added soon after modification. Although the enzyme's activity is abolished in the first half minute, the activity gradually decreases when hydroxylamine is present. This may reflect the time required for the intermediates of Bamberger cleavage to build up . The recovery of activity with hydroxylamine is further evidence for the involvement of histidine residues.

7.7 Time course of inactivation by DEP in the presence of substrates.

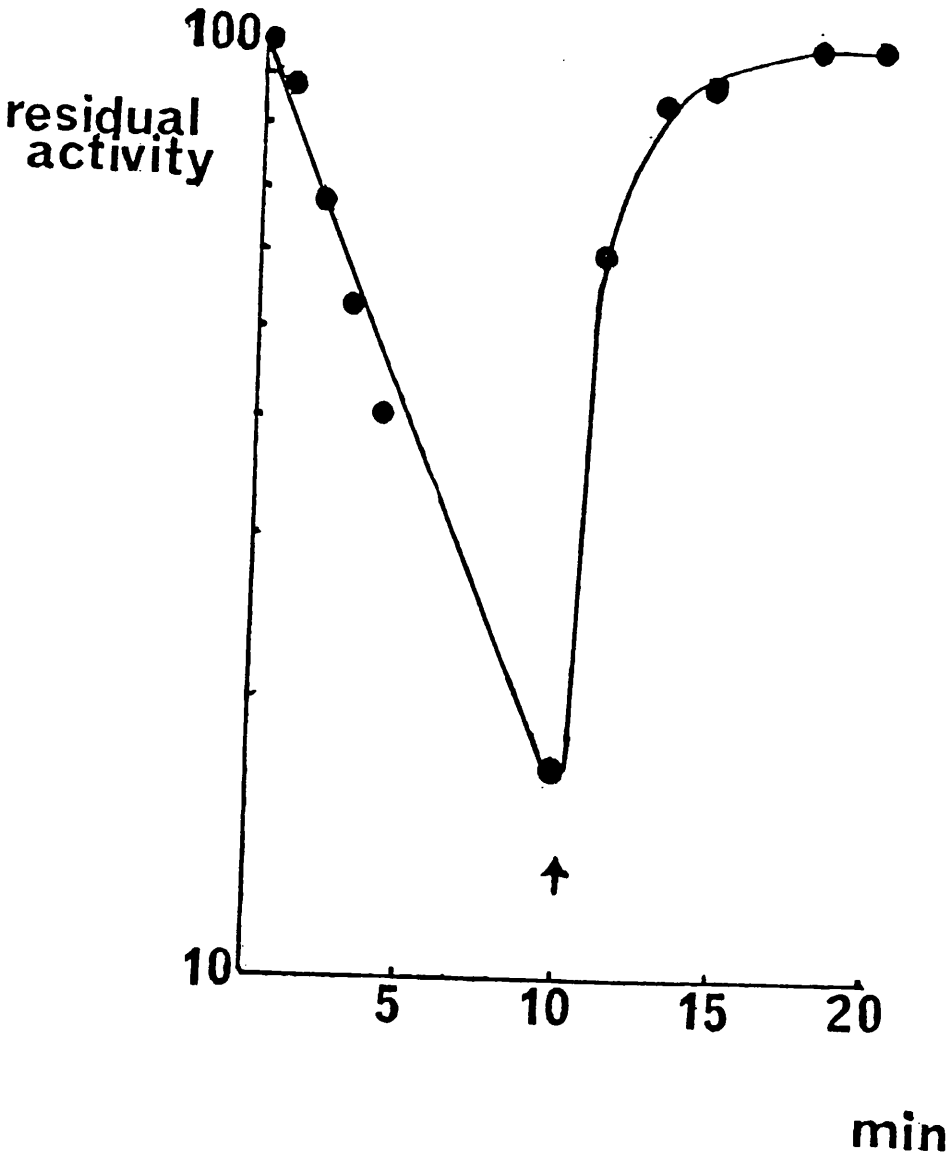
Protection experiments with chloramphenicol (Methods 2.20) (Fig.32) revealed a loss of activity with a pseudo first order rate constant of 0.17 min^{-1}. Addition of 0.05M hydroxylamine caused complete restoration of activity over a 10 minute period. A total of one carbethoxy group was added to the protein which was completely removed on addition of hydroxylamine. This data would appear to suggest that the'slow' rate of loss of activity seen in Fig. 30 is due to the modification of a residue outside the chloramphenicol binding site. Chloramphenicol is able to protect against the 'fast' loss of activity. Since full activity can be regenerated when chloramphenicol is present it would appear that the lack of recovery seen under certain conditions is due to the modification of an essential histidine residue in the chloramphenicol binding site.

No protection was seen with acetyl-S-CoA, acetonyl-S-CoA and methyl-S-CoA. In the case of the latter a slight but reproducible acceleration of the rate of loss of activity was observed. This will be discussed further in the following chapter.

Fig.32.

Protective effect of Chloramphenicol on the inhibition of CAT_{III} by DEP.

CAT_{III} (0.8mg/ml) was incubated at room temperature in sodium phosphate buffer (50mM, pH 6.0) with chloramphenicol (0.25mM) and DEP (0.25mM). Samples were taken at intervals, diluted into standard buffer and assayed by the standard method. After 10 minutes hydroxylamine (0.05M ↑) was added and further measurements made. A control of enzyme and chloramphenicol alone showed no loss of activity.


100
residual activity
10
5
10
15
20
min

Chapter Eight

Chemical Modification Studies with Methyl p-Nitrobenzene Sulphonate

8.1 Introduction.

Although diethylpyrocarbonate (DEP) proved a useful reagent to modify the CAT_{III} enzyme, it had the disadvantage of modifying two classes of histidine residues and also irreversibly modifying the histidine residue of interest. Whilst it is possible in principle to identify the residue(s) modified using radioactive DEP (105,106), it is neither convenient nor reliable to do so. An alternative strategy was employed using a reagent which can be prepared in a radioactive form and yields a stable radio-labeled product.

Nakagawa and Bender (107) described the synthesis and use of the reagent methyl p-nitrobenzene sulphonate (MNBS) (Fig.33). This reagent, which was observed to methylate the N3 position of His57 in chymotrypsin is in many respects an ideal modifying reagent. The product of the reaction with chymotrypsin was shown to be stable and the small size of the attached group minimizes problems of inhibition due solely to steric hindrance and consequent secondary conformational effects. Since the group modified was an essential histidine it was perhaps surprising to find that the methylated chymotrypsin could not only bind substrates as well as native chymotrypsin, but also retained catalytic activity albeit at a low level (108). It was suggested that since the substrate was bound to the enzyme with the same affinity as to the native chymotrypsin that this confirmed that the modification was a relatively minor one and no gross reorganisation of the polypeptide chain had taken place. Small local changes in the precise positions and orientations of the side chains of His57 and Ser195 probably explained the retention of the catalytic rate.

Since it is postulated that chloramphenicol binds in a hydrophobic pocket (56) near the active site of CAT proteins and a reactive histidine is postulated to be near or at the active site this reagent was deemed to be a suitable reagent to synthesize and use. The similarity of the

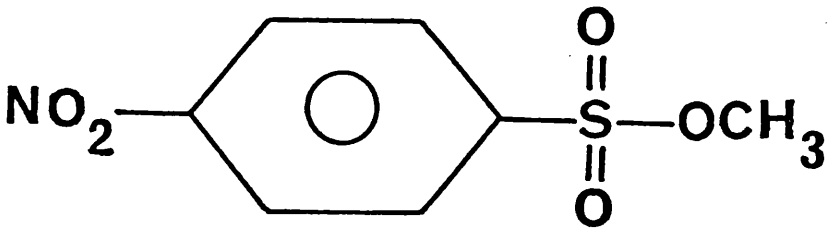


Fig.33.

Structure of Methyl p-Nitrobenzene Sulphonate.

structure of chloramphenicol and the reagent also made this reagent attractive.

8.2 Inhibition of CAT_{III} with MNBS.

Commercially purchased MNBS was recrystallized from petroleum ether (100-120°C). The reagent was dissolved in acetonitrile to make a stock solution. The ultraviolet absorption spectrum in standard buffer showed a λmax of 255nm and ε_{255} of 13.4×10^3 $M^{-1}cm^{-1}$ was obtained. (Lit. 253nm, 13.1×10^3 $M^{-1}cm^{-1}$).

Initial studies indicated that when CAT_{III} was incubated with MNBS (Methods 2.21) the enzyme activity was inhibited. The activity of the enzyme was not restored after overnight dialysis against standard buffer, suggesting that covalent modification had taken place.

The ultraviolet spectrum of the modified protein showed no shift in peak absorbance (280nm). Nakagawa and Bender have taken such a result to support the view that methylation rather than sulphonylation of the enzyme had taken place. When they added p-nitrobenzene sulphonic acid to an equimolar amount of native chymotrypsin a shift in λmax occured from 281nm to 270nm.

8.3 Inhibition of CAT_{III} in the presence of Chloramphenicol, Acetonyl-S-CoA, Acetyl-S-CoA and Methyl-S-CoA.

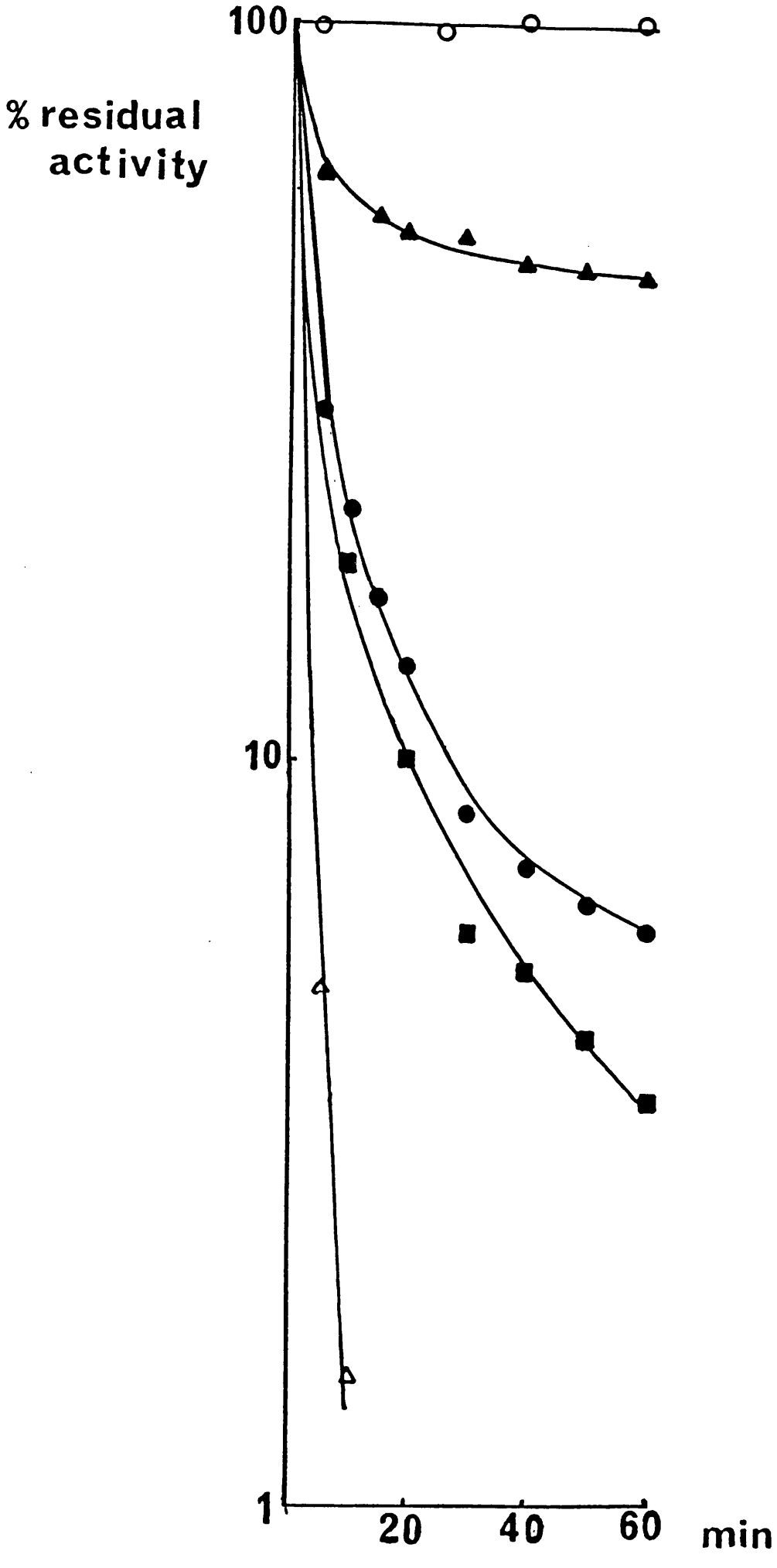
Inhibition studies were carried out as described in Methods 2.21. Enzyme (0.2mg/ml) was incubated at 37°C in standard buffer. The loss of activity of the enzyme was monitored after addition of MNBS. The data was plotted according to the equation $\ln \frac{E}{E_o} = -kt$ (Fig.34). Protection experiments were carried out as described above except that prior incubation of the substrates took place before addition of the inhibitor (Fig.34).

Whilst chloramphenicol is, under the conditions used in the assay, able to protect against the loss of activity to some degree acetonyl-S-CoA was unable to do so. In fact acetonyl-S-CoA produced a slight but reproducible acce leration of the rate of inhibition an effect which was dramatically

Fig.34.

Time course of the inhibition of CAT_{III} by MNBS in the presence of substrates and substrate analogues.

CAT_{III} (0.1mg/ml) was incubated in standard buffer at 37°C with MNBS ■ (0.4mM), in the presence of chloramphenicol ▲(0.2mM), acetonyl-S-CoA ● (0.4mM) or methyl-S-CoA Δ(0.4mM). The control consisted of enzyme and acetonitrile ○. Samples were taken at intervals, diluted into standard buffer and assayed by the standard method (Methods 2.4).


100
% residual
activity
10
1
20
40
60
min

evident with methyl-S-CoA.

Inhibition of CAT_{III} in the presence of chloramphenicol and acetonyl-S-CoA and chloramphenicol and methyl-S-CoA was also carried out (Methods 2.21). In both cases the degree of protection seen by chloramphenicol was reduced (Fig.35).

A small acceleration in the rate of inhibition of the enzyme was also observed in the presence of acetyl-S-CoA, which also reduced the degree of protection seen by a fluorinated chloramphenicol derivative in which fluorine replaces the hydroxyl at the C-3 position (Fig.36). Since the reagent does resemble chloramphenicol structurally and chloramphenicol is able to afford some degree of protection, it would be reasonable to presume that the reagent binds to the enzyme in the chloramphenicol binding site. One possible explanation, therefore, of the phenomena observed with methyl-S-CoA is that the conformation of the chloramphenicol binding site might have been altered thus allowing easier access of the reagent to the modified residue(s).

8.4 Determination of the dissociation constant for Chloramphenicol in the presence of Methyl-S-CoA.

To test the hypothesis that methyl-S-CoA binding may influence the binding of chloramphenicol (and by inference that of MNBS) the dissociation constantfor chloramphenicol was determined in the presence of methyl-S-CoA. In the absence of methyl-S-CoA an average dissociation constant of $16.7 \pm 0.9 \times 10^{-6}M^{-1}$ was obtained whilst in its presence an increase of approximately two fold was observed ($K_d = 34.1 \pm 4.5 \times 10^{-6}M^{-1}$).

The presence of methyl-S-CoA hence has an effect on the interaction of chloramphenicol with the enzyme indicating that the two binding sites are not independent. Any further conclusions with respect to the possible interaction of chloramphenicol and acetyl-S-CoA cannot be made however, since methyl-S-CoA is not the true substrate for the enzyme. Whilst it would be impossible to repeat the binding experiments in the presence of

Fig.35.

Time course of the inhibition of CAT_{III} by MNBS in the presence of combinations of substrates and substrate analogues.

CAT_{III} (0.1mg/ml) was incubated at $37^{0}C$ in standard buffer with MNBS (0.4mM) ● , in the presence of chloramphenicol (0.2mM) and acetonyl-S-CoA (0.4mM) ■ , and chloramphenicol (0.2mM) and methyl-S-CoA (0.4mM) ▲ . Samples were taken at intervals and assayed by the standard method (Methods 2.4).

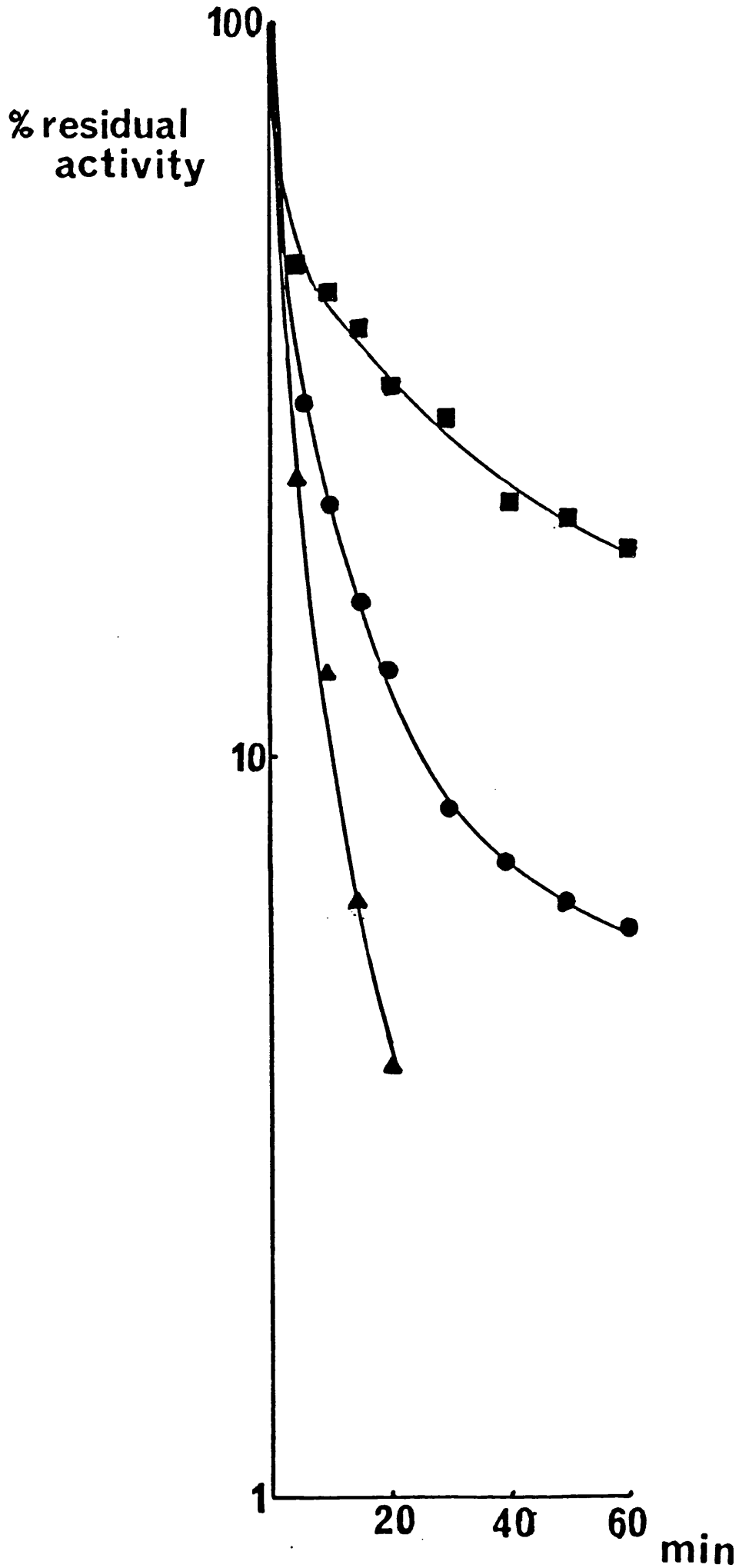
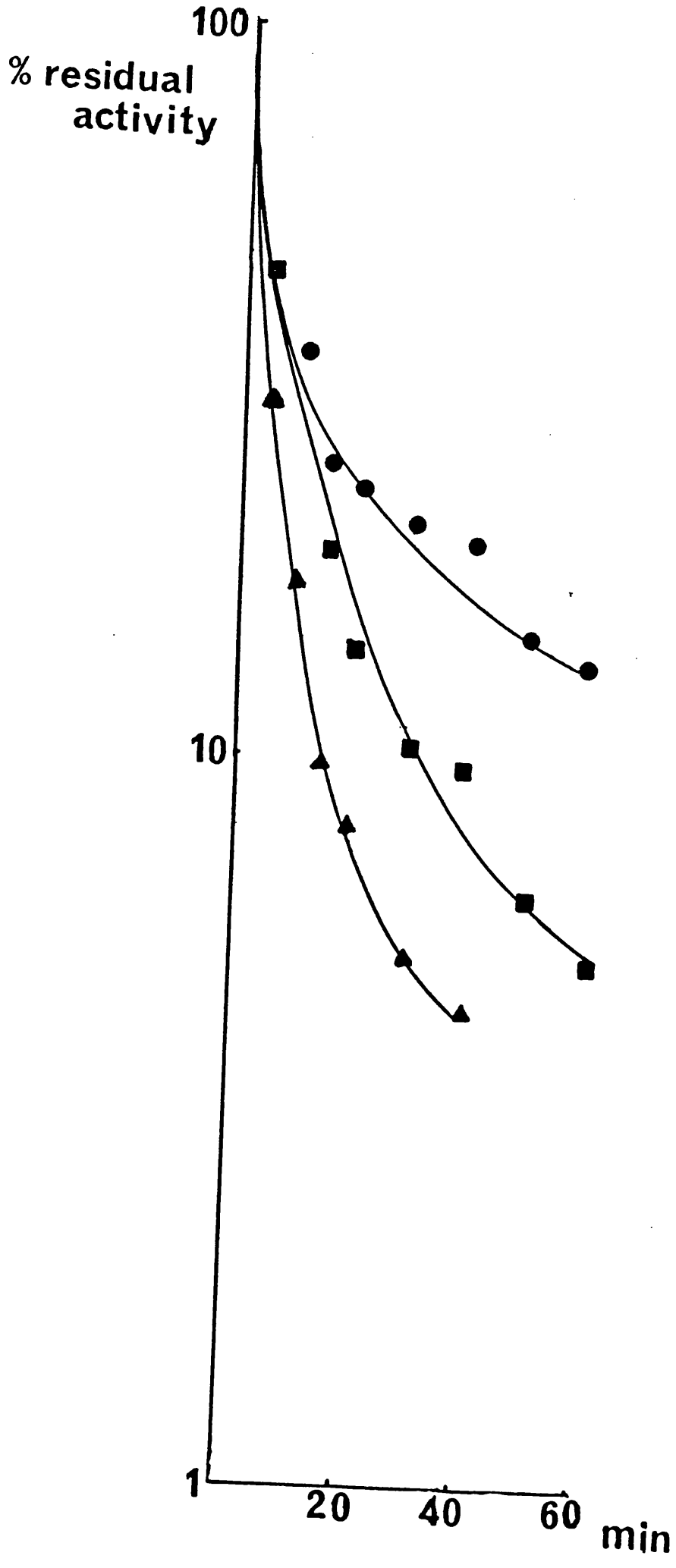

100
% residual activity
10
1
20
40
60
min

Fig.36.

Time course for the inhibition of CAT_{III} by MNBS in the presence of Acetyl-S-CoA and 3-Fluorochloramphenicol.

CAT_{III}(0.1mg/ml) was incubated at 37°C in standard buffer in the presence of MNBS (0.4mM) and acetyl-S-CoA (0.4mM) ▲ , MNBS (0.4mM) and 3-fluoro-chloramphenicol (0.2mM) ● , and MNBS (0.4mM), acetyl-S-CoA (0.4mM) and 3-fluorochloramphenicol (0.2mM) ■ . Samples were taken at intervals and assayed according to methods (Methods 2.4). The fluorinated chloramphenicol derivative has a fluorine atom instead of the hydroxyl group at C3 position.


100
% residual
activity
10
1
20
40
60
min

acetyl-S-CoA, an investigation of the effect of acetonyl-S-CoA on the binding of chloramphenicol may indicate whether the result obtained with methyl-S-CoA is typical.

8.5 Synthesis of [^{14}C] Methyl p-Nitrobenzene Sulphonate.

In order to investigate the residue(s) labelled in the presence and and absence of methyl-S-CoA [^{14}C] MNBS was synthesized. Since the evidence pointed to the enzyme being methylated rather than sulphonylated, the label had to be introduced into the methyl group of the reagent. Radio-labelled methanol was used to prepare [^{14}C] MNBS by a method based on that of Morgan and Cretcher (109).

108mgs of sodium hydride (50% dispersion in oil) was washed 3 times in petroleum ether (40^{0}-60^{0}C) just prior to use, in order to remove the oil, and then was dissolved in 5ml of freshly distilled ether (distilled over lithium aluminium hydride). 500µCi [^{14}C] methanol was vacuum distilled from its vial into a larger reaction vessel using the apparatus described by L.C. Packman (Ph.D Thesis 1978 pg 38). The radioactive methanol and cold methanol (95µl, freshly distilled) were trapped in the reaction vessel using liquid nitrogen. The vessel was allowed to warm to room temperature after which the freshly washed sodium hydride in ether was added. The reaction mix was stirred for thirty minutes before the dropwise addition of 500mg p-nitrobenzene sulphonyl chloride in ether. The reaction course was tested at intervals by spotting 1-2µl onto a silica plate and developing the chromatogram in benzene:chloroform (90:10). Starting material and commercially purchased product were also applied to the plate as standard (RF 0.83, 0.49 respectively).

From experiments carried out with unlabelled methanol alone, it was known that the reaction only went approximately 70% to completion under these conditions (data not shown).

The reaction mixture was then evaporated to dryness at reduced pressure to remove the ether and the product dissolved in water. The aqueous

layer was extracted 3 times with chloroform. The chloroform layers were dried over anhydrous magnesium sulphate, filtered and evaporated to dryness under reduced pressure.

The product was recrystallized using petroleum ether (100^{0}-120^{0}C) At all stages prior to ester formation it was necessary to be extremely careful not to introduce water into the reaction since this causes formation of the sulphonic acid rather than the ester. For this reason freshly distilled methanol and ether were used and the vacuum distillation apparatus was thoroughly evacuated over P_2O_5 before use.

The purity of the recrystallized product was examined on a thin layer silica plate developed using benzene:chloroform (90:10). A spot (95%) was seen at a position corresponding to the commercially purchased product, whilst a faint trace of material was observed corresponding to the position of sulphonic acid. The IR absorption spectrum was performed on a Perkin Elmer 540 spectrophotometer in Nujol and showed absorptions at 1540, 1370, 1190, 980, and 790 cm^{-1}. The ultraviolet spectrum in standard buffer showed a max of 255nm. NMR spectra of the starting material and product are shown in Fig. 37. The relative area ratio of the quartet to the singlet in the product is 4:2.9.

Radioactivity was determined as described in Methods 2.17. The chemical yield of product was 50% and the radioactive yield 34% giving a specific activity of 0.17μCi/umole. The discrepancy between the chemical yield and radioactive yield has been observed before in analogous experiments and has been attributed to loss of radiolabel in the distilation step (L.C. Packman Ph.D Thesis 1978).

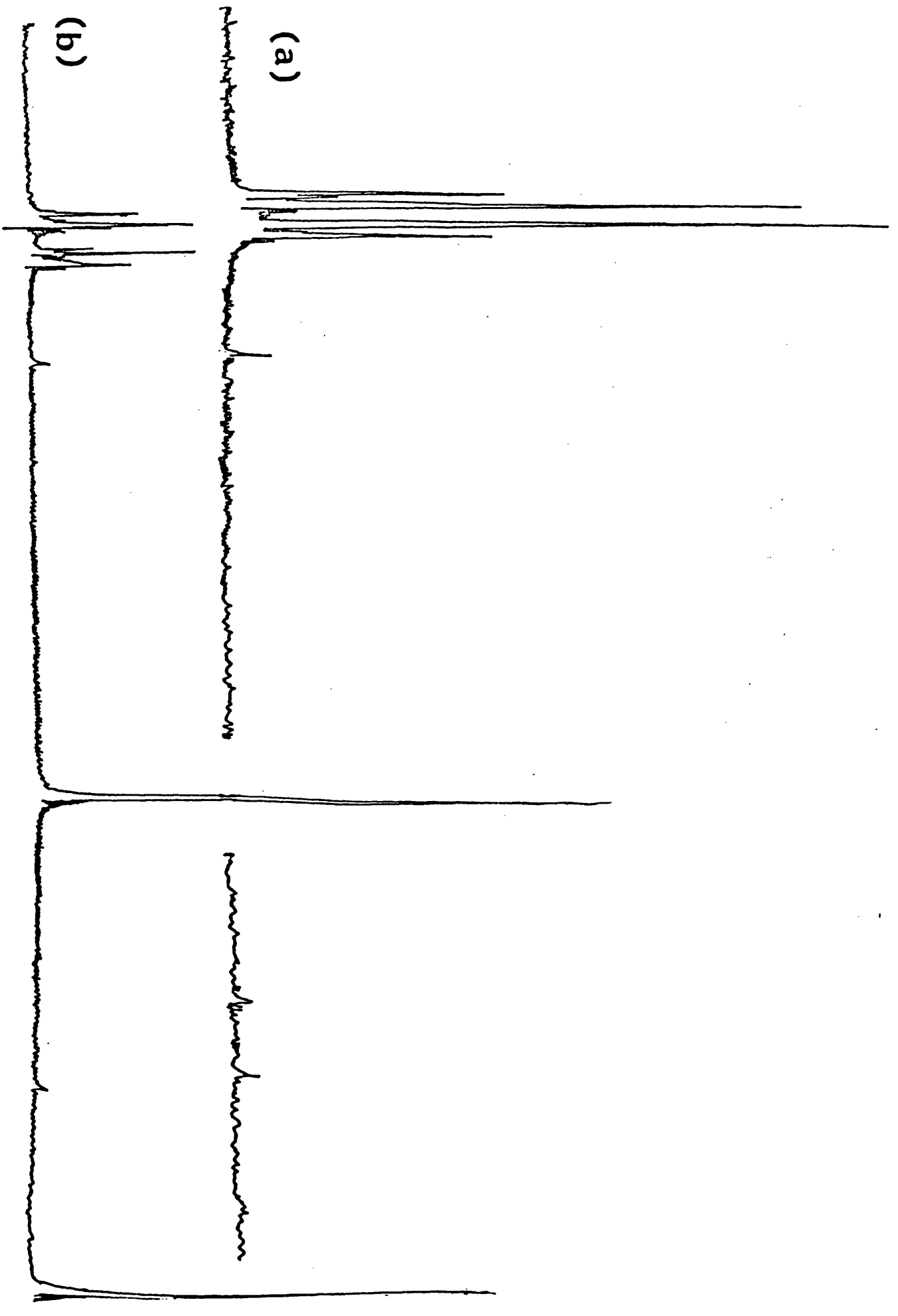
The difficulty in removing all remaining traces of the sulphonic acid even following several recrystallizations led to the decision to test the effect on activity of incubating the enzyme with the acid and the starting material p-nitrobenzene sulphonyl chloride. The former had no effect on the activ ity of the enzyme, whilst the latter rapidly

Fig.37.

NMR spectra of p-Nitrobenzene Sulphonyl Chloride and Methyl p-Nitrobenzene Sulphonate

(a) NMR spectrum of p-nitrobenzene sulphonyl chloride.

(b) NMR spectrum of methyl p-nitrobenzene sulphonate.


(a)
(b)
10
5

inhibited the enzyme. This was investigated further.

8.6 Inhibition of CAT_{III} by p-Nitrobenzene Sulphonyl Chloride.

CAT_{III} (0.1mg/ml) was incubated at $37^{0}C$ in standard buffer with a range of p-nitrobenzene sulphonyl chloride concentrations (Methods 2.21). The results are shown in Fig.38. Extremely rapid inhibition of the enzyme was observed at very low concentrations of the inhibitor.

Protection experiments were carried out (Methods 2.21) in the presence of acetyl-S-CoA, acetonyl-S-CoA, methyl-S-CoA and chloramphenicol. Table 14 shows the final activity observed after incubation of the enzyme and inhibitor plus substrate or substrate analogue for 5 minutes.

The protection seen with acetyl-S-CoA would appear to be artifactual since no protection is seen with acetonyl-S-CoA or methyl-S-CoA.

When inhibition studies were carried out in the presence of CoA protection was again seen. It is postulated that CoA is able to react with the inhibitor and hence give an apparent protection effect. Since acetyl-S-CoA is present in excess over enzyme then it is likely that the acetyl-S-CoA is turned over to produce CoA which then reacts with the inhibitor, giving the apparent protection effect.

The extent of inhibition seen in the presence of acetyl-S-CoA was dependent on how long the acetyl-S-CoA had been preincubated with enzyme. The longer the preincubation time the greater the protection seen, presumably because more CoA was present. Since neither acetyl-S-CoA therefore, nor chloramphenicol protect against the inhibition the modification would appear to be outside the substratebinding sites.

When mercaptoethanol was added to the modified enzyme full activity was restored over a 45 minute period. This would appear to suggest that a sulphydryl group outside the substrate binding site has been modified.

Further investigations werenot carried out, but it is apparent that a trace of the highly reactive starting material could produce unwanted inteference in the MNBS inhibition studies. (If MNBS were used at 1mM

Fig.38.

Time course for the inhibition of CAT_{III} by p-Nitrobenzene Sulphonyl Chloride.

CAT_{III} (0.1mg/ml) was incubated at 37°C in standard buffer with p-nitrobenzene sulphonyl chloride; 2.5μM ● , 5μM ▲ , 7.5μM ■ ,10μM ○ , 12.5μM △ . Samples were taken at intervals, diluted into standard buffer and assayed by the standard method (Methods 2.4). The control consisted of enzyme alone.

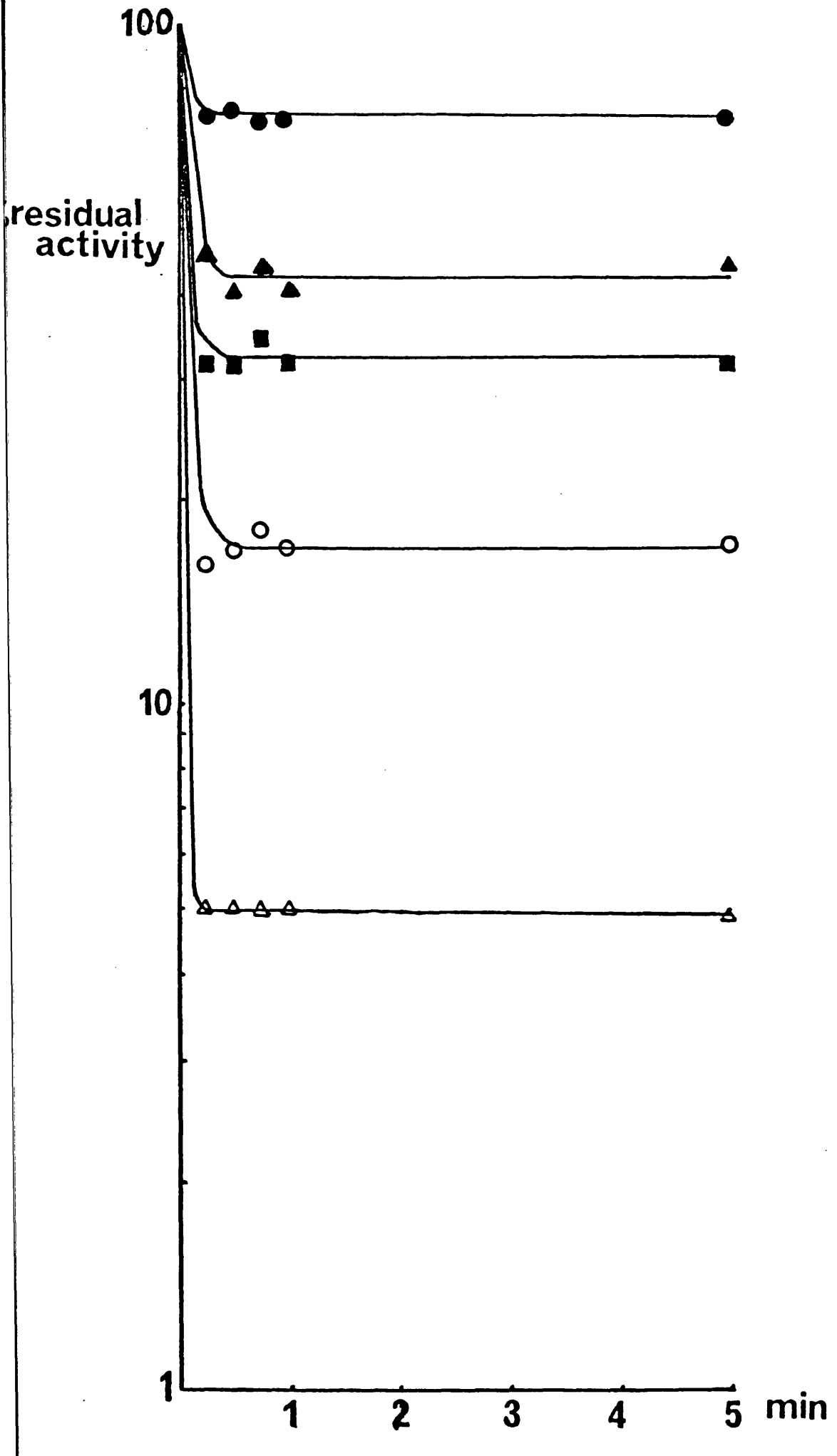

100
residual activity
10
1
1
2
3
4
5
min

Table 14

Effect of substrates and substrate analogues on the inhibition of CAT_{III} by p-Nitrobenzene Sulphonyl Chloride.

CAT_{III} (0.1mg/ml) was incubated at 37°C in standard buffer with p-nitrobenzene sulphonyl chloride in the presence of chloramphenicol (0.03mM), acetonyl-S-CoA (0.08mM), acetyl-S-CoA (0.08mM) and methyl-S-CoA (0.08mM). CoA was also incubated with the enzyme in the presence of reagent (0.08mM). Samples were taken at intervals and the residual activity measured as described in Methods 2.4.

CAT_{III} +	Final Activity(%)
NBSC	11.5
NBSC + Chloramphenicol	14.7
NBSC + Acetyl-S-CoA	59.0
NBSC + Acetonyl-S-CoA	15.4
NBSC + Methyl-S-CoA	13.7
NBSC + CoA	55.0

NBSC = p-Nitrobenzene Sulphonyl Chloride

then a 1% contamination with p-nitrobenzene sulphonyl chloride (10μM) could rapidly inhibit the enzyme). Experiments were carried out in which MNBS was added to buffer 5 minutes prior to the addition of enzyme. Since the sulphonyl chloride is rapidly hydrolysed to sulphonic acid (data not shown), then it would be destroyed in this period. No difference was observed in the inhibition pattern for MNBS after this treatment, indicating that the loss of activity previously recorded was genuine.

8.7 Inhibition of CAT_{III} by [^{14}C]MNBS.

CAT_{III} (0.5mg) was incubated in standard buffer with 1mM [^{14}C]MNBS, in the presence and absence of methyl-S-CoA. Samples were taken at intervals, diluted into standard buffer and assayed by the standard method (Methods 2.4). After 90% inactivation had been reached the sample was applied to a Sephadex G10 column (1 x 25cm), which had been preequilibrated with water. Protein was located by measuring the absorbance of the fractions collected at 280nm. The pooled sample was lyophilized, then half the sample counted for incorporation of label and the other half of the sample was hydrolysed by the standard method (Methods 2.10). After hydrolysis the sample was applied to a thin layer silica plate as a small spot and run in a Shandon electrophoresis tank at pH 6.5 for 120 minutes at 350V (Methods 2.15c). Standards, arginine, histidine, serine, 3-methyl histidine and 1-methyl histidine were applied. Cysteine and methyl cysteine (made by reacting methyl iodide with cysteine) were also applied as standards. After staining to locate the amino acids, the radioactive spots were located by autoradiography (Fig.39). A total of 0.85 moles of [^{14}C]label was incorporated per mole of enzyme monomer, whilst 0.87moles were incorporated in the presence of methyl-S-CoA.

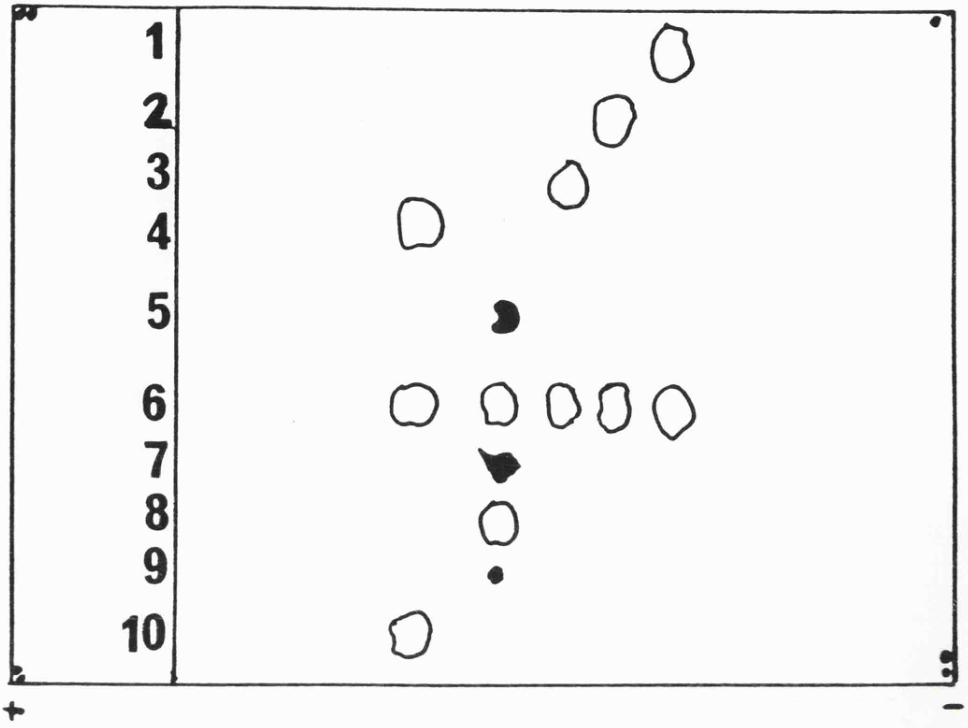
On inspection of the autoradiogram it can be seen that the radioactivity was located at the same position of 3-methyl histidine . On some occasions a small trace of radioactivity was located in the 'neutral' amino acid position. No difference was observed, therefore, either for the location

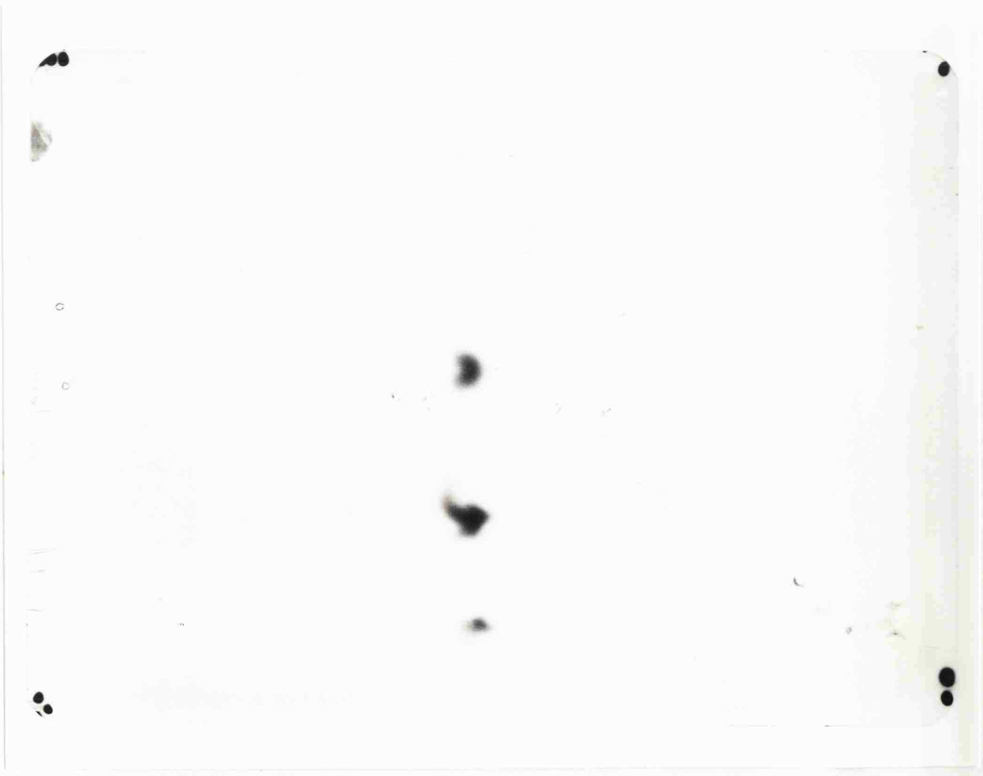
Fig.39.

Identification of the residue modified by $[^{14}C]$MNBS

CAT_{III} (0.5 mg) was incubated in standard buffer with 1mM $[^{14}C]$MNBS. After 90% inactivation had occurred excess reagent was removed on a Sephadex G10 column and the sample lyophilized. Half the sample was hydrolysed (Methods 2.10) and applied to a thin layer silica plate and run at 350V for 120 minutes in pH 6.5 buffer. Standards were applied to the plate as indicated below. After location of the standards using fluorescamine, the radioactive were located by autoradiography.

1. Arginine
2. 1-methyl Histidine
3. Histidine
4. Cysteine and methyl Cysteine
5. CAT_{III} modified with $[^{14}C]$MNBS
6. Arginine, 1-methyl Histidine, Histidine, 3-methyl Histidine, Serine
7. CAT_{III} modified by $[^{14}C]$MNBS in the presence of methyl-S-CoA
8. 3-methyl Histidine
9. CAT_{III} modified with $[^{14}C]$ MNBS in the presence of chloramphenicol
10. Serine


1
2
3
4
5
6
7
8
9
10
+
−



of radioactivity or for the incorporation of label in the presence and absence of methyl-S-CoA. This result would seem to suggest that the acceleration of the rate of inhibition observed in the presence of methyl-S-CoA is not due to the modification of an alternative residue. The result would also be consistent with the idea that the 'active site' is opened up by the analogue making the reactive residue more accessible to the reagent. A more rigorous identification of the modified residue is required, however, before an unequivocal assignment to a histidine residue could be made.

Incorporation of the reagent in the presence of chloramphenicol (0.5mM) reduced the incorporation to 0.42 moles per mole of enzyme monomer. The inability of chloramphenicol to fully protect against the inhibition (Fig. 39 lane c) was disappointing, but may reflect the fact that the inhibitor was structurally related to the substrate and hence may compete very effectively with the binding of chloramphenicol.

Further work is obviously required to locate the position of the modified residue in the CAT_{III} amino acid sequence and to confirm that a histidine residue was modified. The reagent, however, appears to be a suitable candidate for comparative studies with the CAT_I, CAT_{II} and CAT_C variants, the results of which may indicate further the role of histidine in the CAT variants.

Chapter Nine

Preliminary Studies with Rose Bengal, Butanedione and 4-Amino-7-Oxa-Bicyclo [4,10] Hept-3-Ene2,5-Dione-3-Ene-2,5-Dione-3-Carboxamide (G7063-2).

9.1 Photooxidation with Rose Bengal.

Introduction.

The lethal effects of visible light on microorganisms treated with certain dyes was first reported by Raab (110). This effect was investigated many years later by Weil as a means to selectively oxidize proteins and amino acids (111,112,113).

Histidine,tyrosine, tryptophane, methione and cysteine residues were all shown to react between pH 4-10. The relative rates at which the residues underwent photodegradation was found to be dependent not only on the pH of the medium but also on the nature of the sensitizer.

Dye sensitized photooxidations have been shown to proceed via several different mechanisms which are a function of the dye and its concentration, the concentration of oxygen and nature of the substrate.

The following schemes are thought to apply:

$$\text{Dye} \xrightarrow{h\nu} \text{dye}^{*} \begin{cases} \xrightarrow{O_2} \text{dye} + O_2^{\bullet} \longrightarrow (\text{substrate}O_2) \longrightarrow \text{substrate}_{ox} \\ \xrightarrow[\text{substrate}]{} \text{dye}_{red} + \text{substrate}_{ox} \end{cases}$$

Two dyes, methylene blue and rose bengal have been extensively used in photooxidation experiments. Of the two methylene blue has been the most used but evidence favours rose bengal (Fig.40) as the more selective for histidine residues (114,115). Since it is anionic (methylene blue is cationic) it is thought to facilitate the latter's specificity by favouring the formation of short lived dye-imadazole complex.

Inactivation of an enzyme resulting from the conversion of histidine to aspartic acid and urea (116) is often suggested by a characteristic pH dependence of the photooxidation spectrum (114,117,118). In fact, in several well documented cases the enzymatic activity loss following photooxidation could be correlated with the destruction of a unique histidine residue. Analysis of the rate of enzyme activity loss and amino acid content of phosphoglucomutase and of αchymotrypsin after

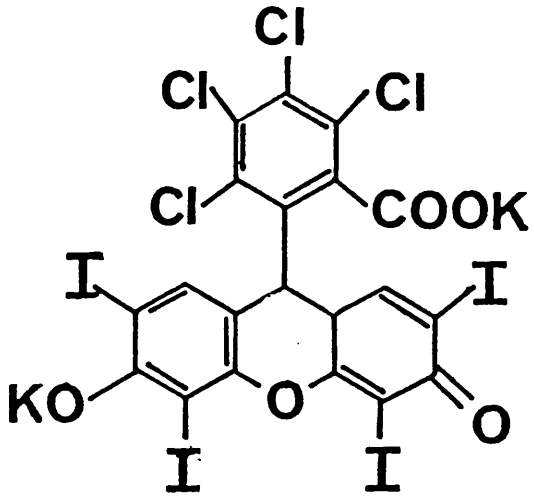


Fig.40.

Structure of the dye Rose Bengal.

photooxidation in the presence of methylene blue allowed Koshland et al. (119) to show the destruction of a single critical histidine residue. Indeed, and perhaps unfortunately the destruction of a histidine residue by photooxidation has been the sole evidence in some cases for the classification of some enzyme as 'histidine' enzymes.

9.2 Inactivation of CAT_{III} with Rose Bengal.

Inactivation of the enzyme (0.2mg/ml in standard buffer) by rose bengal (15μM) was carried out as described in Methods 2.22. A rapid inhibition of activity (Fig.41) was seen with 90% of the initial activity lost in 4 minutes. Incubation of the enzyme with the dye in the absence of light or in the presence of light but absence of dye yielded no loss of activity over the time course of the experiment. A pH profile for the rate of inactivation is also shown (Fig.42). A pK_a of 6.4 was obtained indicating the possible involvement of a histidine residue.

9.3 Inactivation of CAT_{III} by Rose Bengal in the presence of substrates.

Incubation of the enzyme and rose bengal in the presence of either chloramphenicol (1mM) or acetyl-S-CoA (1mM) (Methods 2.22) yielded very different results. Whilst acetyl-S-CoA protected against the loss of activity chloramphenicol slightly acce lerated the rate of inhibition (Fig.43).

Since rose bengal is anionic, it was expected that acetyl-S-CoA may protect against the inhibition. In the following section tentative evidence exists for the presence of arginine residues in the acetyl-S-CoA binding site. It could be postulated that the arginine groups present stabilize the enzyme-dye complex.

Fig.41.

Time course of inactivation of CAT_{III} by Rose Bengal.

CAT_{III} (0.2mg/ml) was incubated at 25°C in standard buffer with Rose Bengal (15μM) (Methods 2.22). Samples were taken at intervals, diluted into standard buffer in covered tubes and the residual activity measured ● . Control experiments were carried out without light ■ , and without dye ▲ .

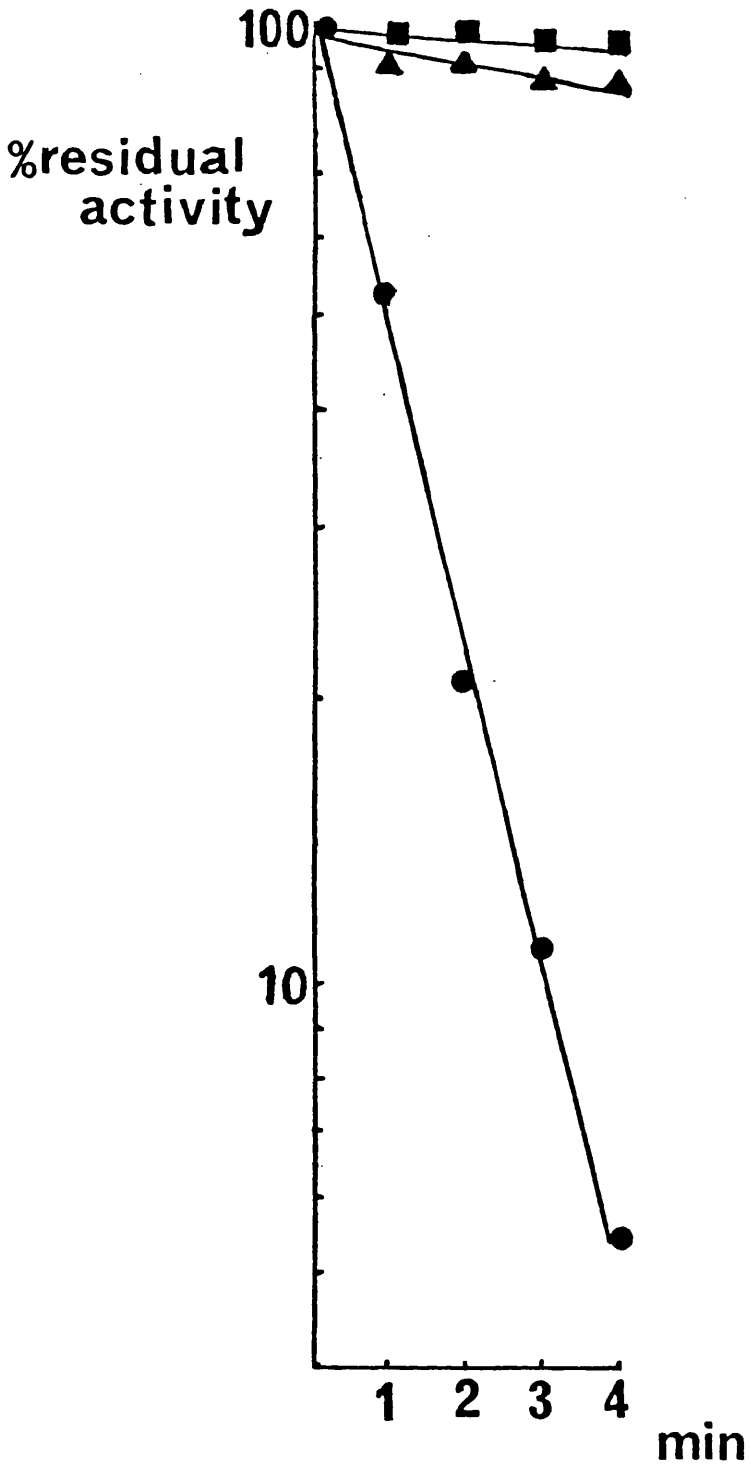

100
%residual
activity
10
1
2
3
4
min

Fig.42.

PH dependence of the rate of inactivation of CAT_{III} by Rose Bengal.

CAT_{III} (0.2mg/ml) was incubated at 25°C in 50mM Tris-Maleate buffer over a range of pH values. The rate of inhibition in the presence of Rose Bengal (5μM) was measured by taking samples at intervals and measuring the residual activity in the standard assay (Methods 2.4). A rate of inactivation was calculated at each pH value using the equation $\ln \frac{E}{E_o} = -kt$.

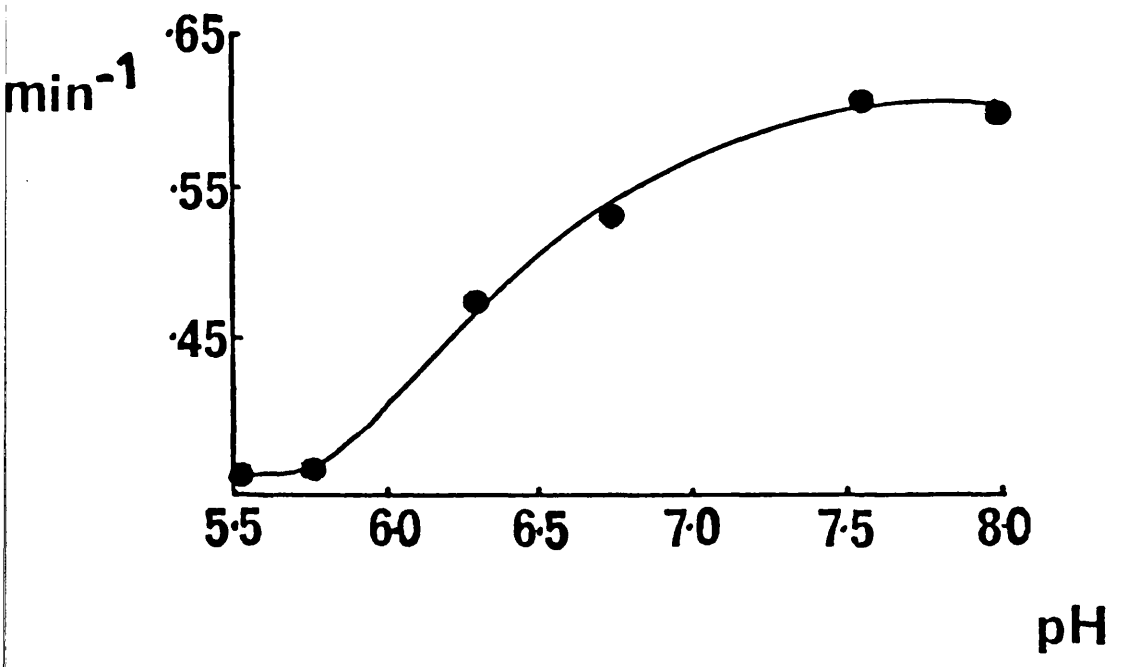
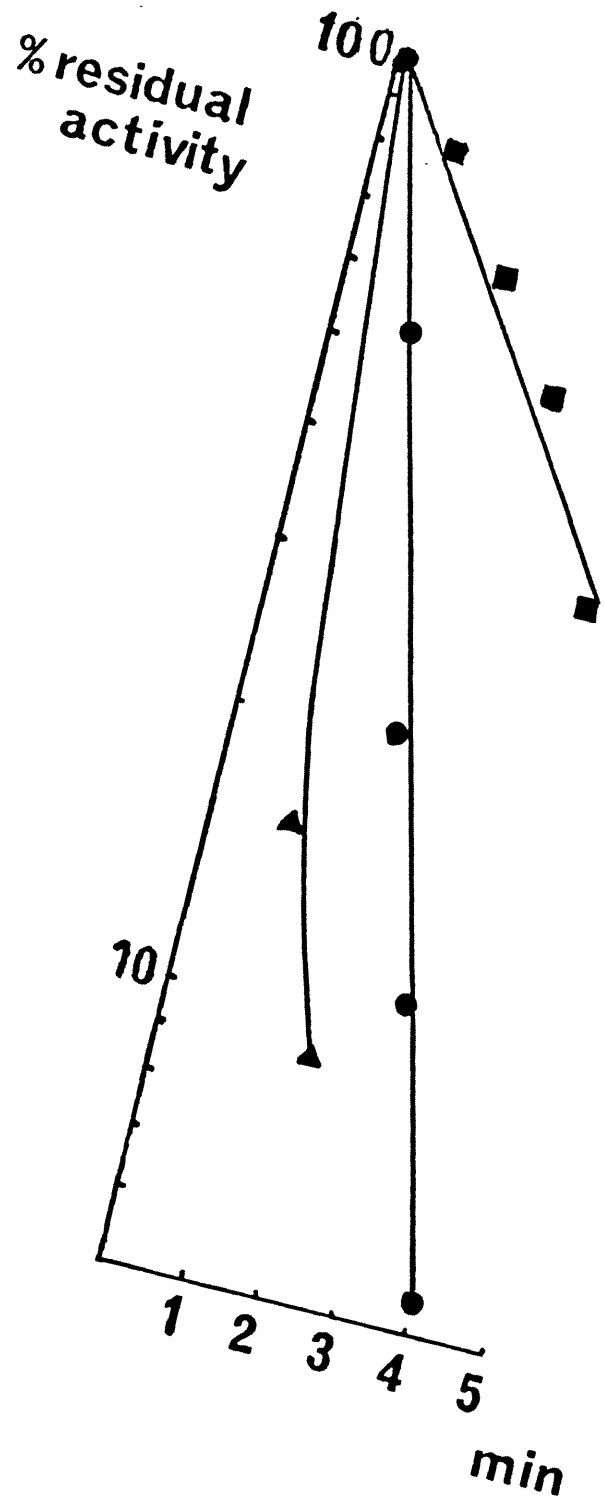

min-1
·65
·55
·45
5·5
6·0
6·5
7·0
7·5
8·0
pH

Fig.43.

Time course of the inhibition of CAT_{III} by Rose Bengal in the presence of substrates.

CAT_{III} (0.2mg/ml) was incubated at $25^{0}C$ in standard buffer with Rose Bengal (15μM) ● . Protection experiments were carried out in the presence of chloramphenicol (1mM) ▲ , and acetyl-S-CoA (1mM) ■ . Samples were taken at intervals and assayed by the standard method.


% residual activity
100
10
1
2
3
4
5
min

9.4 Inhibition studies with Butanedione.

Introduction.

Arginine was one of the last reactive amino acids for which specific modifying reagents were found. This was mainly due to the fact that the guanidium group of arginine fails to react with reagents known to modify lysine, at least at neutral pH in aqueous solution and at low temperatures, conditions favoured for the study of functional proteins. Lysine can generally modified under slightly alkaline conditions where some of the free base form is present but the guanidium group is protonated over the entire pH range of protein stability.

Nowadays a large number of α-dicarbonyl compounds, dialdehydes, keto aldehydes and diketones exist to modify arginyl residues in proteins.

Butanedione (Fig. 44) has been extensively used as an arginine modifying reagent (120,121,122,123). These studies have also indicated that other types of amino acids are either unaffected or react very slowly with butanedione under mild conditions (120). A one hundred - fold slower rate of reaction with lysine and an even slower rate of reaction with histidine residues hasbeen observed.

Arginine has been shown to be part of the binding site for several enzymes which have negatively charged substrates (for example 121,122, 123). Furthermore, in many dehydrogenases an arginine residue has been located within the nucleotide binding site (124,125). CAT has a negatively charged nucleotide as a substrate (acetyl-S-CoA) so to test the hypothesis that arginine residues were involved in binding acetyl-S-CoA experiments using butanedione as an arginine modifying reagent were carried out.

The precise mode of action of butanedione is not yet clear. Riordan (123) found that in the case of carboxypeptidase A the rate of inhibition was specifically enhanced by borate,leading him to suggest that Compound I (formed on addition of butanedione to arginine) (Fig. 44b) complexes rapidly and reversibly with borate to give Compound II. Hence, the

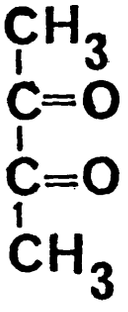
Fig.44a.

Structure of Butanedione.

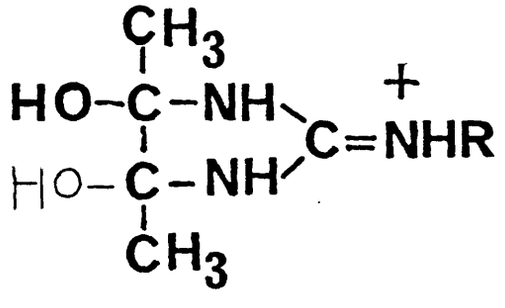
Fig.44b.

Postulated reaction for product stabilization by Borate.

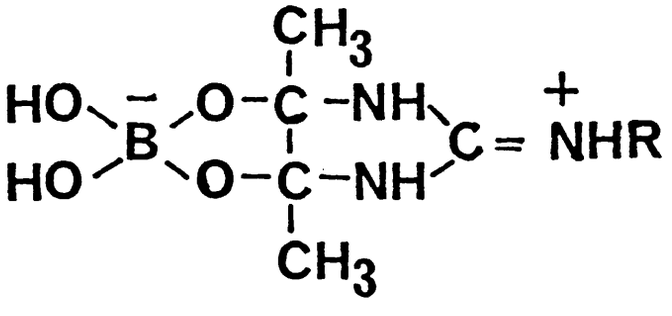
Riordan (129) has postulated that Compound I, formed on addition of butanedione to arginine complexes reversibly with borate to give Compound II.



(a)



(I)



(II)

(b)

reaction of butanedione with arginine is postulated to proceed faster due to product stabilization. Riordan also postulated that Compound I was moderately stable. If the reaction was carried out in the absence of borate and then borate added Compound II was thought to form instantaneously. Hence it was thought that borate should be included in the reaction mix to stabilize the product of the reaction and prevent spontaneous restoration of activity. This phenomena has been observed by others (125) and has been taken as evidence for the involvement of arginine residues.

Other studies, however, have yielded no evidence for recovery of activity, even in the absence of borate (121,126,127). In fact, it has been suggested that the inclusion of borate is not desirable where enzymes using pyridine nucleotides are studied because of the formation of complexes between borate and the ribose moiety of the pyridine nucleotide (128). In these cases it is possible that internal secondary reactions could lead to formation of irreversibly modified arginine residues. Even Riordan (129) noted that if carboxypeptidase A was inactivated for more than an hour then the addition of borate had no affect.

9.5 Inhibition of CAT_{III} by Butanedione.

Initial experiments were carried out in order to determine whether the enzyme could be inhibited by butanedione. CAT_{III} (0.2mg/ml) was incubated in either 50mM sodium phosphate buffer (pH 7.8) or 50mM borate buffer (pH 7.8). Samples were taken at intervals, diluted into standard buffer and assayed by the standard method (Methods 2.23). CAT_{III} was rapidly inhibited by butanedione. Unlike Riordan, however, no increase in the rate of inhibition of CAT was seen with the addition of borate.

9.6 Inhibition of CAT_{III} by Butanedione in the presence of substrates.

Inhibition studies were carried out in the presence of either acetyl-S-CoA (1mM) or chloramphenicol (1mM) as described in Methods 2.23. Table 15 summarizes the data from Fig. 45. Pseudo first order rate constants for the fast and slow rates in the presence and absence of chloramphenicol and acetyl-S-CoA were calculated. The data is compatible with the view that at least two classes of arginine residues may have been modified. The fast rate observed is ten fold that of the slow rate. Chloramphenicol is able to protect against the fast rate of loss of activity to some degree whilst acetyl-S-CoA is able to protect against both in varying amounts.

9.7 Attempted reactivation of CAT_{III} inhibition by Butanedione.

As already stated, in many previous studies borate has been included in the reaction mixture to stabilize the product and to prevent spontaneous restoration of activity. No increase in the rate of inhibition was seen in the presence of borate (data not shown). Despite this, after inhibition in the presence of borate a sample of the reaction mix was applied to a G10 Sephadex column equilibrated with 50mM sodium phosphate buffer (pH 7.8) to remove excess reagent and borate. The results are seen in Table 16.

No restoration of activity was seen at the end of this treatment.

Fig.45.

Time course for the inhibition of CAT_{III} by Butanedione in the presence of substrates.

CAT_{III} (0.2mg/ml) was incubated at 37°C in sodium phosphate buffer (50mM, pH 7.8) with butanedione (285mM) ● and in the presence of chloramphenicol (1mM) ■ , or acetyl-S-CoA (1mM) ▲ . Time samples were taken, diluted into standard buffer and assayed by the standard method.

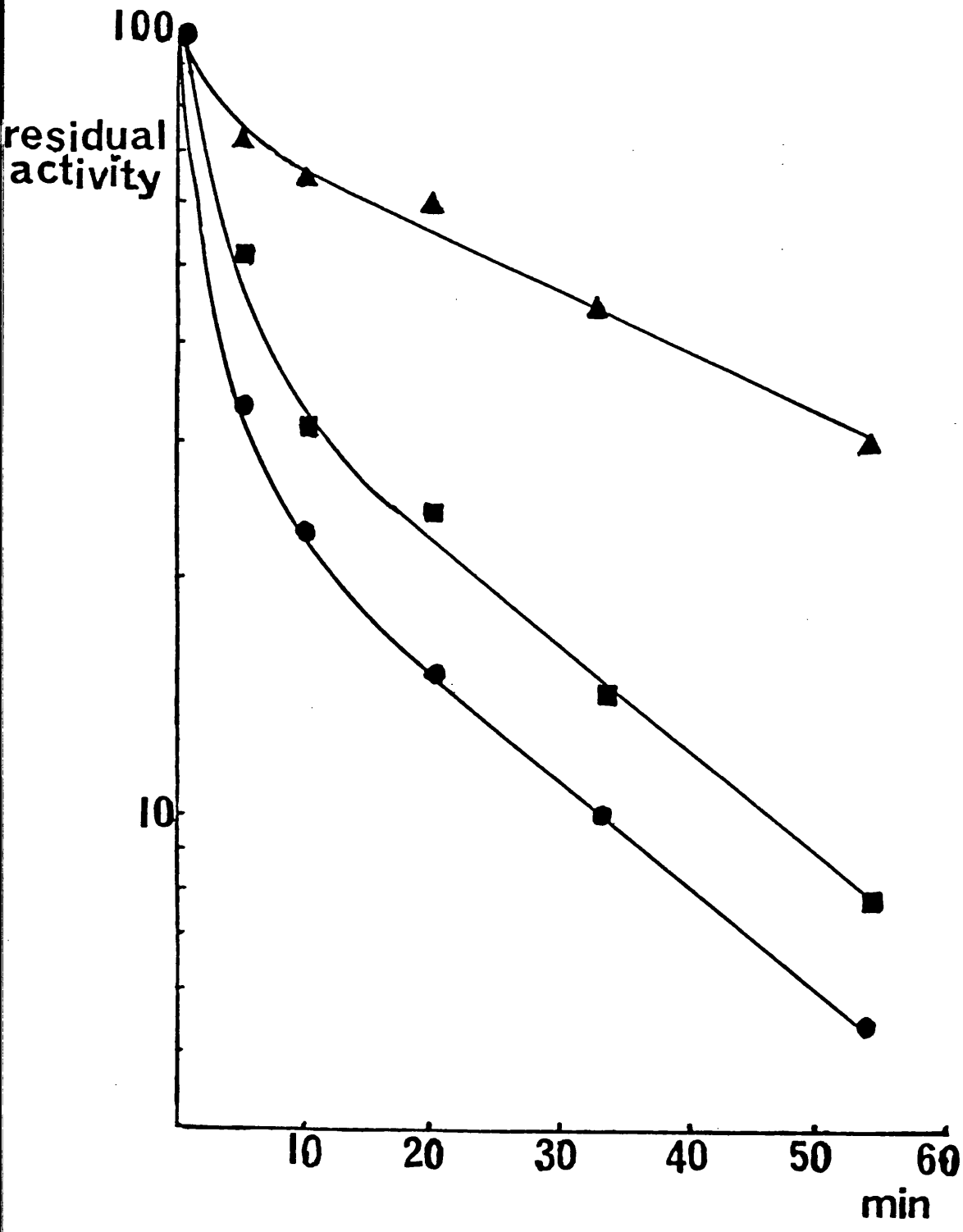

100
residual activity
10
10
20
30
40
50
60
min

Table 15

Pseudo first order rate constants for the inhibition of CAT_{III} by Butanedione in the presence of Chloramphenicol and Acetyl-S-CoA.

Enzyme +	'Slow' $10^3 min^{-1}$	Protection %	'Fast' $10^3 min^{-1}$	Protection %
Butanedione (285mM)	33		370	
Butanedione + (285mM) Chloramphenicol (1mM)	32	3	227	39
Butanedione + (285mM) Acetyl-S-CoA (1mM)	15	53	139	62

Table 16

Activity of CAT_{III} modified by Butanedione after removal of excess reagent and Borate by gel filtration.

	Activity relative to control (%).
Before application to G10 column.	5
Time after application: 2 hours	13
20 hours	10
40 hours	5

The control was an identical sample of unmodified CAT_{III}.

9.8 G7063-2 Introduction.

In 1978 it was reported by Imagawa et al. (131) that they had isolated an antagonist of chloramphenicol I851 (antiphenicol). The compound (Fig.46b) named by the authors 2-amino-3-carboxy-1,4 benzoquinone 5,6-epoxide was isolated from Streptomyces fulvoviolaceus. It showed a broad antibacterial spectrum with weak activity. The antagonistic activity seen against chloramphenicol was limited for other inhibitors of protein synthesis. Antiphenicol was found to be active against Gram-positive and Gram-negative bacteria including Pseudomonas species. Yeast and fungi were not affected at a concentration less than 100µg/ml. Attempts to obtain samples of this compound to see whether it had any affect on CAT_{III} were unsuccessful. However, samples of a very similar compound (Fig. 46a) named by the authors 4-amino-7-oxa-bicyclo[4,1Ø] hept-3-ene-2,5-dione-3-ene-2,5-dione-3-carboxamide (132) were obtained (gift from Glaxo). This compound (known as G7063-2) was isolated from Streptomyces.

In in vitro tests it was moderately active against both Gram-positive and Gram-negative bacteria and weakly active against fungi. G7063-2 inhibited the uptake of [^{3}H]thymidine into DNA, [^{14}C]uracil into RNA, [^{3}H]phenylalanine into protein and [^{14}N]acetylglucosamine into cell walls and inhibited growth of the organism all at the same time; i.e it appeared as if the compound was acting as a general enzyme poison. Antibiotic activity was enhanced if the medium used for assay contained only glucose and minimal salts. When various nutrients were added to the assay medium it was found that activity was antagonized by cysteine and methionine (M. Noble personnal communication).

9.9 Inhibition of CAT_{III} with G7063-2.

Initial experiments indicated that G7063-2 did inhibit CAT_{III} (Fig.47). Inclusion of both chloramphenicol (1mM) or acetyl-S-CoA (1mM) in the reaction afforded some degree of protection. Fig.48 shows the rate of loss of activity (k) as a function of the inhibitor concentration. The

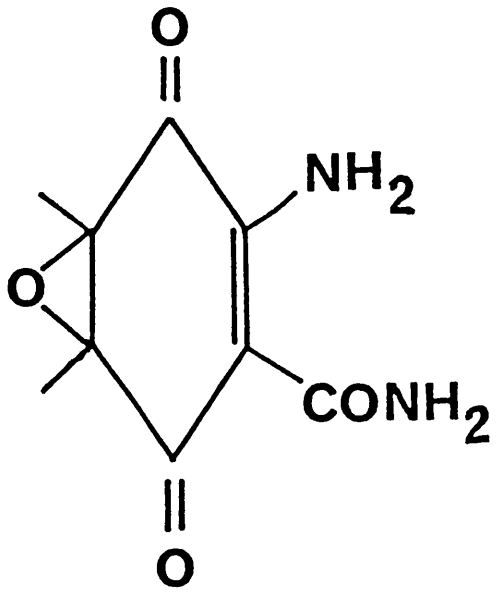
Fig.46a.

Structure of G7063-2.

Fig.46b.

Structure of I851 (Antiphenicol).

(a)



(b)

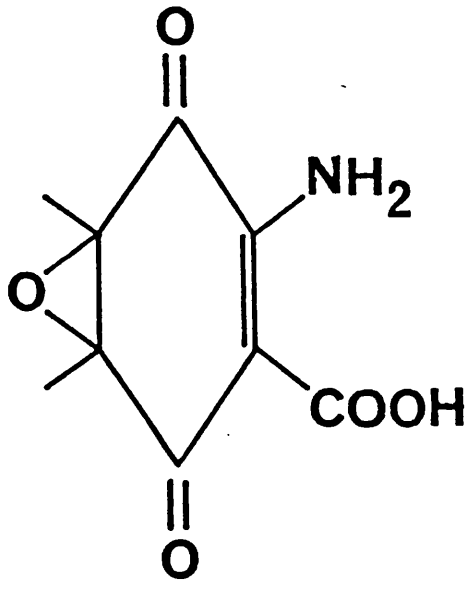


Fig.47.

Time course for the inhibition of CAT_{III} by G7063-2 in the presence of Chloramphenicol and Acetyl-S-CoA.

CAT_{III} (0.2mg/ml) was incubated at 37°C in standard buffer with G7063-2 (10mM) ● , in the presence of chloramphenicol (1mM) ▲ , and acetyl-S-CoA (1mM) ■ . Samples were taken at intervals, diluted into standard buffer and assayed by the standard method.

Fig.48.

Concentration dependence of the rate of inactivation of CAT_{III} by G7063-2.

Inactivation experiments were carried out using a range of G7063-2 concentrations. CAT_{III} (0.2mg/ml) was incubated at 37°C in standard buffer with G7063-2 and samples taken at intervals for assaying by the standard method. Pseudo first order rate constants were calculated for each concentration used, using the equation $\ln \frac{E}{Eo} = -kt$.

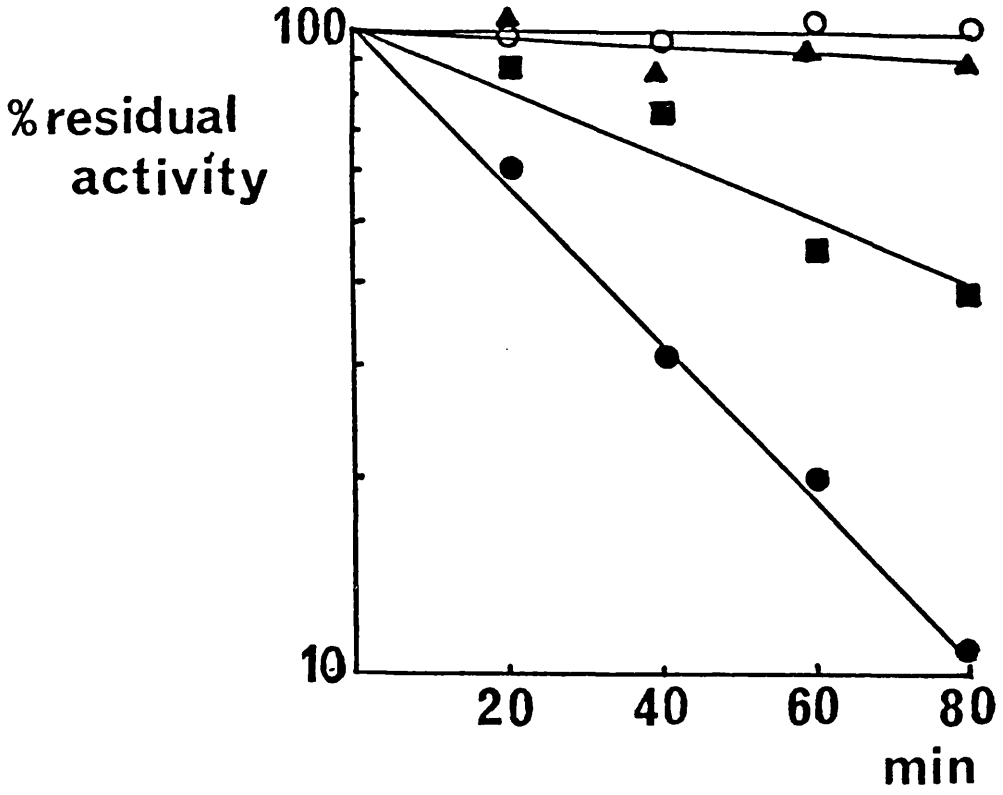

100
% residual activity
10
20
40
60
80
min

Fig.47.

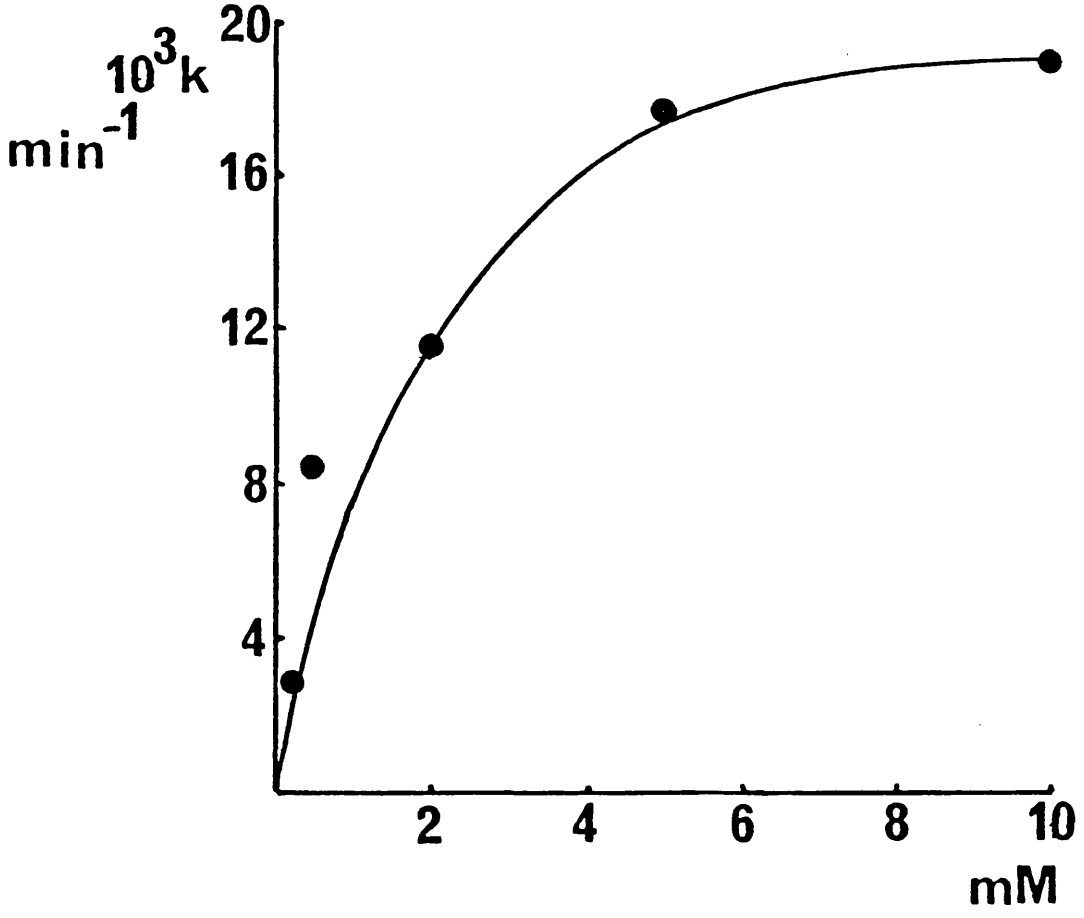

20
$10^3 k$
min^{-1}
16
12
8
4
2
4
6
8
10
mM

Fig.48.

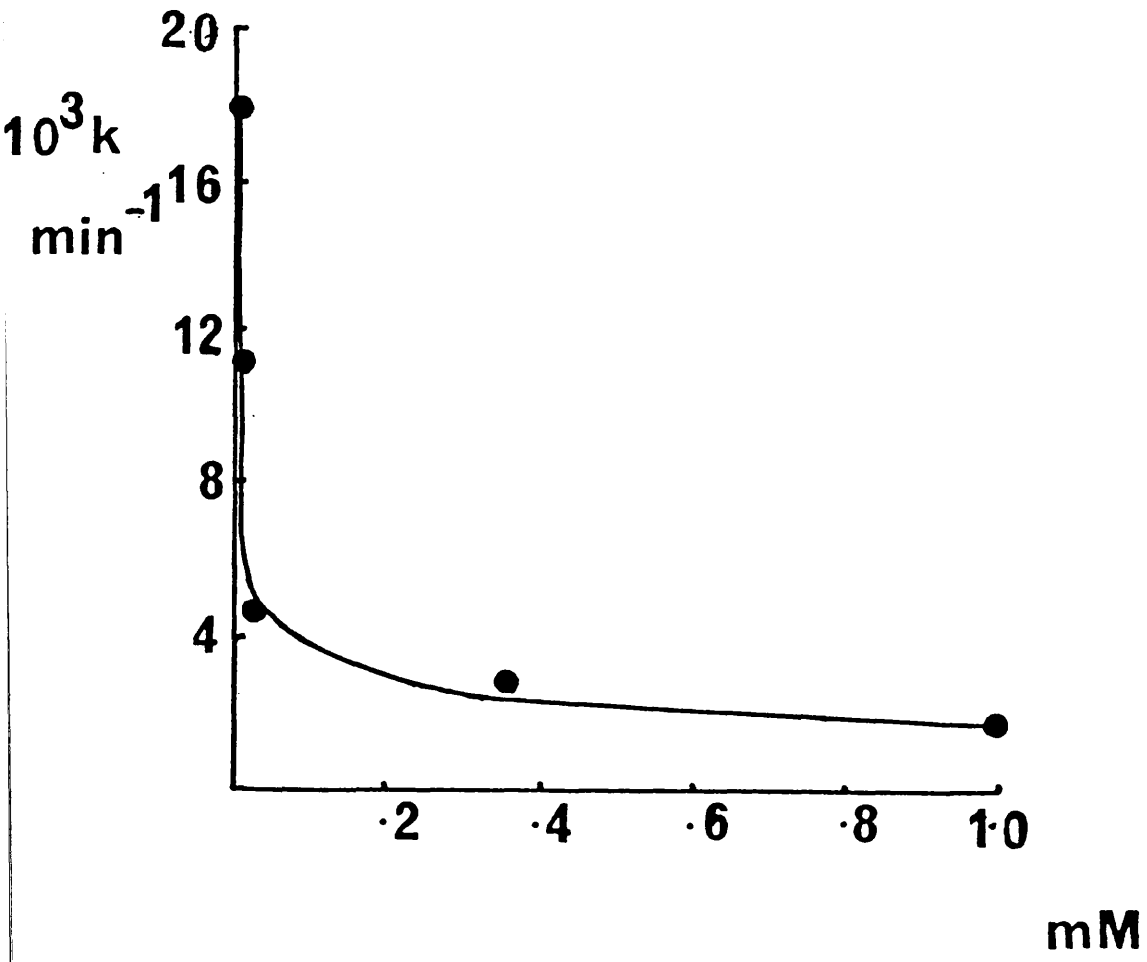
average pseudo first order rate constant (k) calculated in this latter series of experiments using 10mM G7063-2 was $17.8 \times 10^{-3} min^{-1}$ as compared to $27 \times 10^{-3} min^{-1}$ obtained in the initial experiments. As indicated in Methods 2.25 the initial stock solution was pale yellow. After storage for a week at $4^{\circ}C$ the solution was brown and from the above data would have appeared to have lost some activity. It has been suggested (D. Noble) that this could be due to hydrolysis of the epoxide ring.

When the protection by chloramphenicol was investigated over a range of concentrations (0.01-1mM) versus 5mM G7063-2 it was found (Fig.49) that very low concentrations of chloramphenicol were able to protect against G7063-2 inhibition. This would appear to indicate that the 'active' concentration of G7063-2 may have beenconsiderably less than its supposed concentration. The lack of inhibitory action of G7063-2 in the poly UG directed protein synthesis assay (N. Payne and E. Cundliffe personal communication) may also have been related to this apparent discrepancy in active constituent present.

Fig.49.

Concentration dependence of the protection by Chloramphenicol of the rate of inactivation of CAT_{III} by G7063-2.

CAT_{III} (0.2mg/ml) was incubated at 37°C in standard buffer with G7063-2 (5mM) with a range of chloramphenicol concentrations (0.01-1mM). Samples were taken at intervals, diluted into standard buffer and assayed by the standard method. For each concentration of chloramphenicol used a pseudo first order rate constant was calculated using the equation $\ln \frac{E}{E_o} = -kt$.


20
10^3k
min^{-1}
16
12
8
4
·2
·4
·6
·8
1·0
mM

Chapter Ten

Discussion

The overall aim of the studies described in this thesis was the use of chemical modification techniques to define active site residues of a chloramphenicol acetyltransferase variant which was under study by X-ray diffraction and for which amino acid sequence data was available. The enzyme variant chosen for study (CAT_{III}) is easily purified in large quantities by selective elution from a highly substituted affinity resin. Overall yields of pure enzyme following purification varied in this study between 65 and 80 percent. Polyacrylamide gel electrophoresis in SDS gave an apparent molecular weight of 25,000 for the protein monomer. Molecular weights determined by gel electrophoresis have tended to be an underestimate for other CAT variants. CAT_I and the protein specified by staphylococcal plasmid pC194 have actual molecular weights of 25,668 (40) and 25,900 (63) respectively, when calculated from amino acid or DNA sequence data, yet have been found to run regularily at 24,000 and 22,000 (38) respectively on gel electrophoresis. This may reflect difficulties in completely unfolding the protein as mentioned in Section 3.4.

CAT_{III} was isolated from a mutant strain of E.coli (carrying plasmid R387) which is able to grow on 700µg/ml of chloramphenicol, a concentration two fold higher than that which normally inhibits the original strain harboring the gene for CAT_{III}. The specific activity of the enzyme isolated from the high resistance strain was 1900µmole/min/mg of protein measured under standard assay conditions. This value is the highest recorded specific activity within the CAT family and is 4 fold higher than the specific activity of the enzyme of the original plasmid-bearing strain. The expectation was that an organism resistant to such high level of chloramphenicol might possess a higher copy number for the plasmid carrying the cat resistance gene, or increased expression of the gene. Whether this is also true for the strain E.coli J53 (R387;Cm700) is not known nor the difference between the original CAT_{III} and the CAT_{III}

purified in these studies. The increase in catalytic efficiency of the 'mutant' is potentially interesting and important, especially in relation to the question of how enzymes evolve. A comparative study of the 'mutant' and the original protein could be undertaken to determine at what level the catalytic differences reside. Possible explanations include a change in an amino acid(s) involved in the catalytic mechanism of the enzyme or a change of an amino acid(s) which alters the conformation of the active site. The former would require the postulation of a change in mechanism and hence seems unlikely. The location of any amino acid change will be determined only by a comparison of the amino acid sequence of the two proteins.

Comparative peptide mapping using S.aureus V8 protease and papain and Ouchterlony immunodiffusion experiments confirmed that CAT_{III} was related but not identical with the CAT_I and CAT_{II} proteins. Equilibrium binding experiments with chloramphenicol indicated a 1:1 binding stoichiometry per monomer with a binding constant of 15μM at 4°C and pH 7.8. Such a result doesnot distinguish between a model wherein each subunit has an active site as compared with one wherein residues from two contiguous subunits constitute a binding site. This point has proved difficult to investigate since CAT is highly resistant to dissociation (L.C. Packman, Univ. of Leicester, Ph.D Thesis, 1978). Michaelis constants for the two substrates of the variant were determined using a modified spectrophotometric assay. The values obtained, 17.6μM for chloramphenicol and 74μM for acetyl-S-CoA are not dissimilar to those reported for other variants (36-39), a result which suggests that there is likely to have been a high degree of conservation of the substrate binding domains in the CAT 'family' of enzymes. The wide range of k_{cat} values (30 sec^{-1} for S.aureus CAT_C to 780 sec^{-1} for CAT_{III} in this study), suggests on the other hand that catalytic efficiency (as distinct from ligand binding) may be determined by rather small but nonetheless critical changes at or near the catalytic

centre. In the absence of even a low resolution structure from X-ray diffraction data, further speculation is meaningless. Although other functional groups are certain to play a role in the mechanism, His 193 must be central in a functional sense and the reactivity of the critical imidazole moiety could be a 'handle' for exploring the catalytic centre. That is, a study of the reactivity of this residue in several CAT variants which differ in catalytic efficiency by almost two orders of magnitude might yield a positive correlation which would inform further speculations.

Preliminary chemical modification experiments were carried out using iodoacetamide and iodoacetic acid. The pseudo first order rate constants calculated for the inhibition were low and very similar to those observed with the CAT_I and CAT_C variants (Table6, 8) despite the fact that only a histidine residue was modified in the latter case. Iodoacetamide was approximately 15 times as effective as an inhibitor as was iodoacetate. Chloramphenicol protected against the inactivation and the incorporation of label, indicating that the residues modified were located within the chloramphenicol binding site/active site of the enzyme. Whilst it could be demonstrated that the presence of acetyl-S-CoA prevented inactivation it was not possible to show that it prevented labelling by $[^{14}C]$ iodo acetamide because of the incomplete removal of reagent by dialysis which occurred in the presence of substrate. Alternate methods for the removal of $[^{14}C]$ iodoacetamide by, for example, gel filtration in the presence of 6M guanidine hydrochloride, were not employed but might well have proved effective.

The degree of protection (70%) against inactivation observed with acetyl-S-CoA was surprising when compared with previous results obtained for other CAT variants (Table 6). The high degree of protection was confirmed, however, using the substrate analogues acetonyl-S-CoA and methyl-S-CoA. Zaidenzaig and Shaw (58) identified three different radio-active peptides-

Gln-Ser-Val-Ala-Gln-CmCys-Thr-Tyr

His-Ala-Val-CmCys-Asp-Gly-Phe

CmCys-Asp-Glu-Trp-Gly-Ala-Gly-Gln

in an analogous labelling experiment with the CAT_I variant. One peptide was identified in the analogous experiment with CAT_C (38)-

His-CmCys-Ala-Val-Cys

Comparison with the data obtained with the CAT_{III} variant-

Arg-Leu-Pro-CmCys-Gly-Phe

His-CmHis-Ala

illustrates some interesting points. Although a cysteine residue exists at position 31 in CAT_I and CAT_{III} this was not found to be the modified residue in the latter case (position 31 corresponds to the cysteine in the first peptide listed above). Instead, a cysteine residue corresponding to residue 57 of the CAT_I sequence was found to be labelled, a result which may reflect differences in folding of the two variants. In this connection it may be useful to recall that amidination experiments with $[^{14}C]$ methylacetimidate identified a uniquely unreactive and different lysine residue in the Type I and Type III variants (Lys 136 of CAT_I and Lys 38 of CAT_{III}) (62).

After modification, both CAT_{III} and CAT_C were found to contain 3-carboxymethylhistidine. Some doubt exists as to the amino acid modified (and labelled) in the corresponding peptide (see above) of CAT_I. A repeat of the modification experiment previously reported with CAT_I gave radioactivity incorporated corresponding to 2 moles of $[^{14}C]$ label per mole of enzyme monomer as compared with 0.7 moles of carboxymethylhistidine and 1.3 moles of carboxymethylcysteine by amino acid analysis (38). Although the positions of the modified residues were never established it seems likely that the difference observed in the labelling may reflect a situation whereby either the histidine or the cysteine residue (but never both together) are carboxymethylated. The region of the primary

structure in which the modified histidine residue is found (192-197) is highly conserved in all variants and has thus been tentatively proposed as a likely candidate for at least a portion of the active site.

The inability of iodoacetate to inhibit the enzyme seemed to support the hypothesis that a negatively charged residue may reside at or near the active site of the CAT_{III} variant, as previously suggested for the CAT_{I} variant (58). DTNMB and DTNT are novel analogues of Ellman's Reagent which were synthesized to test the hypothesis that removal of the negative charge would allow modification of the enzyme. Prior to quantitative modification experiments with the new compounds it was necessary to characterize them as thiol-specific reagents and for their ability to inhibit the activity of CAT_{III}. In this respect DTNMB behaved in the expected manner, giving a λmax and extinction coefficient very similar to that of DTNB. DTNT, however couldnot be reduced with 2-mercaptoethanol (a 10,000 fold molar excess was tried to no avail) and didnot inhibit the enzyme. By way of contrast the DTNMB analogue was approximately 500 times as effective as an inhibitor as was DTNB at a 10 fold lower concentration of reagent. The stoichiometry of the inactivation process with DTNMB (as judged by the liberation of the thiolate anion concurrent with inhibition) was one mole of thiolate anion released (and hence one mole of modified enzyme) per mole of CAT monomer. Inactivation was virtually complete and was reversed by the addition of 2-mercaptoethanol. The lack of solubility of the compounds in these experiments caused some difficulty and prevented experiments to try and modify a second sulphydryl group. Lack of solubility also probably accounted for the lack of reaction seen with DTNT. This compound was only sparingly soluble in a range of solvents, gave an uncharacteristic absorption spectrum, and failed to produce the expected yellow colour on addition of 2-mercaptoethanol.

As the position of the residue modified by DTNMB was not determined, it is not possible to say whether the cysteine residue modified by iodoacet-

amide was also modified by DTNMB. The sequential treatment of CAT_{III} with DTNMB followed by [^{14}C] iodoacetamide (data not shown) led to a reduction in radioactivity incorporated as compared with the control which was not pretreated with the chromogenic disulphide. Although it is tempting to infer that modification of the sulphydryl by DTNMB prevented its reaction with [^{14}C] iodoacetamide, it is also possible that steric hindrance by the bulky thionitrobenzoate group may have generally prevented incorporation.

Preliminary studies suggested that both chloramphenicol and acetyl-S-CoA were able to protect CAT_{III} from inhibition by DTNMB. The apparent protection by acetyl-S-CoA was investigated further with the analogues acetonyl-S-CoA and methyl-S-CoA. Use of the unreactive thioethers demonstrated that the protection seen with acetyl-S-CoA was likely to be due to hydrolysis of the substrate and generation of the mixed disulphide of DTNMB and CoA in situ, yielding a pseudo-protective effect. The slow turnover of the thioester may thus provide 'buffer' thiol to react with the DTNMB in preference to the reactive enzyme thiol. The latter must be part of or close to the chloramphenicol binding site in view of the protection seen with the antibiotic, a view expressed previously in connection with the reaction of thiol reagents with the CAT_I (58) and CAT_{II} (43) variants.

Although the three enteric variants (CAT_I, CAT_{II} and CAT_{III}) appear to possess at least one reactive sulphydryl at or near the chloramphenicol binding site, the staphylococcal variant (CAT_C) appears to be indifferent to thiol-specific reagents. The presence of a reactive cysteine in CAT variants from Gram negative bacteria has often complicated the interpretation of experiments aimed at elucidating the possible role of a histidyl residue in the catalytic mechanism.

In an attempt to investigate the role of histidine residues in the CAT_{III} protein without interference from cysteine residues, DEP was

chosen initially as a 'histidine reagent' because not only is it fairly specific for histidine residues but also preliminary experiments with the CAT_C variant showed that it inhibited this variant (38). The pH of the buffer (pH 6.0) was chosen to maximize the reactivity of the imidazole group. It seems certain that the CAT_{III} variant contains at least two classes of reactive histidine residues with DEP since two rates of loss of activity could be defined under the conditions used. Initial attempts to reverse the loss of activity by the addition of hydroxylamine were unsuccessful, but conditions were later found in which the rapid loss of activity was prevented by hydroxylamine. The reversal of DEP inhibition by hydroxylamine decreases with time, suggesting a slow build up of the Bamberger intermediates I and II (Fig.31). Two histidine residues were modified by DEP and the modification of one, the 'fast' residue, was protected by the addition of chloramphenicol. The latter was unable, however, to protect against the 'slow' rate of loss of activity indicating that this residue was outside the chloramphenicol binding site. Apparent second order rate constants calculated for the fast and slow inactivation reactions yield values of 180 $M^{-1}sec^{-1}$ and 10 M^{-1} sec^{-1} at 25°C respectively. The former is dramatically higher than that for free histidine (24 $M^{-1}sec^{-1}$ at 20°C (91)) whilst the lower value for DEP reacting with CAT_{III} resembles that for the free amino acid more closely. Pseudo first order rate constants obtained with the CAT_{III} variant are remarkably similar to those obtained with the CAT_C (2.1 min^{-1}, 0.49 min^{-1}) (38) and CAT_{II} (2.8 min^{-1}, 0.10 min^{-1}) variants (W.V. Shaw, personal communication). Under conditions similar to those employed in these experiments complete protection was afforded to the fast rate of loss of activity in the presence of chloramphenicol for CAT_C and 75% in the case of CAT_{II}.

No attempt was made to determine the position of the residues modified by DEP using a radiolabelled reagent. Although experiments with [^{14}C]

reagent (105) and [^{3}H] DEP (106) have been described, these compounds have not been widely used, probably because carbethoxyhistidine is unstable under acidic conditions. In circumstances where Bamberger cleavage takes place the label is not lost, but the imidazole group is destroyed. Despite this complication it might have been useful to have used radioactive DEP to confirm the amino acid analysis which was subject to some variation.

An alternative to the use of DEP for modification of the histidine residues of CAT_{III} was the reagent MNBS, an inhibitor used by Bender to modify His 57 of chymotrypsin (107). Preliminary studies revealed that this reagent did inhibit the enzyme irreversibly. Chloramphenicol protected against inhibition of CAT_{III} but acetyl-S-CoA, acetonyl-S-CoA and methyl-S-CoA failed to do so, further evidence for the location of a histidine residue within or contiguous with the chloramphenicol binding site. The presence of methyl-S-CoA actually accelerated the rate of inhibition. Methyl-S-CoA also reduced protection seen with chloramphenicol as did acetonyl-S-CoA. Likewise protection afforded by the unreactive 3-fluorochloramphenicol was reduced by the presence of acetyl-S-CoA.

One hypothesis to explain or reconcile these observations involves a model wherein distortion of the active site geometry takes place with methyl-S-CoA but not (at least to the same degree) with the binding of acetonyl-S-CoA or acetyl-S-CoA. An increase (approximately two fold) was observed for the dissociation constant for chloramphenicol in the presence of methyl-S-CoA over that with chloramphenicol alone. It would be tempting to invoke this as evidence that the binding of methyl-S-CoA causes a conformational change in the chloramphenicol binding site allowing easier access for the reagent MNBS. The unusual effect seen with methyl-S-CoA may, on the other hand, indicate a requirement for the carbonyl group of an acyl-S-CoA ligand for proper binding.

Since MNBS inhibited the enzyme, it was synthesized in radioactive form from p-nitrobenzene sulphonyl chloride. The latter compound rapidly inhibited the enzyme, an effect which was not explored further since chloramphenicol, acetonyl-S-CoA and methyl-S-CoA all failed to protect against the loss of activity. Since full activity of the enzyme could be restored on addition of 2-mercaptoethanol, a reactive sulphydryl group outside the substrate binding sites seems likely to have been modified.

When CAT_{III} was modified by $[^{14}C]$ MNBS no difference was observed between samples modified in the presence or absence of methyl-S-CoA, whilst chloramphenicol reduced the level of incorporation. The incorporation of $[^{14}C]$-methyl into the protein confirmed the previous spectrophotometric observation that the compound (MNBS) was methylating rather than sulphonylating the protein. Analysis of a hydrolysed sample of enzyme on a silica plate showed that the radioactivity was to be found in 3-methyl histidine rather than 1-methylhistidine. The presence of methyl-S-CoA did not alter the position of the incorporated label, a result which would indicate that the increase in rate of inactivation of the CAT_{III} by MNBS in the presence of methyl-S-CoA was not due to the modification of another residue. The exact location of the modified residue in the primary structure has yet to be determined definitively. It would also be useful to examine further the catalytic properties (K_m and K_{cat} values) of the modified enzyme. In the case of chymotrypsin the substrates were bound with the same affinity after modification and the enzyme retained a low level of catalytic activity (108). Small local changes in the precise position and orientations of the side chains of His 57 and Ser 195 were thought to account for the observed catalytic rate. A careful kinetic examination of the modified protein may therefore reveal whether a similar case exists for the CAT_{III} variant.

The results of the experiments with DEP and MNBS led to the conclusion that histidine(s) were important in the catalytic mechanism of the CAT_{III}

protein. This was further tested using the dye rose bengal, an anionic dye which is known to be selective for histidines (114,115). The protein was inhibited by the dye in the presence of light and since the inhibition was dependent on the ionization of a group with a pK_a of 6.4 it would appear that a histidine group has indeed been modified. Acetyl-S-CoA protected against the loss of activity, whilst chloramphenicol failed to do so. These results can be contrasted with those obtained with the CAT_C variant with which methylene blue was used (59). In this case, chloramphenicol rather than acetyl-S-CoA afforded protection. This may reflect the fact that methylene blue is cationic whilst rose bengal is an anionic dye. The results of the experiments with the dye rose bengal led to the hypothesis that positively charged groups within the acetyl-S-CoA binding site may be influencing the binding of the dye. Experiments were thus devised to see whether these groups were located in the acetyl-S-CoA binding site, and hence could be inferred in the binding of the substrate,a not uncommon occurrence for negatively charged substrates (121,122,123). Two classes of reactive groups were observed on modification of CAT_{III} with the reagent butanedione. Acetyl-S-CoA protects against the inhibition, indicating that positive charges are, indeed, located within the acetyl-S-CoA binding site. Specific functions of active site arginines can, however, vary from direct to partial to almost no participation in coenzyme binding. Since acetyldephospho-S-CoA is not a substrate for the enzyme (29) this would imply that arginine groups are required for the binding of the substrate in the case of the CAT variants. It would be useful to locate the arginine groups modified in the primary structure. $[^{14}C]$ Butanedione is not available, however, since it is very unstable and rapidly polymerises via a process thought to be initiated by the emission from radioactive decay (129). The alternative arginine modifying reagent phenylglyoxal can be obtained in a stable radioactive form so this reagent is often used

in conjunction with butanedioneto identify modified groups. It is less selective for arginine than butanedione and some lability of the label has been noted in subsequent fragmentation and isolation procedures (130). Phenylglyoxal need not necessarily modify the same arginine residues that were labelled with butanedione, but it would be useful, however, to extend these preliminary studies by using phenylglyoxal.

The final modification described in these studies with the epoxide antibiotic G7063-2 is not really related to the previously described studies but did yield some interesting if unexpected results. Despite difficulties with the stability of the compound it was found to be a good inhibitor of CAT_{III} and full protection could be afforded by chloramphenicol. The protection by acetyl-S-CoA was not investigated further due to lack of availibilty of reagent. Further investigations of this protection using the non reactive analogues, acetonyl-S-CoA and methyl-S-CoA are required in order to substantiate whether the protection was 'real' or 'apparent' (that is, due to the interference of the inhibitor by acetyl-S-CoA). Initially it may appear curious that the reagent reacted with the enzyme in such a specific manner. Tanaka *et al* (56), however, have reported the inhibition of CAT_I by basic triphenyl methane dyes and CAT_I is also able to bind fusidic acid, a steroid which bears little obvious structural relationship to chloramphenicol. Interestingly another case has been reported in which an epoxide antibiotic inhibits an enzyme in a specific manner. The antibiotic pentalactone isolated from Streptomyces can inhibit glyceraldehyde 3-phosphate dehydrogenase. Pentalactone, an epoxide, was found to covalently bind an essential thiol group of the enzyme (133). It seems feasible that G7063-2 interacts with the reactive thiol group in the chloramphenicol binding site of the CAT_{III} protein.

In conclusion, therefore, these studies have identified a reactive sulphydryl group (identified by studies with iodoacetamide and DTNMB)

and a reactive histidine (identified by studies with iodoacetamide, DEP, rose bengal and MNBS). In both cases the residue must be located either at or near the active site since the substrates of the enzyme, chloramphenicol and acetyl-S-CoA are able to protect against the loss of activity. Prior to this work reactive thiol groups had been identified within or near the CAT_I and CAT_{II} active sites, but when the experiments with the sulphydryl reagents were repeated with CAT_C (see Table 6) no significant loss of activity was observed. The CAT_C variant was, however, found to be inhibited by DEP and the dye methylene blue (59) indicating a reactive histidine in the protein. From these results, therefore, it was unclear whether the Gram negative and Gram positive variants use different catalytic mechanisms based on sulphydryl and histidine residues respectively or whether they share a common mechanism using one of these residues. The CAT_{III} variant, however, has been found to be sensitive to reagents which modify imidazole and sulphydryl groups and hence these results indicated that Gram negative variants were sensitive to histidine directed reagents. Lately this observation has been confirmed since CAT_I and CAT_{II} have both been found to be sensitive to inhibition by DEP (W.V. Shaw, personal communication). Interestingly the pseudo first order rate constants for the CAT_{III}, CAT_{II} and CAT_C (with respect to inhibition by DEP) are remarkably similar. There is, however, great variability in the rates of inactivation observed for the CAT variants when treated with sulphydryl directed reagents.

A schematic representation of the 'active site' of a CAT variant is shown in Fig.50. Site A is likely to consist of hydrophobic (aromatic) functional groups that interact with the C_1 substituent of chloramphenicol. E corresponds to the reactive thiol group
Site B would correspond to His 193, whilst Site C would correspond to the arginine residues identified in experiments with butanedione. Three positions of interaction are shown with the anionic portion of acetyl-S-CoA but the actual number of arginine residues within the acetyl-S-CoA

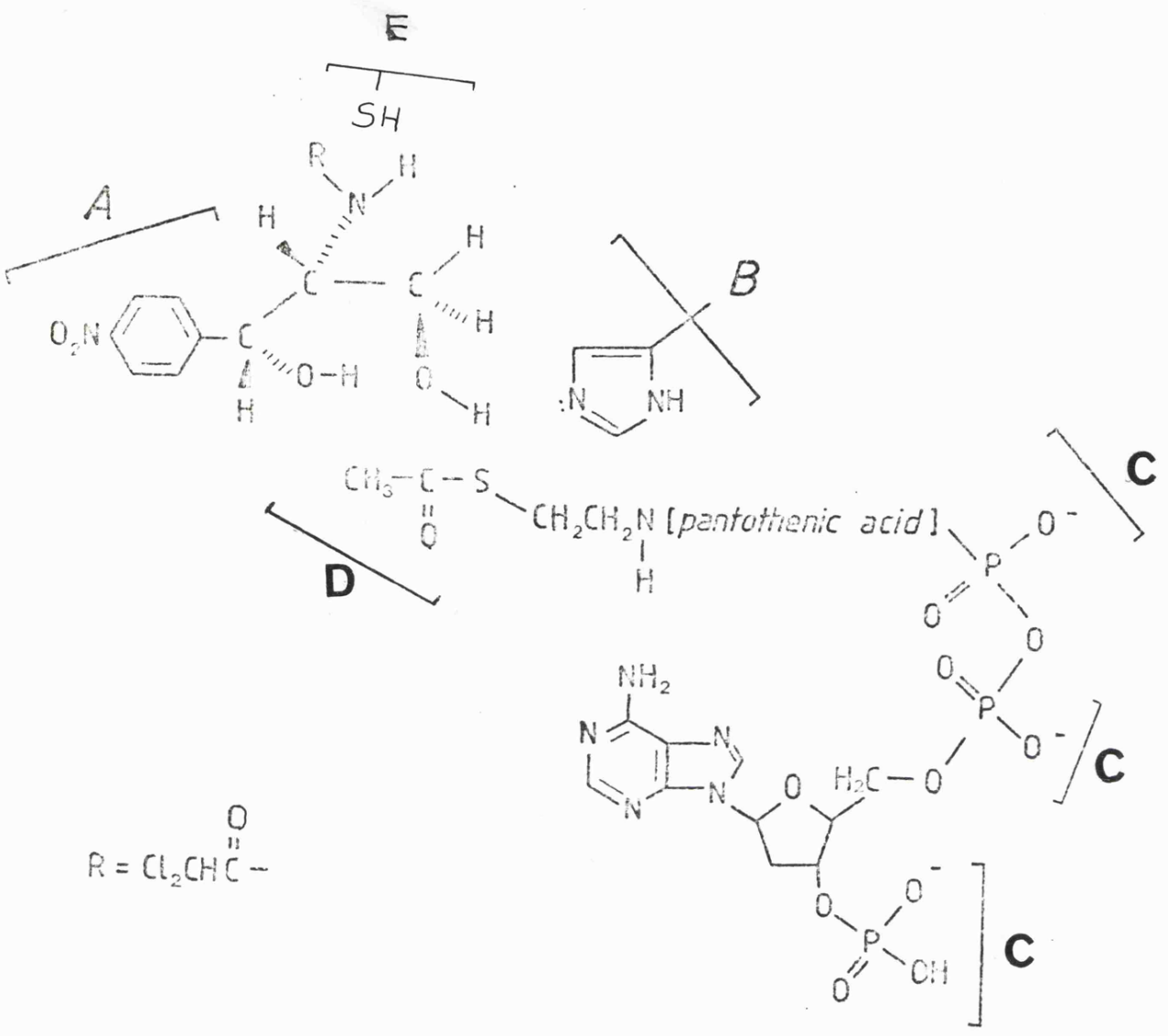


Fig.50.

Representation of an active site of a Chloramphenicol Acetyltransferase variant.

binding site and their precise role is unknown. A constraint on the size of the acyl substituent probably exists at Site D since transfer drops off for substituents longer than the propionyl moiety (54).

Histidine 193 modified by iodoacetamide in CAT_{III} is conserved in all CAT variants studied so far, indicating the importance of this region but without really specifying a particular role. A general base mechanism would be compatible with the known kinetic data and would hence assign a role to this uniquely reactive histidine residue. It could be postulated that the reaction mechanism proceeds by one of two routes-either the proton of the C_3 hydroxyl is labilised or a stepwise mechanism occurs with an initial attack at the C_2 carbon of the acetyl group. Both of these mechanisms would allow for the slow turnover of acetyl-S-CoA when it is incubated with CAT in the absence of chloramphenicol, a situation wherein water acts as the acetyl acceptor or source of the nucleophile attacking the carbonyl of acetyl-S-CoA. Futher experiments using different techniques (NMR spectroscopy and X-ray diffraction analysis)are required to confirm the presence of and the role of the histidine in the active site.

Further chemical modification experiments could take two directions. A detailed examination and comparison of the other enteric (Gram negative) variants modified with MNBS, DEP and rose bengal would provide useful information on the role of histidine in these variants. Chemical modification studies of other CAT proteins(for example, those from Proteus and Haemophilus species) would also contribute to the overall understanding of the CAT family.

A second and perhaps more promising approach would to continue to investigate CAT_{III} as an enzyme 'in its own right'. Identification of the location of the reactive arginine residues, for example, would contribute to a more detailed understanding of the geometry of the active site, and whilst the sulphydryl groups may not be involved directly in the catalytic mechanism further experiments may reveal whether they play

any role at all in the active site of the enzyme. A small modification (analogous to the methylation of the histidine residue by MNBS) of the reactive sulphydryl group in the chloramphenicol binding site may clarify its role. Most of the sulphydryl modifying reagents used with the CAT system have been bulky (NEM, DTNB,DTP) but Zaidenzaig and Shaw (58) have reported the modification of CAT_I using the reagent potassium cyanide. CAT_I was modified with a 1.5 fold excess of DTP such that 95% inactivation occurred. After dialysis to remove excess reagent the modified enzyme was treated with a 5 fold molar excess of $[^{14}C]$ cyanide. 0.74 moles of CN^- were introduced per mole of enzyme monomer but no reactivation of the enzyme was noted. The lack of reactivation of the enzyme suggested either a reduced cysteine was essential for catalytic activity or the conformational constraints on the cysteine sequence were critical and could not even accommodate a CN^- substitution, or that the initial reaction produced an irreversible conformational change. The use of the reagent methane thiosulphonate (a reagent which methylates sulphydryl groups) described by Bloxham would allow reexamination of these hypotheses.

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Studies on a Chloramphenicol Acetyltransferase Variant

by Angela Jane Corney

An E.coli Chloramphenicol Acetyltransferase Type III variant has been purified and characterized. The variant has been shown to be related to, but not identical, to the E.coli Type I and Type II variants.

Inhibition studies with the reagents iodoacetamide and iodoacetate revealed that iodoacetamide was 15 times as effective as an inhibitor as was iodoacetate. Experiments with [^{14}C] iodoacetamide gave an incorporation of approximately 1 mole of [^{14}C] label per mole of enzyme monomer. Two unique radioactive peptides were isolated and sequence analysis indicated that a histidine and a cysteine residue were modified. The substrates, chloramphenicol and acetyl-S-CoA were both able to protect against the loss of activity.

To test the hypothesis that a negatively charged residue was near the active site of the variant the 5,5' dithiobis(2-nitrobenzoic acid) analogues, 5,5' dithiobis(2-nitromethylbenzoate) and 5,5' dithiobis(2-nitrotoluene) were characterized. The latter was found not to be suitable as an inhibitor but the former rapidly inhibited the enzyme. Chloramphenicol was able to protect against the loss of activity, supporting the hypothesis that a reactive sulphydryl group existed near or in the chloramphenicol binding site.

The histidine residue was further investigated using the reagents diethylpyrocarbonate (DEP) and methyl nitrobenzene sulphonate (MNBS). DEP modified two histidine residues, one within the chloramphenicol binding site and one outside the substrate binding sites. Radioactively labelled MNBS was synthesised and analysis of the modified enzyme revealed the formation of 3-methylhistidine. The acetyl-S-CoA analogues, acetonyl-S-CoA and methyl-S-CoA, were synthesised and characterised. Methyl-S-CoA was found to accelerate the rate of inhibition seen with MNBS and increased the dissociation constant of chloramphenicol with respect to the enzyme.

Preliminary studies were also carried out with the reagents rose bengal, butanedione and an epoxide antibiotic.

The data suggests that a reactive histidine and cysteine residue exists within or near the active site of the variant. A proposed reaction mechanism with the histidine residue acting as a general base is postulated.