THE ACTIVE SITE GROUPS OF N-ACETYLNEURAMINIC ACID LYASE

A thesis submitted to the University of Southampton for the degree of

MASTER OF PHILOSOPHY

by

FRAGISKOS N. KOLISIS

Department of Physiology and Biochemistry

The University of Southampton 1974.

T O M Y P A R E N T S

ACKNOWLEDGEMENTS

I wish to express my sincerest thanks to my supervisor, Dr. J.E.G. Barnett for his guidance, advice and friendly encouragement and tolerance throughout this work.

My thanks are also extended to Professor K.A. Munday for the use of the facilities of his department and to anyone else who helped me in the fulfilment of this work.

Finally I wish to thank my father Nikolaos for his financial support that made my studies and stay in Southampton possible.

A B S T R A C T

FACULTY OF SCIENCE

PHYSIOLOGY and BIOCHEMISTRY

Master of Philosophy

THE ACTIVE SITE GROUPS OF

N-ACETYLNEURAMINIC ACID LYASE

by Fragiskos N. Kolisis

N-acetylneuraminic acid lyase from Clostridium perfrigens was rapidly inactivated by the substrate analogue chloropyruvate at pH 7.2 and 37°C. At 5°C, 0.5 mM-chloropyruvate reacted with the enzyme about 10 times as fast as bromopyruvate. In contrast, at pH 6.0 and 9°C, chloropyruvate reacted with N-acetyl-cysteine 7 times more slowly than bromopyruvate. The rate of inactivation of N-acetylneuraminic acid lyase by chloropyruvate was diminished in the presence of pyruvate, indicating that the inhibitor acted at, or close to, the pyruvate binding site.

A brief, 2 min. incubation of the enzyme with 1.0 mM (^{14}C) chloropyruvate gave an inactive enzyme in which 4.5 moles of (^{14}C) chloropyruvate were bound per molecule of enzyme. It was shown that borohydride-reduction of the enzyme - (^{14}C) pyruvate Schiff's base complex gave 3.7 pyruvate residues covalently bound to the enzyme. This confirmed that there were probably 4 active sites per molecule of enzyme.

The enzyme inactivated by radioactive chloropyruvate was reduced by sodium borohydride and the hydrolysate gave a radioactive substance which was electrophoretically identical with S-(3 lactic acid)-cysteine. This confirmed the previous suggestions that there is one "essential" cysteine residue per active site.

It is suggested that, for this enzyme, chloropyruvate can be

selectively used to alkylate the active site residues, whereas it has previously been shown that bromopyruvate cannot.

The apparent molecular weight of the enzyme prepared by two slightly different methods was determined by Sephadex G-200 chromatography. The molecular weights were 100,000 and 250,000 respectively. The later value has been used to calculate the number of pyruvate residues bound to the enzyme and the probability that these are dimeric and tetrameric forms of the enzyme is discussed.

CONTENTS

INTRODUCTION

THE ALDOL CONDENSATION

i) Catalysis by acid or base

The aldol condensation can be regarded as a nucleophilic attack on a carbonyl group. Like other nucleophilic reactions of this group, the aldol condensation may be catalysed by acid, either by formation of conjugate acid of the carbonyl compound

or by the formation of a hydrogen bond

The hydration of acetaldehyde belongs to the latter type of reaction, and the following mechanism was suggested (Fig.1) (Bell,1949)

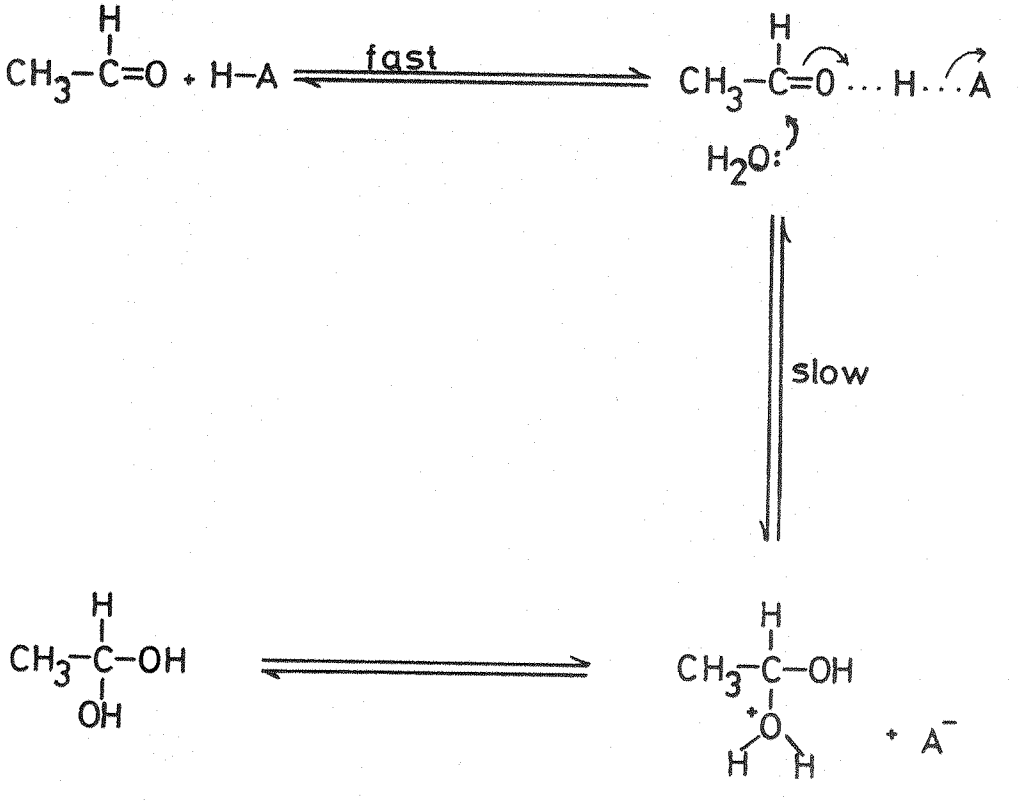


Fig.1.

Nucleophilic attack on the carbonyl group is also subject to base catalysis. Thus the hydration of acetaldehyde is also subject to general base catalysis and in the presence of a base, the mechanism

changes as follows (Fig.2)

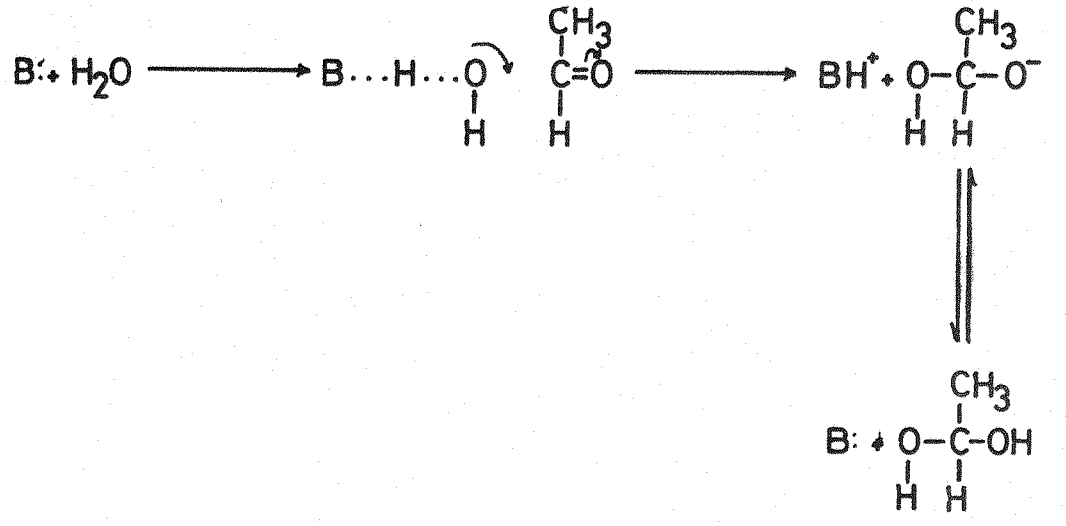


Fig.2

This catalysis is called general base catalysis because all bases in the solution contribute to the reaction. In specific base catalysis only one specific base can catalyse the reaction.

The aldol condensation is another example of a base-catalysed reaction involving attack on a carbonyl group. This action of bases results in the formation of a stabilized carbanion which can attack another carbonyl group forming a new carbon-carbon bond (Fig.3) (Gray,1971).

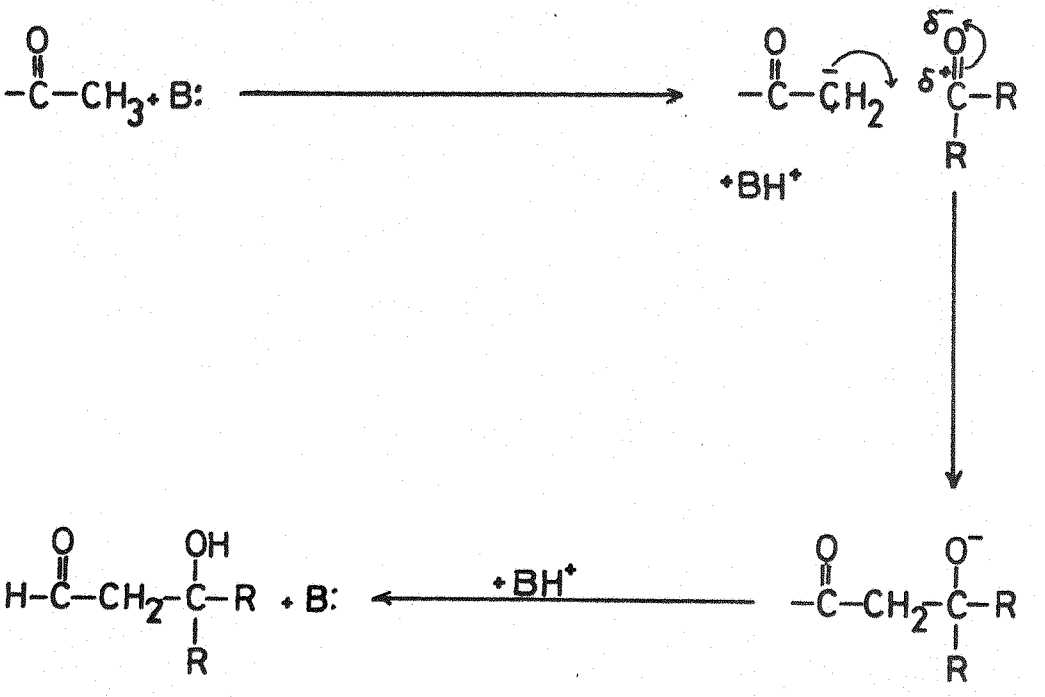


Fig.3

ii) Catalysis by formation of a Schiff's base

The oxygen of a carbonyl group attracts electrons strongly, acting as an "electron sink". This renders the carbon of the carbonyl group susceptible to attack by nucleophilic agents. In the case in which the nucleophile is an amine, RNH_2, a series of proton transfers can take place to give a Schiff's base (Fig.4).

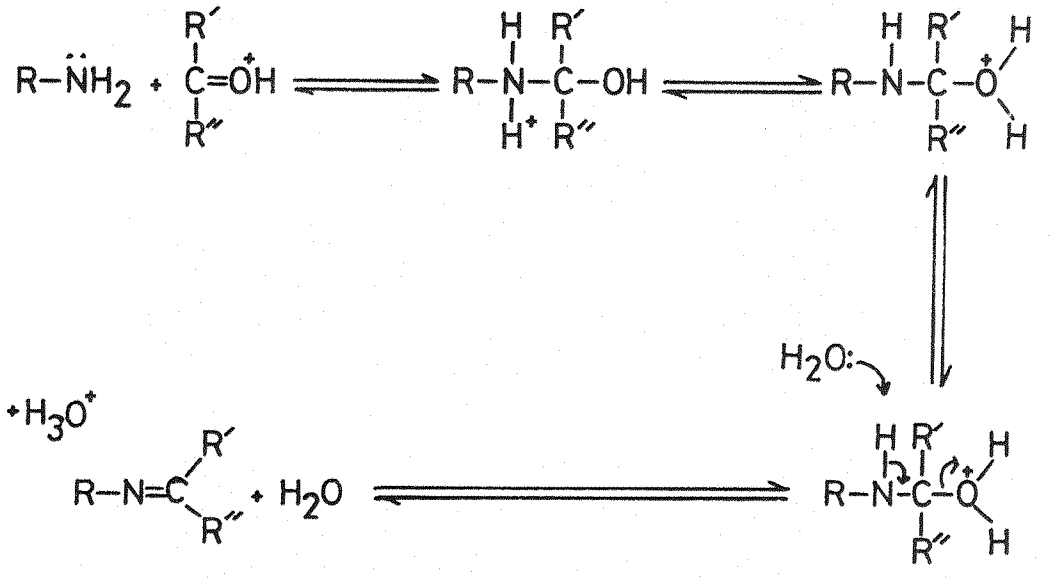


Fig.4

The Schiff's base formed, when protonated, is an even better "electron sink". Schiff's bases are intermediates in many enzyme-reactions. Non-enzymic aldol condensations, when catalyzed by primary amines, appear to proceed by an enamine intermediate (I) (Yasnikov et al., 1966,1967). The aldolization of acetone is a good example (Fig.5).

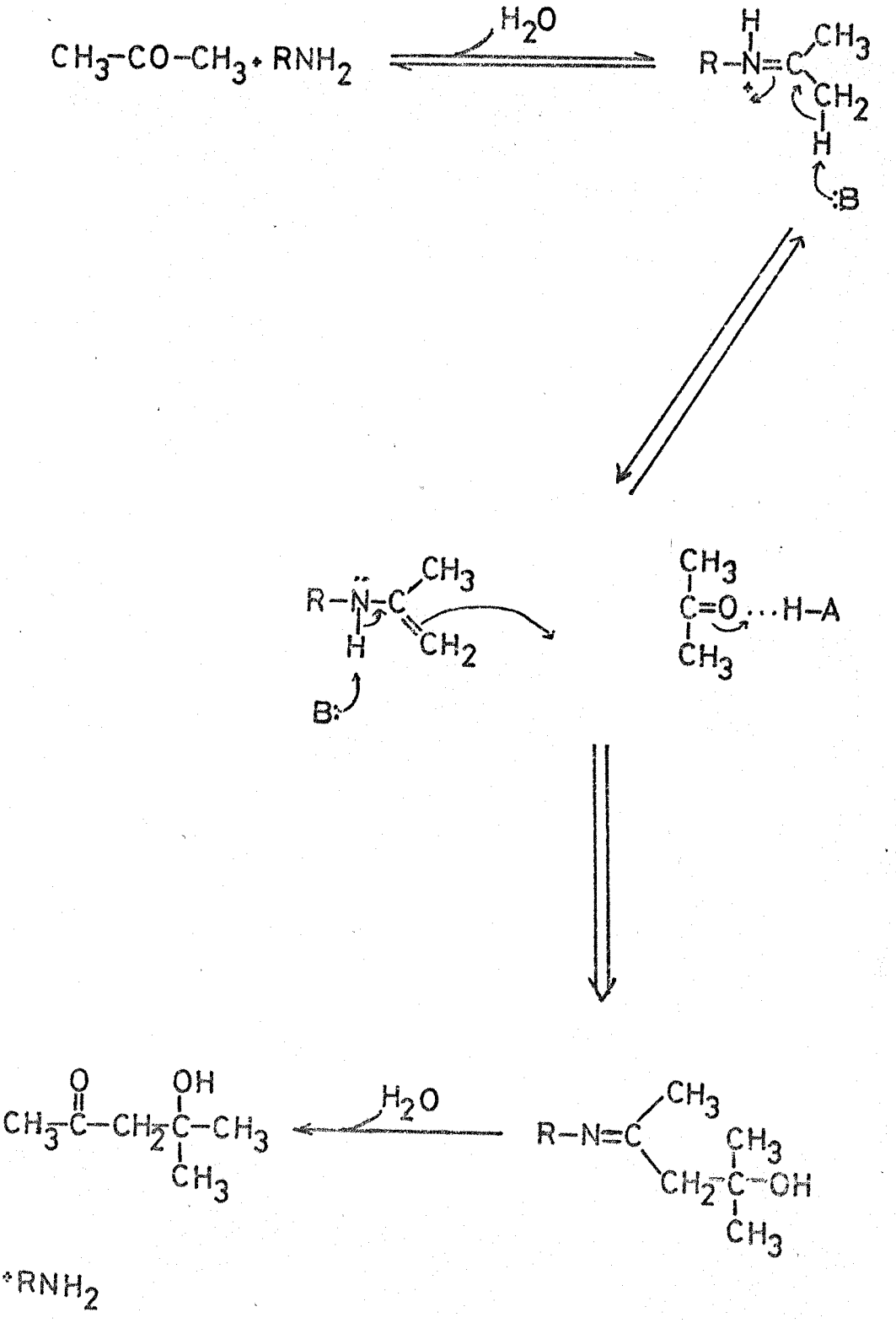

CH3-CO-CH3 + RNH2
H2O
R-N=C
CH3
CH2
H
B:
R-N-C
CH3
CH2
H
B:
CH3
C=O···H-A
CH3
O
OH
CH3-C-CH2-C-CH3
CH3
H2O
R-N=C
CH3
CH3
CH2-C-OH
CH3
*RNH2

Fig.5

ALDOLASES

Aldolases are the enzymes which catalyze aldol cleavage or condensation reactions. The first aldolase to be discovered and studied was fructose-1-6 diphosphate aldolase. This enzyme was discovered by Meyerhof and Lohman (1934) in mammalian muscle and catalyses the cleavage of fructose 1,6-diphosphate to yield dihydroxyacetone phosphate and glyceraldehyde phosphate according to the following scheme Fig.6.

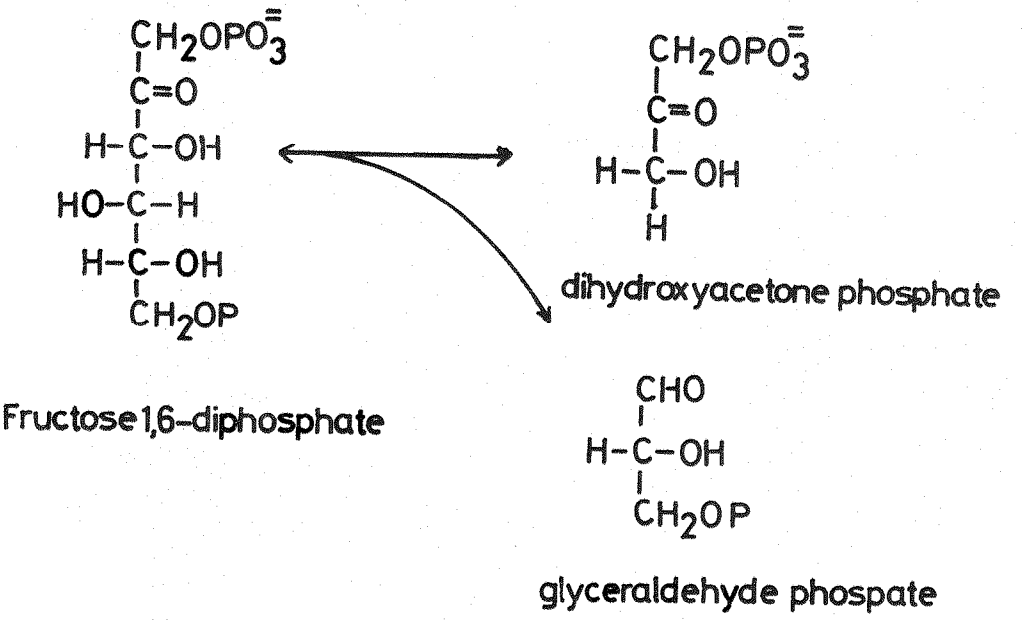


Fig.6

Fructose-1-6-diphosphate aldolase can accept a large number of aldehydes as substrates, including L-glyceraldehye, D- and L-erythrose, D- and L-threose, formaldehyde, acetaldehyde, glycolaldehyde and propionaldehyde, but is far more specific for the ketone. The three reactions which are catalyzed with the highest efficiencies are:

DHAP + D-glyceraldehyde-3-P $\longleftrightarrow$ FDP

DHAP + D-glyceraldehyde $\longleftrightarrow$ F-1-P

DHAP + D-erythrose-4-P $\longleftrightarrow$ sedoheptulose-7-P

The distribution of fructose 1,6-diphosphate aldolase in nature is very wide. It appears to be present in all animal and plant tissues. Aldolase is an intracellular enzyme usually found in the soluble fraction of the cytoplasm, however the aldolase of brain appears to be strongly bound to

the mitochondrial fraction along with hexokinase, and to a lesser extent, other glycolytic enzymes (Brumgraber-Abood, 1960).

A low aldolase activity is found in serum from normal animals but in certain pathological conditions (cancer, hepatitis, muscular dystrophy, myocardial infarction, etc.) the serum aldolase level is markedly elevated.

Warburg and Christian (1943) found that fructose 1,6-diphosphate aldolases may be divided into two classes depending on their properties.

The enzyme isolated from yeast is active only in the presence of bivalent metal ion. In contrast aldolase from muscle is not inhibited by chelating compounds such as cysteine, pyrophosphate or EDTA, and does not require metal ions for activity. Therefore aldolases may be divided according to their inhibition by chelating agents and the reversal of this inhibition by addition of bivalent metal ions (Table I.page 7).

Rutter (1964) classified aldolases from higher plants and animals as Class I, non metal-ion dependent, and the aldolases from fungi and bacteria which require metal ions as Class II. The muscle enzyme (Class I) has subsequently shown to form a Schiff's base at the active site with the substrate (Horecker, 1963; Grazi, 1963). This is discussed in detail later (p.10). In yeast aldolase (Class II) the role of the metal ion (Fig.7) is similar to that of the Schiff's base in promoting the aldol reaction.

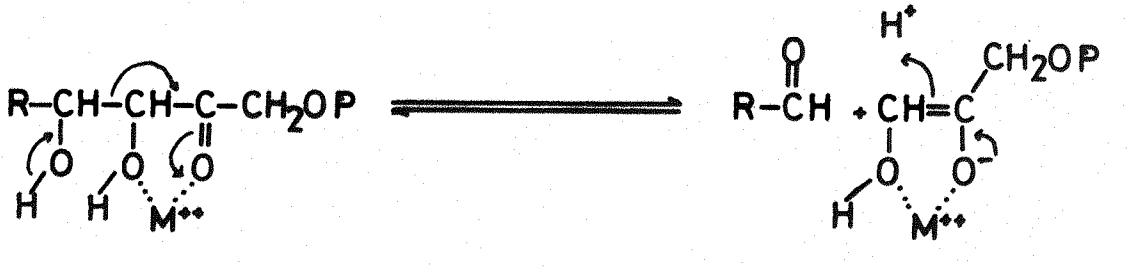


Fig.7

TABLE I

METAL IONS AND ALDOLASE ACTIVITY

Group	Source	Inhibition by chelating agents	Recovery of activity by addition of metal ion	Divalent metal present in isolated enzyme	Ref.
1	Muscle	No	-	None	a
	Liver	No	-	None	b
	Pea	No	-		c
2	Yeast	Yes	Zn^{++}, Fe^{++}, Co^{++}, Cu^{++}	1 Zn/ 50,000 g.	d
			Stimulation by K^{+}		e
	Aspergillus niger, oryzae, wentii	Yes	Zn^{++}, Fe^{++}, Co^{++}	1 Zn/ 50,000 g.	f
	Clostridium perfringens	Yes	Fe^{++}, Co^{++}		g
	Lactobacillus bifidus	Yes	Zn^{++}, Fe^{++}, Co^{++}		h
	Brucella suis	Yes	Fe^{++}, Mn^{++}		i
	Escherichia coli		Mn^{++}		k
	Mycobacterium tuberculosis (BCG)	Yes			l

TABLE I

References

a Warburg and Christian (1943)

b Peanasky and Lardy (1958)

c Stumpf (1948)

d Warburg and Christian (1943)
Rutter and Ling (1958)

e Richards (1960)

f Jagannathan and Singh (1954,1956)

g Barod and Gusalus (1950)

h Kuhn and Tiedeman (1953)

i Gary et al (1955)

k Knox et al (1948)

l Su and Ling (1956)

Both the muscle (Class I) and yeast aldolase (Class II) have now been isolated in a high state of purity. The properties of the final preparations seem to be independent of the method of purification. Some of these preparations are shown in Table II. (page 9). Other differences between those two classes of aldolases which have been studied so far are: the molecular weight of the Class I enzyme from animal sources is 150,000 - 160,000 daltons. Estimates of the MW after dissociation under a variety of conditions indicate the presence of three or four subunits. The Class II enzymes from plant sources are somewhat smaller with molecular weight of 120,000-140,000 d. The Class II aldolases are less sensitive to inhibition by sulphydryl-binding reagent than are the Class I enzymes. Class I aldolases also differ from those of II in their substrate specificity and kinetic parameters. The metaloaldolases (Class II) are unaffected by treatment with borohydride while Schiff's base forming (Class I) enzymes are irreversibly inactivated by borohydride treatment.

Class I Aldolases

Meyerhof and his collaborators have studied the nature of the functional groups and the specificity of fructose 1,6-diphosphate aldolase from rat muscle, which was the first isolated and crystallized by Warburg and Christian (1943). These studies on the specificity of the enzyme showed that the condensation reaction was specific for dihydroxyacetone phosphate while glyceraldehyde-3-P could be replaced by a number of aldehydes.

Topper and his coworkers (1957) obtained spectrophotometric evidence for the existence of an enzyme-substrate complex. This was later shown to be a Schiff's base derivative between the dihydroxyacetone phosphate and a specific lysine residue at the active site of the enzyme (Grazi et al., 1962; Horecker et al., 1963; Speck et al.,1963). These authors showed that the Schiff's base intermediate could be reduced with

TABLE II

ISOLATION AND PHYSICAL PROPERTIES OF ALDOLASES[a]

Source	Purification procedure	Activity* 30° moles FDP/ min./mole enzyme	Molecular weight
Skeletal muscle Rabbit	a	3580	
Rat	b**	5650	
Rabbit	c**	1770 5050	149,000
Rabbit	d**	1790	180,000
Rabbit	e**	5740	
Liver			
Ox	f**	400	159,000
Yeast	g**	1950	
	h**	(7000)[e]	65,000 - 75,000
	i	6800[e]	65,000 - 75,000
Aspergillus niger	k	1950	50,000 ***

* Based on molecular weight of 149,000 for muscle, 159,000 for liver, 50,000 for yeast and Aspergillus niger.

** Crystalline preparations.

*** Minimum molecular weight based on zinc content.

a Herbert et al (1940)

b Warburg and Christian (1943)

c Taylor and Green (1948)

d Glikina and Finogenor (1950)

e Beisenherz et al (1953)

f Pennasky and Lardy (1958)

g Warburg et al (1954)

h Vanderheiden (1956)

i C.Richard (1960)

k Jagannathan et al (1956)

sodium borohydride, yielding a stable secondary amine which was isolated and characterized. They also found that, when this aldolase was treated with $NaBH_4$ in the presence of ^{14}C or ^{32}P labelled dihydroxyacetone phosphate, there was incorporation of radioactivity into the enzyme molecule, with concomitant loss of aldolase activity. After acid hydrolysis the labelled amino acid derivative was isolated and identified as N^6-β-glycerolysine. All this led to the suggestion that the formation of a Schiff's base intermediate and its reduction with $NaBH_4$ for fructose-1,6-diphosphate aldolase from rabbit muscle proceeded according to the following equation (Fig.8)

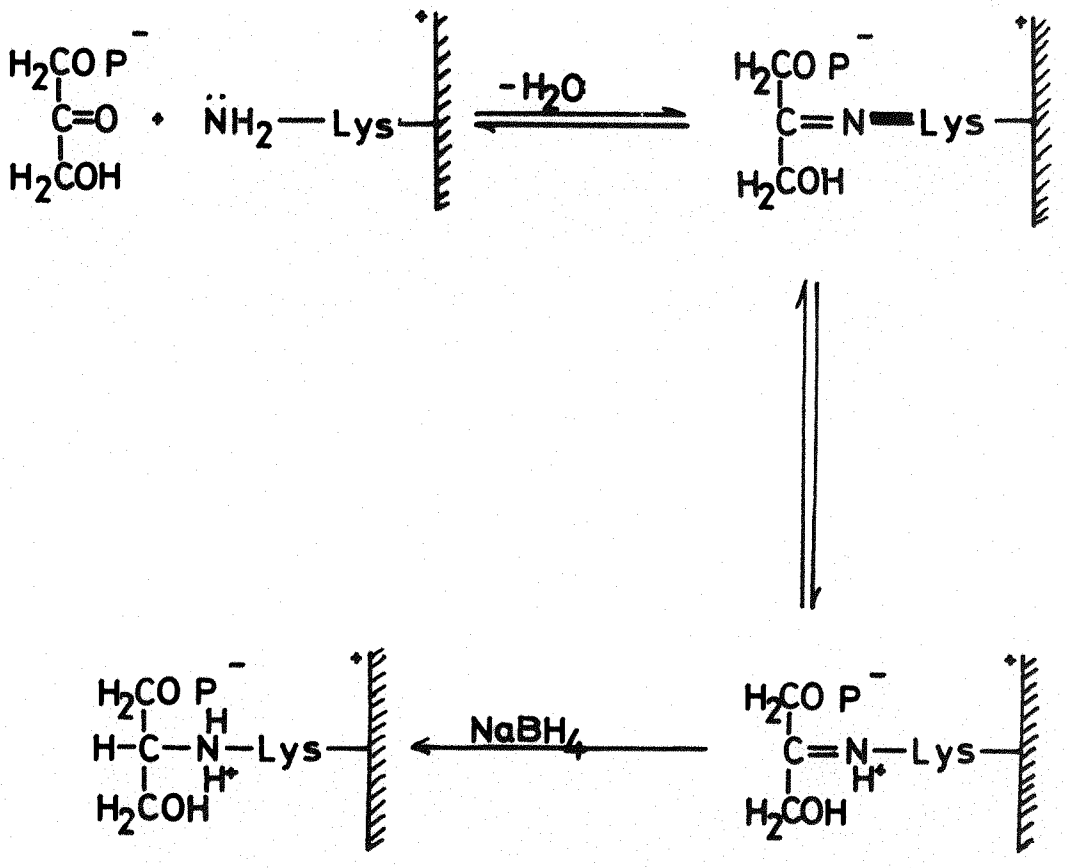
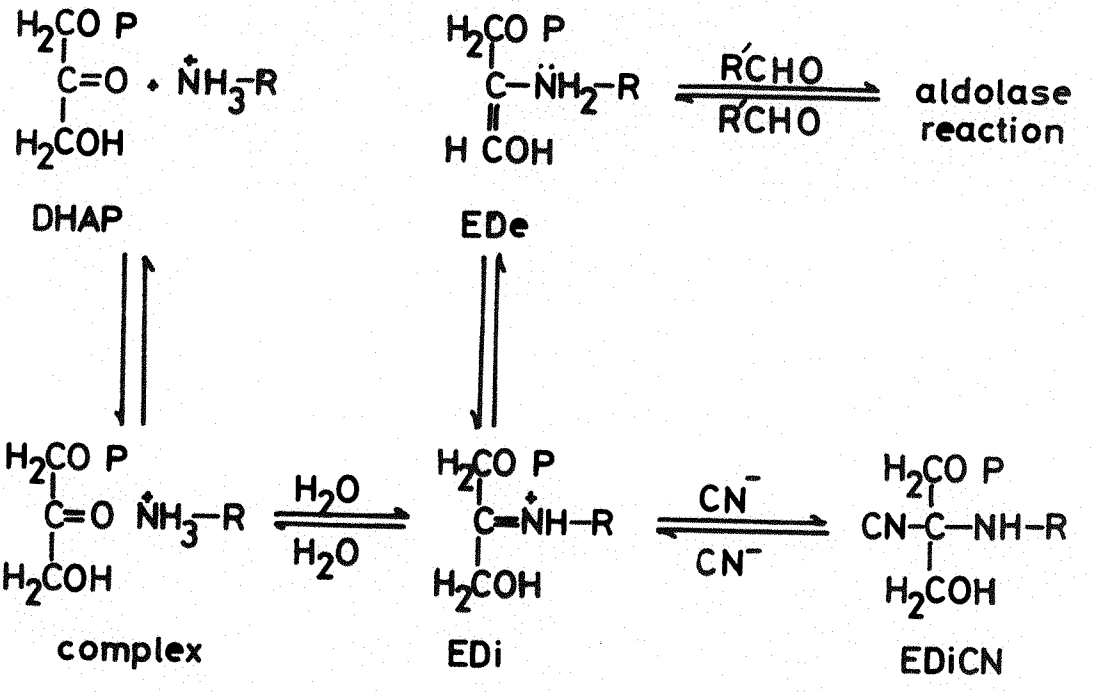


Fig.8

Additional evidence for the Schiff's base intermediate have been reported, based on the formation of the cyanide addition produce. Hence it has

been shown by Cash and Wilson (1966) that aldolase is slowly inhibited in the presence of dihydroxyacetone phosphate and hydrogen cyanide. Activity was restored on removal of either of these reagents. Neither dihydroxyacetone phosphate or hydrogen cyanide separately nor dihydroxyacetone with cyanide inhibited aldolase. Inhibition of aldolase by hydrogen cyanide in the presence of the specific substrate dihydroxyacetone phosphate, but not in the presence of dihydroxyacetone supports the general hypothesis that an amine derivative (Schiff's base) is a catalytic intermediate in aldolase reactions (Fig.9).



EDi: protonated imine
EDiCN: aminonitrile
EDe: enamine

Fig.9

The discovery of the Schiff's base intermediate has not only provided evidence as to the mechanism of the enzyme-catalyzed reaction, but it also provided a convenient experimental basis for the classification of aldolases.

The enzyme-catalyzed reaction is stereospecific. After incubation of dihydroxyacetone phosphate and enzyme in tritiated water only one of the two apparently equivalent C-3 hydrogen atoms was exchanged. This

was shown to be the 3R hydrogen atom and shows that the enzyme catalyzes exchange of that hydrogen atom which is removed on condensation. The new carbon-carbon bond is formed with the same stereochemistry. Fig.10

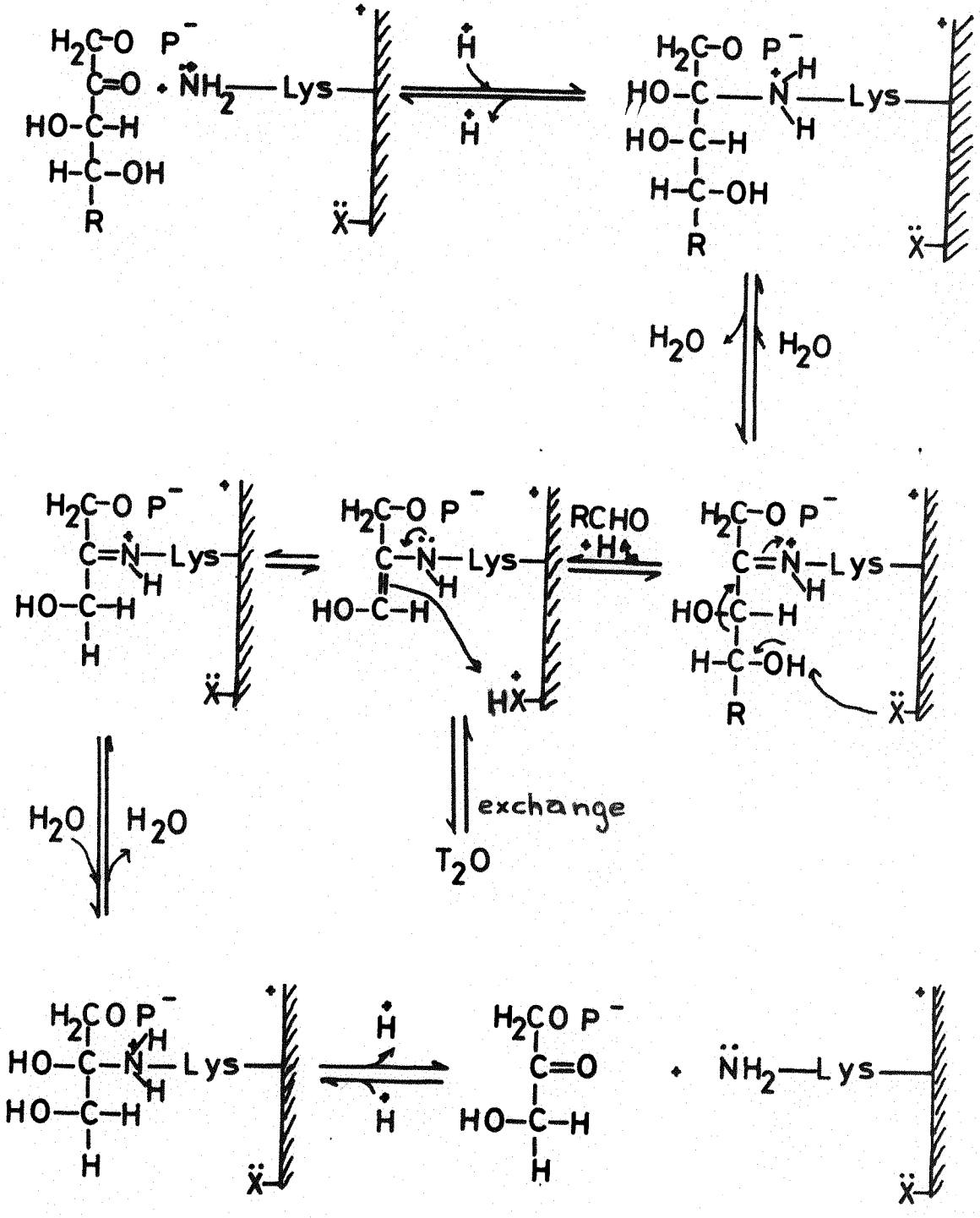


Fig.10

This indicates that the aldol condensation reaction involves a primary stereospecific activation of dihydroxyacetone phosphate.

All these results led to the conclusion that in the Class I aldolases, the protonated nitrogen of the Schiff's base acts as an electron sink, giving an enamine intermediate. The proton lost is stereospecifically abstracted by an adjacent base. The mechanism of action of Class I aldolases is illustrated in Fig.10. As is shown the aldolase mechanism involves two essential base-catalyzed and proton-transfer reactions. These could possibly be performed by the same group.

Possible nucleophilic species acting as either of these base catalysts are; the hydroxide ion of water, the thiolate ion of cysteine, the hydroxylate ion of serine, the phenolate ion of tyrosine, the carboxylate ion of aspartic or glutamic acid, and the imidazole of histidine.

In rabbit muscle aldolase it has been shown by Hoffee et al.(1967) that one (or both) of the protonated reactions is carried out by a histidine residue.

Isoenzymes of fructose 1,6 diphosphate aldolase in mammals

Three aldolase isoenzymes have been identified in animal tissues. These isoenzymes have been classified as aldolase A, the form found in muscle; aldolase B, the predominant form in liver and kidney; and aldolase C, which is found in brain together with aldolase A. All these three forms of aldolase form a Schiff's base intermediate with DHAP. Later studies by Lebherz and Rutter (1969) have established the presence of the 3 primary aldolase isoenzymes in a large number of animals, including fish, amphibia, birds and mammals.

Other Schiff's base forming aldolases

The aldol reaction is a general one occurring widely in nature. The various aldolases found can also be separated into Schiff's base (Class I) or metal-ion-dependent (Class II) aldolases. Other Schiff's

base aldolase are: the transaldolase from Candida utilis (Horecker,1961). 2-deoxy-D-ribose-5-phosphate aldolase from Lactobacillus plantarum (Grazi, 1963), 2-keto-3-deoxy-6-phosphogluconate aldolase from Pseudomonas fluorescens (Grazi,1963), 2-keto-4-hydroxy-glutarate aldolase from rat or beef liver (Kobes, 1966; Rosso, 1966), fructose-1-6-diphosphate aldolase (Horecker,1959,1963), N-acetylneuraminic acid aldolase from Clostridium perfringens (Barnett et al., 1971). All of these enzymes appear to use the amino group of a lysine as the amino group which forms the Schiff's base.

Class II Aldolases

Fructose 1,6-diphosphate aldolase

The Class II aldolases (metaloaldolases) which are bacterial in origin, are similar to the Class I enzymes with respect to their specificities, but are clearly different in their mode of action in that they require the presence of metal ions.

The metal ion at the active centre is thought to catalyze the reaction by forming a partial bond with the carbonyl group of the substrate (Rutter,1964). Thus the ternary complex formed between DHAP, metal ions and the enzyme would be equivalent to the Schiff's base intermediate formed in Class I aldolase. In the direction of condensation this would labilize the proton at the C-3 carbon atom and facilitate the nucleophilic attack on the aldehyde substrate. The metal ions appear not only to form a co-ordination bond with the carbonyl group of DHAP, but also to co-ordinate at the C-1 phosphate binding site. It has been studied by Ingram (1967) in Class II aldolase from Fusarium oxysporum that the proton exchange at the C-3 carbon atom of DHAP appears to be facilitated by the β-carboxyl group of an aspartic acid or α-carboxyl group of a glutamic acid residue. The proposed mechanism of action of metaloaldolase is illustrated in Fig.11.

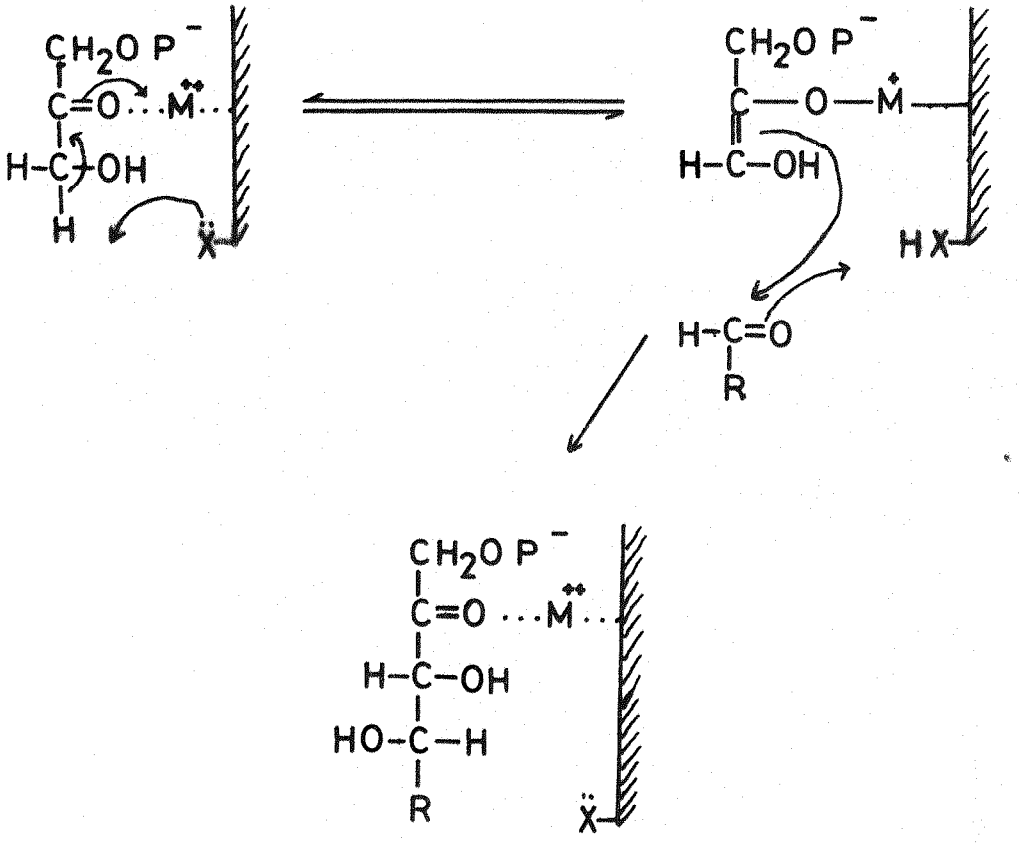


Fig.11

Metaloaldolases are completely inhibited by metal chelators (EDTA). The inhibition is reversed by the addition of excess of a divalent metal ion. They are also unaffected by treatment with borohydride.

Other Class II aldolases

In the Class II aldolases (metaloaldolases) also fall, fuculose-1-phosphate aldolase isolated from Escherichia coli (Chalambor et al., 1962), 2-keto-4-hydroxy-4-methylglutarate aldolase from rabbit muscle (Stellwagen, 1962), 3-hydroxy-3-methylglutaryl CoA cleavage enzyme from pig heart (Bachawat,1955).

N-ACETYLNEURAMINIC ACID LYASE

N-Acetylneuraminic acid aldolase is the enzyme that catalyzes the cleavage of N-acetylneuraminic acid to pyruvate and the corresponding N-acetyl-D-mannosamine according to the following reversible reaction (Fig.12) (Brunetti et al.,1962)

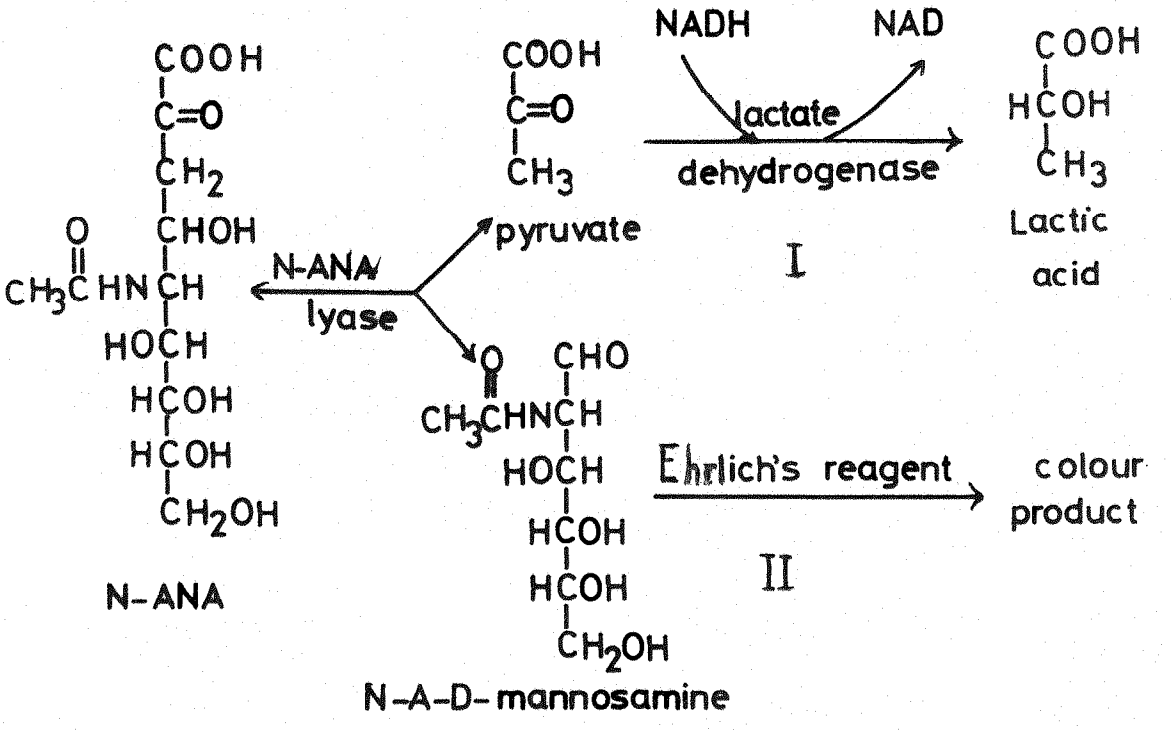


Fig.12

Other names used for the same enzyme are N-acetylneuraminate lyase and N-acetylneuraminate pyruvate lyase (E.C.4.1.3.3.). There are two major methods used to determine the enzyme activity. These are by measuring the rate of formation, from N-acetylneuraminic acid, either of pyruvate by using NADH and lactate dehydrogenase (Fig.12, I) or of N-acetyl-D-mannosamine by using a suitable colorimetric method (Fig.12, II). The pyruvate assay is not satisfactory with crude extracts obtained from animal tissues or when pyruvate analogues are used as inhibitors (Barnett, 1967), since pyruvate and NADH are metabolized by many of the crude extracts, while halogenopyruvates are substrates for lactate dehydrogenase.

The formation of N-acetyl-D-mannosamine is assayed by a modified Morgan-Elson colour reaction as described by Brunetti et al.(1962)

Occurrence and distribution

The N-ANA lyase is widely distributed in animal tissues. Brunetti and his co-workers (1962) have purified the enzyme from hog kidney cortex, they have also studied the distribution of N-ANA aldolase in animal tissues. The results obtained with crude extracts of various tissues are given in Table III.

TABLE III

Distribution of the N-acetylneuraminic acid aldolase in animal tissues.

Species	Specific activity of crude extract (X 100)						
	Kidney	Liver	Spleen	Brain	Muscle	Lung	Testis
Rat	4.0	1.4	2.3	0.51	0.32	0.90	1.9
Hog	4.3	0.23	2.8	Trace		2.4	
Guinea pig	17	0.77	5.0	2.1	0.32	0.42	8.3
Cow	14	0.32	0.24	0		Trace	
Rabbit	5.3	0.25	0.83	0	0	Trace	
Frog		44			0	3.3	

In general, the kidney seems to be richest in N-acetylneuraminic acid lyase, where activity appears to be confined to the cortex, although the high activity found in frog liver should be noted. Just as other enzyme activities change in frog liver with changing physiological states (Brown, 1959), so changes occur in the case of N-acetylneuraminic acid lyase. Brunetti et al. have found the following specific activities in the tissues tested (the specific activities are expressed as micromoles of N-acetylneuraminic acid lyase cleaved in 15 minutes at 37°C per mg.of protein, and the numbers express specific activity x 100): rat bone marrow, 1.0; lysed rat red cells, 0.3; hog lymph mode, 4.7; hog heart, ovary pancreas, thyroid and hypophysis, no detectable activity; rabbit

heart, no detectable activity. A number of other tissues including hog stomach, mucosa and the submaxillary glands from several species, provided negative results. Sirbasky and Binkley (1970) have prepared and purified the enzyme from kidney cortex.

N-Acetylneuraminic acid lyase has also been isolated from certain strains of bacteria. Comb and Roseman (1960) have prepared the enzyme from Clostridium perfringens and from Escherichia coli K-235. Gautt and his co-workers have prepared the enzyme from Cl. perfringens (A.T.C.C. 10543, Cassidy strain) by following the method of Comb. Sheldon (1973) reported that those corynebacteria related to Corynebacteria diphtheriae exhibited N-ANA aldolase activity. Drzeniek (1973) reported that the concentration of N-ANA aldolase is higher in the cell free culture medium of Pasteurella multosida than in the bacterium. Mueller (1973), by paper chromatography characterization, indicated the occurrence of N-acetylneuraminic acid aldolase (C.E.4.1.3.3) in Streptococcus sangnis, which was isolated from the blood culture of a patient with sepsis.

Studies on the substrate specificity of N-acetylneuraminic acid lyase

Comb and Roseman (1958) have shown that N-glycolneuraminic acid is cleaved at 65% of the rate of N-acetylneruaminic acid, whereas N,O-diacetylneuraminic acid cleaves at 14% of the rate. That means that the acyl group can be acetyl or glycolyl. In other respects, the enzyme is very specific in its substrate requirements. The enzyme is inactive with methoxyneuraminic acid, 2-keto-3-deoxy-6-phospho-D-gluconate, 2-keto-3-deoxy-D-octonate, and 2-keto-3-deoxy-8-phospho-D-octonoate They have demonstrated that in the reverse direction the enzyme is active with pyruvate and either N-acetyl-D-mannosamine or N-glycolyl-D-mannosamine, but it is inactive when phospho-enol-pyruvate is substituted for pyruvate. The following hexosamine derivatives have been found to be inactive with either pyruvate or phospho-enol-pyruvate:

N-acetyl-D-glucosamine, N-acetyl-D-galactosamine , D-glucosamine, D-mannosamine, the 6-phosphate esters of either the free hexosamines, or the corresponding N-acetyl derivatives.

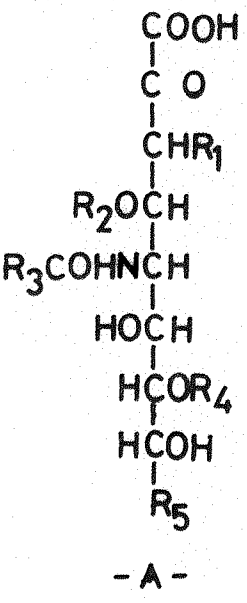
Additional studies on the substrate specificity have been made by Suttajit et al. (1971). They have studied the action of N-acetyl-neuraminic acid aldolase (EC.4.1.3.3) on 7-C and 8-C analogues of N-acetylneuraminic acid. The 8-C analogue (5-acetamido-3,5-dideoxy-D-galacto-octulosonic acid) was slowly and reversibly cleaved by the aldolase to yield 2-acetamido-2-deoxy-D-lyxose and pyruvic acid. The 7-C analogue (5-acetamido-3,5-dideoxy-L-arabinoheptulosonic acid) was not cleaved by the enzyme. The 8-C analogue competed with N-acetyl-neuraminic acid for the active site of the enzyme, but the 7-C analogue exhibited no inhibitory effect even when present at 10-fold greater concentration than N-AN acid.

DeVries and Binkley (1972) synthesysed 3-hydroxy-N-acetyl-neuraminic acid by the condensation of N-acetyl-D-mannosamine with bromopyruvate or hydroxypyruvate in yields of 24.4% and 12.7% respectively. They reported that this substituted sialic acid was a weak non-competitive inhibitor of N-ANA aldolase. Schauer et al. (1971) have also studied the substrate specificity of N-acetylneuraminic acid lyase using the enzyme from Cl.perfringens. They have shown that different substituents of the amino group of neuraminic acid (N-glycolyl, N-monochloroacetyl, N-formyl and N-succinyl groups)are of little influence on the enzyme activity when compared with N-acetyl-neuraminic acid. In contrast, N-monofluoroacetylneuraminic acid is cleaved at a considerably lower rate. D-acetylation in position 4 of the C-9 chain of N-AN acid reduces the cleavage rate by about 80%, and an O-acetyl group in position 7 decreases the cleavage rate by about 40%. However, the same substituent in position 8 has no marked influence. N-acetyl-neuraminic acid methyl ester, 2-deoxy-2,3-di-dehydro-N-acetyl or

N-butyrylneuraminic acid are resistant to the action of N-acetyl-neuraminate aldolase. N-acetyl-4-D-acetyl and N-monofluoroacetyl-neuraminic acid can inhibit the enzyme competitively.

As a conclusion to all these studies on the substrate specificity of the N-acetylneuraminic acid lyase we could point out, that there are only a few substituents of the original substrate N-acetylneuraminic acid which have been shown to have a little or no influence on the enzyme activity. These substituents are N-glycolylneuraminic acid, the 8-C analogue of N-acetylneuraminic acid 5-acetamido-3, 5-dideoxy-D-galacto-octulosonic acid, and the substituents of the amino group, N-monochloroacetyl, N-formyl and N-succinyl groups. In other words the enzyme seems to be fairly specific in its substrate requirements.

All these substituents of the N-acetylneuraminic acid are illustrated in Fig.13.



if R_1 : H
R_2 : H
R_3 : CH_3
R_4 : H
R_5 : CH_2OH
A≡ANA

R_1 : H
R_2 : CH_3CO
R_3 : CH_3
R_4 : CH
R_5 : CH_2OH
A≡substrate

R_1 : H
R_2 : H
R_4 : H
A≡substrate

R_1 : H
R_2 : H
R_3 : CH_3
R_4 : H
R_5 : H
A≡substrate

R_1 : H
R_2 : H
R_3 : Hor FH_2
R_4 : H
R_5 : CH_2OH
A≡poor substrate

R_3 : $ClCH_2$ or
CH_3CH or
CH_2CH_2COOH
R_5 : CH_2OH

Fig.13

Studies on the mechanism of N-acetylneuraminic acid lyase

Studies by Barnett et al. (1971) showed that EDTA had no effect on the enzyme activity and that sodium borohydride in the presence of the substrate, pyruvate, inactivated the enzyme. These results indicate that the enzyme is a Class I aldolase forming a Schiff's base-enzyme complex. By using (U-^{14}C) pyruvate they found that the substrate pyruvate is bound to the enzyme as a Schiff's base with lysine. They have proposed a possible mechanism for N-ANA aldolase which is illustrated in Fig.14.

In this mechanism of aldolase catalysis requires a group (X) close to the point of condensation, able to accept the proton liberated. Such a group should also act as a nucleophile. Using radioactive bromopyruvate, as an analogue of the substrate pyruvate, they showed that this group may be a cysteine residue. But this finding was somewhat ambiguous because of extensive labelling of the enzyme at areas other than the active site.

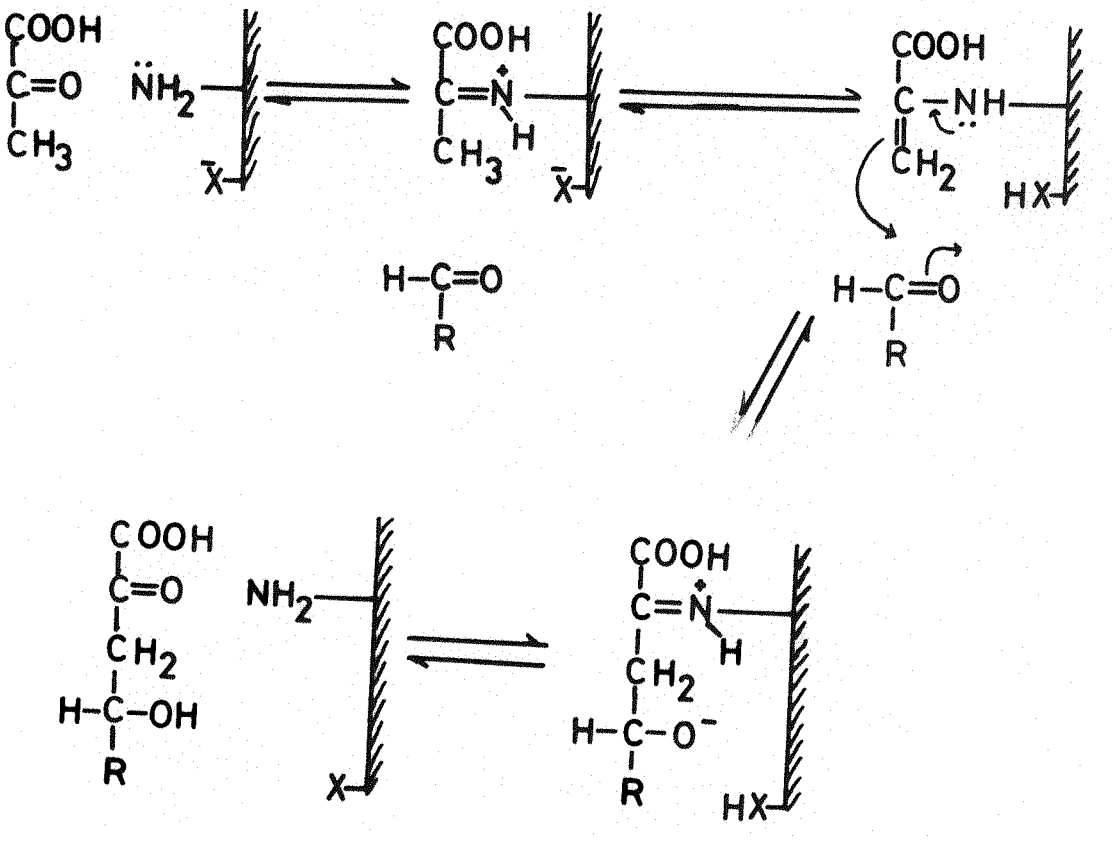


Fig.14

Roland Schauer et al. (1971) have independently confirmed the presence of a Schiff's base with substrate. They also showed that borohydride reduced the activity of the enzyme irreversibly (to about 10%) only in the presence of the substrates (N-ANA or pyruvate). After reduction of the enzyme-(1-^{14}C)pyruvate or N-(1-^{14}C)acetyl-neuraminate complex the radioactivity remained firmly bound to the enzyme protein. These authors did not identify the amino acid residue at the active site. Treatment of the enzyme-pyruvate complex with tritium-labelled borohydride resulted in incorporation of tritium into the enzyme. In the presence of both the substrate and cyanide the enzyme activity decreased to about 25%, but was unaffected by preincubation with cyanide alone. They also showed, using photo-oxidation experiments, that a histidine residue was important for enzyme activity.

Studies on the molecular weight and the subunit structure

Recently DeVries and Binkley (1972) reported the best purification of N-acetylneuraminic acid lyase from Clostridium perfringens so far.

Using Sephadex chromatography they have shown that their purified enzyme had a molecular weight of 92,000 and consisted of two protein components each one of which has enzyme activity.

By the same method, using a less purified perparation, Barnett et al. (1971) had previously suggested that the molecular weight was 250,000 with four subunits.

Because both groups used Sephadex chromatography for molecular weight determination it is possible that the enzyme can form both dimers and tetramers.

So far, although the type of mechanism of N-acetylneuraminic acid lyase is known, some ambiguity remains concerning the nature of the active site groups. Methods for identifying these are discussed in the next section.

THE ACTIVE SITE

The active site, in general, is that portion of the enzyme which is in contact with, or in very close proximity to, the substrate during the reaction.

Koshland (1960) proposed that the active site amino acids should be defined as those which are at some point only one bond distance removed from some part of the substrate molecule. Amongst the amino acids comprising the active site will be found those groups which take a direct part in the enzyme catalysis. Such groups are called the catalytic groups.

The identification of the active site of the enzyme is very important in studies of enzyme mechanism. A variety of techniques have been used to identify the active site of enzymes. Most valuable methods involve formation or stabilisation of a covalent bond between the enzyme and its substrate or substrate analogue.

Labelling by substrate or cofactor

Many enzymes form an enzyme substrate or enzyme cofactor covalent bond as part of the normal enzyme mechanism. The existence of such a covalent intermediate during the course of enzyme action allows the possibility of labelling an amino acid in the active site by stabilization of a radioactive substrate-enzyme complex. Stabilization can be achieved by either physical or chemical means. For example, condition of pH may be found in which covalent bond formation is still rapid but decomposition is very slow. Balls and his coworkers (1955,1956) have shown that an acetylated chymotrypsin intermediate can be isolated at low pH, but it decomposes at high pH.

Alternatively a chemical method which can be used is the stabilization of an enzyme-substrate complex by reduction with sodium borohydride. For example, the Schiff's bases formed between fructose 1,6-diphosphate aldolase of muscle and DHAP (Grazi, 1962),

transaldolase and fructose-6-phosphate (Horecker, 1961), 2-keto-3-deoxy-6-phosphogluconate aldolase and pyruvic acid (Jordan 1965) and many others, have been reduced with sodium borohydride to form stable secondary amines (Fig.15.)

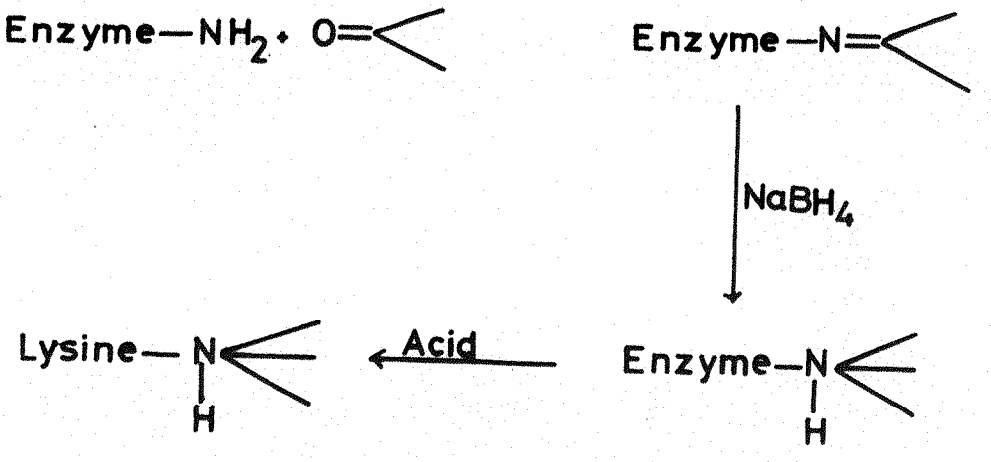


Fig.15

Labelling by quasi-substrate

An alternative method for labelling the active site of enzymes is by use of a quasi-substrate. A quasi-substrate is a substrate that is similar to the normal substrate and forms the covalent enzyme-substrate complex, but it is sufficiently different so that this intermediate does not decompose or decomposes only slowly. For example, diisopropylfluoro-phosphate (DFP) (Figure 16) can be used like a quasi-substrate for labelling of the active site of the esterases and proteases (Ball, 1952, Glander, 1958; Jansen, 1949; Jordan, 1965), because the phosphoryl group of DFP is similar to the carbonyl group of the substrate (Aldridge, 1953; Hartley, 1956; Mounter, 1957) to form a bond with serine, but sufficiently different, so that the phosphoryl serine hydrolyses at a very slow rate.

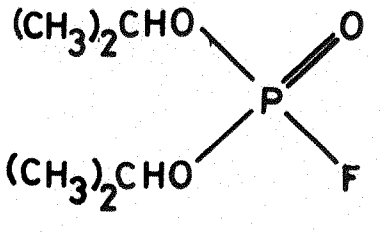


Fig.16

Alkylating agents

Non-specific alkylating agents have been used in an attempt to identify active site residues by the reaction of an enzyme with a reagent specific for a given amino acid. The following types of reagents have been used: α-haloacids, activated benzyl halides, sulphenyl halides. But unless precautions are taken it is difficult to prove that reaction has taken place at the active site.

Protection of the active site by competitive inhibitor or substrate and use of non-specific alkylating agents

The enzyme is treated with an excess of the alkylating reagent in the presence of competitive inhibitor. The unreacted reagent and substrate or competitive inhibitor are removed by dialysis or by Sephadex (G-10) chromatography. Then the enzyme, if it is still active, is treated with the same reagent but in this case radioactive. The hope in this case is that the residues which will react with the radioactive alkylating agent are all at the active site, which had been masked by the substrate or competitive inhibitor during the first incubation.

Affinity labelling

In this method the alkylating agent is designed so that the substrate or competitive inhibitor binding requirements of the enzyme are satisfied, but after formation of the enzyme-substrate complex the enzyme is alkylated by the reagent to give a stable covalent complex. The residue attached by the reagent can then be identified. An example from this kind of identification is N-toluene sulphonyl phenylalanine chloromethyl ketone (Fig.17) Shaw et al.,(1963) for the inhibition of chymotrypsin, which has indicated the involvement of a histidine group in chymotrypsin.

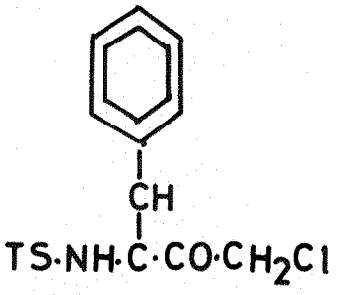


Fig.17

Identification by non-covalent methods:

Alternative ways of identifying amino acids at the active site involve indirect method which do not involve covalent labelling. These are rather unsatisfactory.

pH Dependence

In this method the identification is built on the variation of some parameter of the reaction, notably the Michaelis constant or maximum velocity, with pH. This method is notoriously unreliable as a method identifying active groups, because it relies on a knowledge of the pH of ionisation of the amino acids in the protein. Depending on the environment the pH can shift ± 3 pH units from the value for the same amino acid in water. This means that even carboxylic acids (pH about 4) can have pH values in proteins (e.g. lysozyme) of 6.5 more characteristic of a histidine in water.

Loss of activity with amino acid modification

Another technique which has been used to identify the residues required for enzymatic activity is to compare the loss of catalytic power with amino acid modification. If there is no loss in enzymatic activity then the groups which react must be the part of enzyme outer structure, but if there is loss of activity, this can be due to one or more of the amino acids which react with the reagent being necessary for enzyme action. This is not to say the residue has anything to do with the enzyme mechanism or with the active site. The protection with substrate or competitive

inhibitor (Hopkins, 1938) may give the clue as to whether the amino acid was at the active site or part of the contributing structure.

THE USE OF BROMOPYRUVIC ACID IN ENZYMOLOGY

3-Bromopyruvic acid has been shown to be a highly effective growth inhibitor of certain yeasts (Zygmunt and Martin, 1964). These authors noted wide differences in the inhibitory activity of the compound among different species and genera of yeast.

Maruasi and Makoto (1972) studied the effect of 3-bromopyruvate on the acetylcholine induced responses of Aplysia california and suggest that the inhibitory effect of 3-bromopyruvate appeared to be non-competitive.

Maldonado and Kyung-Jo 1972 studied the effect of bromopyruvate on the catalytic properties of the pyruvate dehydrogenase complex from E.coli. They reported that bromopyruvate inactivated the pyruvate dehydrogenase complex in a thiamine pyrophosphate (TPP) dependent process. The catalytic activities of the individual enzyme components within the complex are not completely destroyed by bromopyruvate under similar conditions, but the activities of the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase components are reduced in a thiamine pyrophosphate independent process. Radioactivity from (2-^{14}C) bromopyruvate is irreversibly bound in the presence or absence of TPP, but the amount of radioactivity bound in the presence of TPP is 3.9 times the amount bound in its absence. TPP-dependent inactivation probably involves the action of bromopyruvate as an irreversible inhibitor which is directed into the complex by specific but not necessarily irreversible interaction with the pyruvate dehydrogenase component, i.e. it forms a normal enzyme-substrate complex.

Meloche et al. (1972) have used bromopyruvate as both a substrate and alkylating agent for kinetic and stereochemical studies on 2-keto-6-phosphogluconic aldolase. They have shown that the substrate analogue bromopyruvate is an active site-specific alkylating agent and that tritium in 3-bromopyruvate exchanges with solvent in the presence

of enzyme indicating that the reagent can act as a substrate. A kinetic expression derived from a model in which exchange and inactivation occur at the same protein site requires that the rate of tritium exchanges per mole of enzyme inactivated should remain constant under different conditions. This model was tested with the reagent over a range of concentrations and the ratio of bromopyruvate detritiated to aldolase inactivated remained constant. The ratio was about 50:1. Excess enzyme removed only half of the tritium of (3R,S)-(^{3}H)bromopyruvate showing the reaction to be stereospecific. Additional experiments by Meloche et al. (1972) showed that the product of reagent detritiation was S-(3-^{3}H) bromopyruvate.

Okamoto and Yoshimasa (1973) have used bromopyruvate for the affinity labelling of aspartate transferase isozymes. They reported that incubation with bromopyruvate did not cause appreciable inactivation of either supernatant or mitochondrial isozyme of asparate aminotransferase from pig heart. In the presence of either L-cysteine sulphinate or L-aspartate, bromopyruvic acid rapidly inactivated both isozymes. Bromopyruvate also acted as a keto acid substrate in the conversion of the pyridoxamine of both enzymes to their pyridoxal forms.

It has been also reported by Chang and Hsu (1973) that bromopyruvate is an alkylating agent of pigeon liver malic enzyme (malate dehydrogenase (decarboxylating)). It combines first with the enzyme to give an enzyme-bromopyruvate complex, then reacts with a proximal -SH group, resulting in the formation of a pyruvate derivative. Bromopyruvate is also a substrate for the reductase partial reaction, and a non-competitive inhibitor of L-malate in the overall oxidative decarboxylase reaction catalyzed by this enzyme. Modification of the -SH group by this compound is accompanied by concomitant loss of both oxidative decarboxylase activity and reductase activity on bromopyruvate.

Berghauser, Falderbaum and Woenchan (1970) have explored the

location of an essential histidine residue of the substrate binding site of lactate dehydrogenase by using bromopyruvate. They found out that lactate dehydrogenase from pig heart catalyses the reduction of bromopyruvate. The Michaelis constant for bromopyruvate is 2×10^{-3}M, which is larger than for the natural substrate, pyruvate. Bromopyruvate is also an inactivator. The rate of this inactivation is increased by the presence of NAD^+ or nicotinamideriboside-5'-diphosphate-(P)-ribose (NMNPR). Inactivation is the result of the specific incorporation of the inactivator into the enzyme-NAD^+-bromopyruvate complex and results in the carboxyketomethylation of an essential histidine residue. In the absence of NAD^+, the binding between inactivator and apoenzyme is five times weaker and a cysteine residue is alkylated in addition to the histidine. Inactivated lactate dehydrogenase binds the same amount of NADH as the native enzyme. Depending on the degree of inactivation however, there is a proportional decrease in the formation of the ternary "dead-end" enzyme-NAD^+-pyruvate complex.

Barnett et al.(1971) have also used bromopyruvate as a potential analogue of the substrate, pyruvate, for N-acetylneuraminic acid lyase, in order to explore the mechanism of action of the enzyme.

In contrast, some workers have used bromopyruvate as a non-specific alkylating agent. Thus Rashed and Rabin (1968) have used bromopyruvate and iodoacetate as alkylating agents for inhibition of yeast alcohol dehydrogenase. Barnett and Atkins (1973) have also used bromopyruvate as a non-alkylating agent for inhibition of Sorbitol dehydrogenase (polyol dehydrogenase, L-iditol-NAD-oxidoreductase,EC 1.1.1.14). They found that inactivation was decreased markedly by NAD^+, and conclude that the bromopyruvate attacks a residue close to the NAD^+ binding site.

Baker and Rabin (1969) have studied the effects of bromopyruvate on the control and catalytic properties of glutamate dehydrogenase.

Despite the extensive work on bromopyruvic acid, the related

halogenopyruvic acid, chloropyruvic acid, does not seem to have been used hitherto as an enzymic inhibitor. This thesis explores the behaviour of chloropyruvic acid as an inactivator of N-acetylneuraminic acid aldolase.

MATERIALS

Growth of Clostridium perfrigens

One tablet of Robinson cooked meat Broth (Oxoid Ltd.) was added to a screwed cap tube with 10 ml of water, and the solution left 15 minutes at room temperature. Then the tube was sterilized at 15 pounds pressure for 20 minutes, and allowed to cool at room temperature.

A tube of freeze-dried Clostridium perfrigens NCIB strain 8875 was marked with a glass-cutter at the middle of a cotton-wool plug. Then it was broken, the plug of cotton wool removed, and under sterile conditions, a small volume of Robinson cooked meat solution was added to dissolve the freeze-dried Clostridium perfrigens. This solution was withdrawn with a sterilized pipette and added to the cooked meat broth. Then it was incubated at 37°C overnight and used for inoculation.

The following solutions were autoclaved for 30 minutes at 15 pounds pressure:126 g Todd Hewitt broth (Oxoid Ltd.) in 3.54 litres of water in a 5 litre flask, 7.5 g of Todd Hewitt broth dissolved in 200 ml of water and then divided into five test tubes, 5.4 g of glucose in 20 ml of water, 9.0 g of NaCl and 6.5 g of K_2HPO_4 in 30 ml of water, and liquid paraffin. The liquid paraffin was used to keep the media anaerobic by adding it while the solutions were still hot. 0.18 g of cysteine was dissolved in 6.0 ml of sterile water and was filtered through a millipore filter into a sterile flask. 0.1 ml of the cysteine, 0.2 ml of glucose, and 0.3 ml of salt solution were added in each tube of Todd Hewitt broth solution with sterile pipettes, while the remaining glucose, salt and cysteine were added into the 5 litre flask. One drop of the dissolved Cl. perfrigens strain 8875 was added to the 4 tubes with a sterile pipette and they were incubated overnight at 37°C. The grown organism was transferred to the bulk of the Todd Hewitt broth. The contents of the conical flask were gently stirred with a magnetic stirrer at 37°C for 4-5 hours. The cells were collected by centrifugation at 32,000 g

for 20 minutes at 0-4°C. The collected cells could be kept for a period of 6-12 months and retained full enzymic activity.

Preparation of Chloropyruvate

In a three necked flask fitted with a magnetic stirrer, dropping funnel, thermometer and reflux condenser protected with a $CaCl_2$ tube were placed 2.83 moles of pyruvic acid, which was redistilled before use. The stirrer was started and 2.92 moles of sulfuryl chloride were added dropwise over a period of two hours. During the addition the temperature was maintained at 25-30°C. The mixture was then stirred at room temperature for an additional 60 hours. The product was transferred to a crystallizing dish and kept in a vacuum dessicator in the cold room for about a week. The crude chloropyruvic acid was distilled in vacuo, b.p. 6 mm, 76°C.

The material solidified in the condenser. The product was very hygroscopic and melted between 51-55°C. It was stored in a vacuum dessicator in the cold room.

Preparation of Radioactive Chloropyruvate

To the sodium salt of (^{14}C) pyruvic acid (2.25μmole, 25 mCi) (The Radiochemical Centre, Amersham, Bucks) were added 0.3 ml of pure sulfuryl chloride. The tube was fitted with a $CaCl_2$ drying tube. The mixture was then shaken at room temperature for 60 hours. The product was then transferred to a vacuum desiccator over KOH pellets for at least one week, dry acetone added and removed, and the residue dissolved in 0.5 ml acetone. This solution was used directly for the inactivation experiments. 5 μl of this solution were taken and assayed according to the method for assay of pyruvic acid and derivatives with lactate dehydrogenase (Barnett et al., 1971), which is described below. (Yield 1.2μmoles, 1.59×10^7 cpm/mole.)

Preparation of S-(3-lactic acid)-cysteine

100 μmoles of N-acetylcysteine were dissolved in 1 ml of 0.1 M NaOH. The pH was adjusted (Radiometer pH-stat) to pH 6.0 using the

autoburette filled with 0.1M NaOH. 100 μmoles of bromopyruvate were dissolved in 1.5 ml water the solution was adjusted to pH 6.0 in the similar way. Then the two solutions were mixed, and the autoburette, filled with 0.1M NaOH, used to maintain pH 6.0 at room temperature. When the reaction was completed 400 μmoles of sodium borohydride were added. After 30 minutes the solution was deionized with Amberlite 1R 120 (H^+) and evaporated to dryness. Addition of methanol and evaporation was repeated several times to remove boric acid. 6.0 ml of 6 N-HCl was added, the tube sealed and heated at 110°C for 24 hours. Then the solution was evaporated to dryness using a vacuum pump, a small volume of water was added and evaporated several times to remove HCl. The residue was dissolved in 1 ml of water and this hydrolysate was used as a reference compound for chromatography of the product of enzyme modification by chloropyruvic acid.

N-acetylneuraminic acid and N-acetyl-D-mannosamine were purchased from Koch-Light Laboratories Ltd.,(Colnbrook,Bucks). Radioactive pyruvate was obtained from the Radiochemical Centre (Amersham,Bucks). Todd Hewitt broth was obtained from Oxoid Ltd.(London,SE1). The scintillation fluid NE 250 was supplied by Nuclear Enterprises Ltd. (Edinburgh). Clostridium perfrigens strain 8875 was purchased from the Torrey Research Station (Aberdeen,Scotland). Sephadex G-200,G-25 and G10 were supplied by Pharmacia (Upsala,Sweden). Polymixine-B-sulphate, cytochrome and urease were obtained from Sigma Chemical Co.(St.Louis, Mo.,U.S.A.). DE52 diethylaminoethyl cellulose was supplied from Whatman Biochemical Ltd.(Springfield Mill, Maidstone,Kent).

Most of the other chemicals which were used in this work were purchased from the British Drug Houses (Poole,Dorset).

METHODS AND EXPERIMENTAL

Assay of pyruvic acid and derivatives

The assay is based on the oxidation of NADH in the presence of lactate dehydrogenase and its substrate pyruvic acid. The same method can be used for halogenopyruvic acids because they are also substrates for lactate dehydrogenase.

Assay

i) Solutions

2mM - pyruvic or halogenopyruvic acid

2mM - NADH

6.5 units/ml lactate dehydrogenase (crystalline suspension)

0.2M, pH 7.3 Potassium phosphate buffer, (Sigma)

ii) Method

0-50 µl 2mM-Pyruvic or Chloropyruvic acid (0-0.1µmol) was added at room temperature to 0.3 ml potassium phosphate buffer, water (1 ml), lactate dehydrogenase (20 µg) and 0.1 ml NADH in a 1 cm light-path cell. The optical density was measured in a Unicam SP.800 spectophotometer at ε 340. The system reached equilibrium in about 1 min.

Assay of N-acetylneuraminic acid lyase

Two ways of determining the enzyme activity were used; by measuring the rate of formation of either pyruvic acid or N-acetyl-D-mannosamine. The latter was generally used.

a) The rate of formation of pyruvic acid was measured by the decrease in absorbancy at 340 nm (Brunetti et al, 1963).

Assay

i) Solutions

0.05 - 0.2 ml enzyme

50 mM - N-acetylneuraminic acid

0.1 M - potassium phosphate buffer, pH 7.2

5mM - NADH

lactate dehydrogenase

ii) Method

0.05 - 0.2 ml of the enzyme were added at room temperature to (0.1 ml, 50 mM-) N-acetylneuraminic acid, (pH 7.2, 2 ml, 0.1M) potassium phosphate buffer (0.5 ml, 5mM) NADH and 0.01 ml of a crystalline suspension of lactate dehydrogenase in a 1 cm light-path cell. The E_{340} was measured in a Unicam SP.800 spectophotometer with a scale expansion by a Smith's Servoscribe recorder. As it has been previously pointed out the pyruvate assay is not satisfactory with crude extracts obtained from animal tissues.

b) The rate of formation of N-acetyl-D-mannosamine was measured by the method of Brunetti et al (1962) using N-acetyl-neuraminic acid as substrate.

Assay

i) Solutions

0.005 - 0.2 ml of enzyme

10 mM-N-acetylneuraminic acid

0.1M potassium phosphate buffer,pH 7.2

0.8M-potassium tetraborate buffer pH9.1

(disolve the required amount of boric acid in KOH)

Erlich's reagent

Erlich's reagent was made up from a solution of p-dimethylaminobenzaldehyde (10g) in HCl (12.5 ml) and acetic acid (87.5 ml) and then diluted with 9 parts of acetic acid. 0.3 ml of the diluted solution was added to the assay mixture.

ii) Method

The enzyme was incubated with 10mM-N-acetylneuraminic acid in 100 mM-potassium phosphate buffer (pH 7.2), in a final volume 5ml, at 37°C for 15 min. The solution was heated at 100°C for 2 min. and then 0.5 ml of water was added and the mixture was centrifuged. The supernatant (0.5 ml) was incubated with 0.4 mM-potassium tetraborate,PH 9.1 (0.5 ml) at 100°C for 10 min. and 3 ml of the diluted solution (Erlich's reagent)

was added. The mixture was incubated at 37°C for 10 min. The E_{585} was measured and was compared with a standard curve made using N-acetyl-D-mannosamine.

Enzyme-activity

One unit of enzyme activity was defined as the amount of enzyme that will hydrolyse 1 μmole of N-acetylneuraminic acid in 15 min. at 37°C. This unit is 15 times that used by Brunetti et al (1962)

Protein determination

Protein was determined by the Biuret method (for high concentrations), by Lowry's et al (1951) method (low concentrations) and by its U.V. absorption at 230nm.

a) Biuret method

Biuret reagent was prepared by dissolving 1.5g of copper sulphate, 6.0g of sodium potassium tartrate in 500 ml water, 300 ml of 10% NaOH free from carbonate and the volume made to 1 litre.

Assay

i) Solutions

5mg/ml of protein solution

4.0 ml Biuret reagent

ii) Method

0.01 - 0.2 ml of protein solution (5mg/ml) was added to the Biuret reagent (4.0 ml), and water in a final volume of 5.0 ml. The mixture was allowed to stand for 30 min. at room temperature with occasional shaking and the extinction was measured at 560 nm and compared with a standard curve of borine serum albumin.

b) Lowry's method

Assay

i) Solutions

A) 2% sodium carbonate in 0.1 M NaOH

B_1) 1% copper sulphate

B_2) 2% sodium potassium tartrate

C) 50 ml of solution A, 0.5 ml of B_2 and 0.5 ml of B_1

D) 1M Folin's reagent (BDH)

300 /µg protein

ii) Method

3.0 ml of solution C was mixed with 0.6 ml of a solution of protein containing no more than 300/µg of protein. The mixture was allowed to stand for 10 min. at room temperature. Then 0.3 ml of diluted B.D.H. Folin's reagent (1 part reagent, 1.3 part water) was added. The mixture was allowed to stand for 30 min. at room temperature with occasional shaking and the extinction was measured at 540 nm. A standard curve of borine serum albumin was used.

The protein's U.V. absorption at 230 nm was generally used for detection of column eluates rather than for quantitative determination.

Preparation and purification of N-acetylneuraminic acid lyase from Clostridium perfrigens

N-acetylneuraminic acid lyase was purified from Cl.perfrigens cell paste by the following procedure.

All centrifugation took place at 2-4°C.

1) Step; Crude extract

The total yield of cell paste (15g) was suspended in 2 volumes of 0.15 M KCl. The cells were sonicated for 30 min. at 5-10°C in a 10-Kc Raytheon sonic oscillator. The solution was then centrifuged at 32,000g for 20 min. The precipitate was discarded.

2) Step; Bentonite clearance

Bentonite was washed with water and then it was added to the crude extracts (75 mg dry weight per ml). The mixture was stirred gently for 10 min. and then centrifuged at 32,000 g for 10 min. The precipitate was rewashed with 0.15 KCl, centrifuged again and finally

the residue was discarded.

3) Step: Polymixine precipitation

An ice-cold 40% solution of polymixine B sulphate, was added dropwise with stirring to the Bentonite supernatant (0.25 ml of polymixine solution per ml of the fluid) to remove the nucleic acids. After 10 min. of stirring, the mixture was centrifuged at 32,000 g for 20 min. The precipitate was discarded.

4) Step: Heat denaturation

The supernatant of the previous step, divided into 10 ml portions, was treated at 60-61°C for 5-6 min. At this temperature NADH oxidase and other proteins are denatured and precipitated . The mixture was then centrifuged at 32,000 g for 20 min. and the residue discarded.

5) Step: Ammonium sulphate fractionation

Powdered ammonium sulphate was added slowly with stirring at 0-4°C to the supernatant fluid until the salt concentration was 60% saturation (35.9 g per 100 ml of original volume). The mixture was stirred for an additional 30 min. and centrifuged at 32,000 g for 30 min. The precipitate was discarded. Powdered ammonium sulphate was then added to the supernatant fluid until it was 90% saturated with salt (an additional 13.7 g per 100 ml of original volume) and the solution again stirred at 2-4°C for 30 min. Then the precipitate was collected by centrifugation at 32,000 g for 30 min. and dissolved in 5 ml of 0.1M potassium phosphate buffer, pH 7.2 and dialyzed, with mechanical stirring against 50 volumes of water with three changes of fresh water, overnight.

6) Step: DEAE-cellulose

DEAE-cellulose (70g) was equilibrated with 0.05M potassium phosphate buffer (pH 7.2) and poured into a 2.6 x 25 cm column. After dialysis the 60% to 90% fraction was applied to the column and a linear gradient of 0-0.5M NaCl in 0.05M potassium phosphate buffer (pH 7.2,

total volume 800-1000 ml) was run through the column at a flow rate of 60 ml per hour and fractions (3ml) were collected. The fractions with the highest specific activities were pooled and concentrated to about 5ml by ultrafiltration.

7) Step: Sephadex G-200 column

Sephadex G-200 was suspended in water and allowed to swell for 3 days. A 2.5 x 52 cm column was equilibrated with 0.15 M KCl. The concentrated enzyme solution was applied to this column and was eluted with 0.15 M KCl at a flow rate of 10ml per hour, and fractions (3 ml) were collected. The fractions containing the highest specific activity were pooled and concentrated to a final total volume of 3 ml by ultrafiltration. The concentrated enzyme was stored at -4°C. The specific activities of a typical fractionation is shown in Table IV P.48

Inactivation of Clostridium perfrigens N-acetylneuraminic acid lyase by bromopyruvic acid

To 0.5 ml of enzyme solution in 0.05M pH7.2 potassium phosphate buffer was added 0.05 ml bromopyruvic acid diluted in acetone (0.1 mM final concentration). This solution was incubated at 37°C. Portions of 50 µl were removed, at required times, and quickly added to the standard assay system, which contained 1 mM dithiothreitol (final concentration) to inactivate the bromopyruvate. The enzyme was measured and compared with controls treated in the same way but lacking bromopyruvate.

Inactivation of frog liver N-acetylneuraminic acid lyase by bromopyruvate

N-acetylneuraminic acid lyase was obtained from the liver of the frog.

Two methods were followed

1) The liver was cut into slices and homogenized with two parts of distilled water in a Waring Blendor for 30 min. at 5°C.

2) The liver was cut into slices and homogenized with two parts of water in a glass homogenizer, for 30' min. at 5°C.

The crude extract from both of the two above methods was treated in the same way. It was centrifuged for 60 min. at 32,000 g at 2-4°C. The residue was discarded. A portion from the supernatant was assayed for enzyme activity according to the method of Brunetti et al (1962), measuring the rate of formation of N-acetyl-D-mannosamine. The enzyme obtained according to the second method had the highest specific activity.

Inactivation by bromopyruvate:

To 3 ml of enzyme solution were added 0.3 ml of bromopyruvate diluted in acetone (final concentration 5 mM). The solution was incubated at 37°C. Portions of 0.2 ml were removed at required times and added to the standard assay system which contained 0.1 ml of ImM dithiothreitol (final conc.) for stopping the reaction.

Inactivation of Clostridium perfrigens N-acetylneuraminic acid lyase by chloropyruvate

0.5 ml enzyme in PH.7.2 potassium phosphate buffer was incubated at 37°C with chloropyruvate diluted in acetone (50 ml, final concentration 0.1 - 1.0 mM). Portions (50 µl) were removed at the required times and quickly added to the standard assay system, which contained 1mM dithiothreitol (0.1 ml). The enzyme activity was measured and compared with controls treated in the same way but lacking chloropyruvate. Because the rate of inactivation was very fast, the experiment was repeated at 5°C. The enzyme assays were carried out at 37°C as usual.

Inactivation of N-acetylneuraminic acid lyase with sodium borohydride in the presence of (U - ^{14}C) pyruvate

The enzyme (2.5 mg of purified protein) was dissolved in 0.05 M potassium phosphate buffer (1 ml) 0.2 ml (U - ^{14}C) pyruvate (25 μCi, 11mCi per mmol) diluted in buffer was added. The mixture was allowed to stand at room temperature for 10 min. and then 0.1 ml of 1M sodium borohydride was added. After 10 min. 50 μl of 2M-acetic acid was added. The process of adding sodium borohydride and acetic acid was repeated 4 times, and when the reaction was vigorous it was cooled in an ice bath. A 20 μl sample was assayed for enzyme activity. The solution was dialyzed against water (800 volumes) for 12 hours with four changes of fresh water. The enzyme was completely inactivated. A control from which the sodium borohydride was omitted lost no enzyme activity.

The radioactive enzyme derivative was freeze-dried, dissolved in 2.0 ml of 0.15 M K Cl, and passed through a sephadex Cr-25 column (1.5 x 20 cm) and eluted with 0.15 M KCl. 3.0 ml fractions were collected and the optical density was measured at 230 nm for the protein. 0.2 ml of each fraction was removed and measured for radioactivity. The radioactive fractions were collected and freeze-dried.

The radioactive enzyme derivatives was again dissolved in 1 ml water. A 0.01 ml sample was added to 10 ml of NE 250 scintillation fluid and the radioactivity counted by using a Beckman scintillator counter. Another 0.1 ml sample was removed and assayed for protein according to Lowry's method.

Reaction of radioactive chloropyruvate with N-acetylneuraminic acid lyase

0.3 ml (^{14}C) Chloropyruvate (25 μCi 11 mCi/mmol) diluted in acetone was added to 2.5 mg purified enzyme diluted in 1 ml of 0.05M potassium phosphate buffer, pH 7.2. The solution was incubated at 37°C for 2 min. The reaction was stopped after the 2 min. by the addition of

1 ml dithiothreitol (f.c. 2mM). A 10 µl portion was removed and added to the enzyme assay system. There was no enzyme activity. The inactivated enzyme was dialyzed overnight against 1 l of water with four changes of water, then it was freeze-dried.

The freeze-dried inactivated enzyme was diluted in 1 ml of water $NaBH_4$ (8 mg) was added to reduce the S-pyruvate residue to S-lactate and chromatographed on a Sephadex G-25 column using 0.15 M-KCl as eluant. The fractions containing the bulk of the radioactivity were pooled and again freeze-dried. This ammount of inactivated enzyme with (^{14}C) Chloropyruvate was used for the calculation of chloropyruvate bound per mole of the enzyme.

Preparation of (^{14}C) chloropyruvate inactivated enzyme complex

0.5 ml of (^{14}C) chloropyruvate (15 µmoles) diluted in acetone was added to the enzyme (4.8 mg purified protein) in 2 ml 0.05M potassium phosphate buffer, pH 7.2, and incubated at 37°C for 2 min. The reaction was stopped by adding 1 mM (final concentration) dithiothreitol. A 20 µl portion was removed and added to the enzyme assay system which contained 0.1 ml of 1mM dithiothreitol (f.c). There was no enzyme activity. The inactivated enzyme was dialyzed with mechanical stirring overnight against 1l water (four changes) and freeze-dried. This preparation was used for identification of the modified amino acid.

Hydrolysis of the (^{14}C) chloropyruvate-inactivated enzyme and identification of the modified amino acid

The freeze-dried (^{14}C) chloropyruvate-inactivated enzyme was dissolved in 1.0 ml of water and was reduced to the corresponding lactate derivative by the addition of sodium borohydride (0.4 mg). The solution was left for 15 min. at room temperature and then 2.0 ml of 6-M HCl was added. After the hydrogen generated had been allowed to escape the vessel

was sealed and heated at 110°C for 24 hours. The hydrolysate was freeze-dried, the residue redisolved in a small volume of water and again freeze-dried. The residue was finally dissolved in 0.5 ml of distilled water and was used for electrophoresis, using synthetic S- (3-Lactic acid)-cysteine as a reference compound.

Rate of reaction of bromopyruvate and of chloropyruvate with N-acetyl-cysteine

The N-acetylcysteine and the bromopyruvate were preincubated at 9°C, the temperature which was to be used for the experiment, and they were adjusted to pH 6 using the pH-start. Then 0.1 ml of 10mM N-acetyl-cysteine was added to 4.8 ml of water in a jacketted cell at 10°C and adjusted to pH 6.0 by starting the autoburette filled with 0.01 NaOH. Then 0.1 ml of bromopyruvic acid (100mM) was added and the pH maintained by automatic addition of 0.01M-NaOH by using a burette of maximum capacity 0.25 ml.

The same experiment was repeated using chloropyruvate instead of bromopyruvate. An attempt to determination of the rate of the reaction of chloropyruvate with N-acetylcysteine at pH 7.0 was impossible because the rate of the reaction was too high.

Paper electrophoresis

Electrophoresis was performed on Whatman no.1 paper with a Shandon vertical electrophoresis tank and a Vokam power pack at constant voltage (300v). The buffers used were: 25% formic acid-acetic acid-water (13 : 29 : 258, by vol.) pH 1.6, pyridine-acetic acid-water (50 : 2 : 948 by vol.) pH 6.0, sodium diethyl barbiturate-diethylbarbituric acid-water (10.3 : 1.84 : 1000 w/w 1v) pH 8.6.

Method

The paper was folded in half and 50-100 µg of the amino acids

or proteins were spotted on the fold, at a distance of 3-4 cm apart, with a micropipette or a capillary tube. The paper was wetted with buffer from a 10 ml pipette which was drawn across one side of the paper in a line about one inch below the fold. This procedure was repeated on the opposite side of the paper. The buffer was allowed to meet by capilliary action. After it was completely wet the constant voltage, 300 v, was applied.

Location of the substances

The amino acids were located by dipping the paper in 0.2% ninhydrin in acetone or using ninhydrin spray. Then it was dried by a hair drier, to give purple spots.

For the location of radioactive areas the paper was cut into pieces (1 cm x 2 cm) which were put in 10 ml of NE250 scintillation fluid and the radioactivity counted by a Beckman scintillation counter.

Chloropyruvate was located by silver nitrate-sodium hydroxide. The paper was dipped in a solution made by 0.4 ml saturated silver nitrate solution in 40 ml acetone just sufficient water was added dropwise to dissolve the precipitate. The paper was dried at room temperature, then dipped through a solution of 0.5% sodium hydroxide in ethanol to develop the spots. The paper was rinsed by washing in a 5% solution of sodium thiosulphate. The spots appeared brown on a white background.

Determination of molecular weight of N-acetylneuraminic acid lyase

The molecular weight of purified N-ANA lyase was determined by the method of Andrews (1965) on a column of Sephadex G-200 (2.5 x 50 cm).

The Sephadex G-200 was suspended in water and allowed to swell for 5 h in water bath 100^{o}C. Then it was cooled in room temperature. The column was filled with the swollen sephadex in the cold room (4^{o}C) and then 1l of 0.05M potassium phosphate pH 7.2 buffer was passed through.

2 mg of N-acetylneuraminic acid lyase was dissolved in 2ml of 0.05 potassium phosphate buffer pH 7.2 together with 1mg of blue dextran 2.5 mg of cytochrone C, 0.1 mg of lactate dehydrogenase, 0.1 mg of malate

dehydrogenase, 0.2 mg of catalase, and 0.1 mg of urease, and applied to the top of the column. The column was equilibrated with the same buffer and fractions of 3ml were collected. Samples from each fraction were tested for protein either by their optical density or by suitable enzyme assays.

Blue dextran: It was determined by its E625 using a cuvvette of 1 cm light path.

Urease: The method used for determination of Urease activity was as follows:

Assay

Solutions

9.6% phosphate buffer, PH 7.2

3% urea

1M HCl

Nessler's reagent

Method

1.0 ml of the effluent was added to 1.0 ml of 9.6% phosphate buffer, pH 7.2 and 1.0 ml of 3% urea and the mixture was incubated at room temperature for 5 min. Then 1 ml of 1M HCl and 2.5 ml of Nessler's reagent were added and diluted to 5 ml with water. E 436 was measured.

The assay of Catalase

Solutions

7.0 potassium phosphate buffer

1M, H_2O_2

10mM or less enzyme

Method

The assay mixture contained 10 µl of the enzyme, 2.69 ml of potassium phosphate buffer, 0.1 ml of H_2O_2. 0.2 ml of the effluent fraction from the Sephadex G-200 column was added to the standard assay system for the enzyme activity. Catalase was estimated by the disappearance of hydrogen

peroxide followed at 240 nm (Chance and Machly, 1955).

The assay of lactate dehydrogenase

Solutions

0.1M potassium phosphate buffer, pH 7.2

6mM NADH

22.7 mM pyruvate

Method

0.2 ml of the effluent fraction were added to 2.6 ml of 0.1M potassium phosphate buffer, 0.1 ml of 6mM NADH 0.1 ml of 22.7 mM pyruvate in a total volume of 3.0 ml and the E340 was measured. The assay was based on the oxidation of NADH in the presence of excess of pyruvate (Konberg 1955).

The assay of Malate dehydrogenase

Solutions

0.1 ml of the enzyme (0.5 µg/ml)

of 0.1 M potassium phosphate buffer pH 7.2

of 6mM NADH

of 6mM oxaloacetic acid

Method

0.1 ml of the enzyme was added to 2.7 ml of 0.1M potassium phosphate buffer, PH 7.2, 0.1 ml of 6mM NADH, 0.1 ml of 6 mM oxaloacetic acid, and the decrease in E340 was determined. The control lacked oxaloacetic acid. 0.2 ml of the effluent fraction was added to the assay system to determine the enzyme activity. Malate dehydrogenase was estimated by the oxidation of NADH in the presence of excess of oxaloacetic acid (Ochoa, 1955).

RESULTS

Purification of N-acetylneuraminic acid lyase

The method of purification is shown in the methods section (p 38). The purification through DEAE-cellulose and Sephadex G-200 is shown in Fig. 18 and Fig.19 The purified enzyme was kept at -5°C and used for the various experiments.

The results of a typical purification are summarized in Table IV.

Table IV Purification of N-acetylneuraminic acid lyase

Fraction	Total units	Specific Activity	Purification
1.Crude extract	906	0.6	1
2.Bentonite	870	1.1	1.8
3.Polymixine	736	0.7	1.1
4.Heat denaturation	630	1.4	2.3
5.60-80% $(NH_4)_2SO_4$	565	15.3	25.5
6.DEAE cellulose	410	36.1	61.8
7.Sephadex G-200	340	68	113

Enzyme Storage

It appears that during the enzyme storage it loses some of its activity. Therefore, the possibility of storing the enzyme in solution was explored. It seems that the enzyme can be stored without appreciable loss for about one week in the deep freeze (-5°C) in the presence of dithiothreitol (0.1mM). The effect of various methods of storage on the ability of the enzyme are given in Fig 20.

The dithiothreitol enzyme cannot be used for the inactivation experiments of N-acetylneuraminic acid lyase with either chloropyruvate or bromopyruvate, because they react rapidly with dithiothreitol.

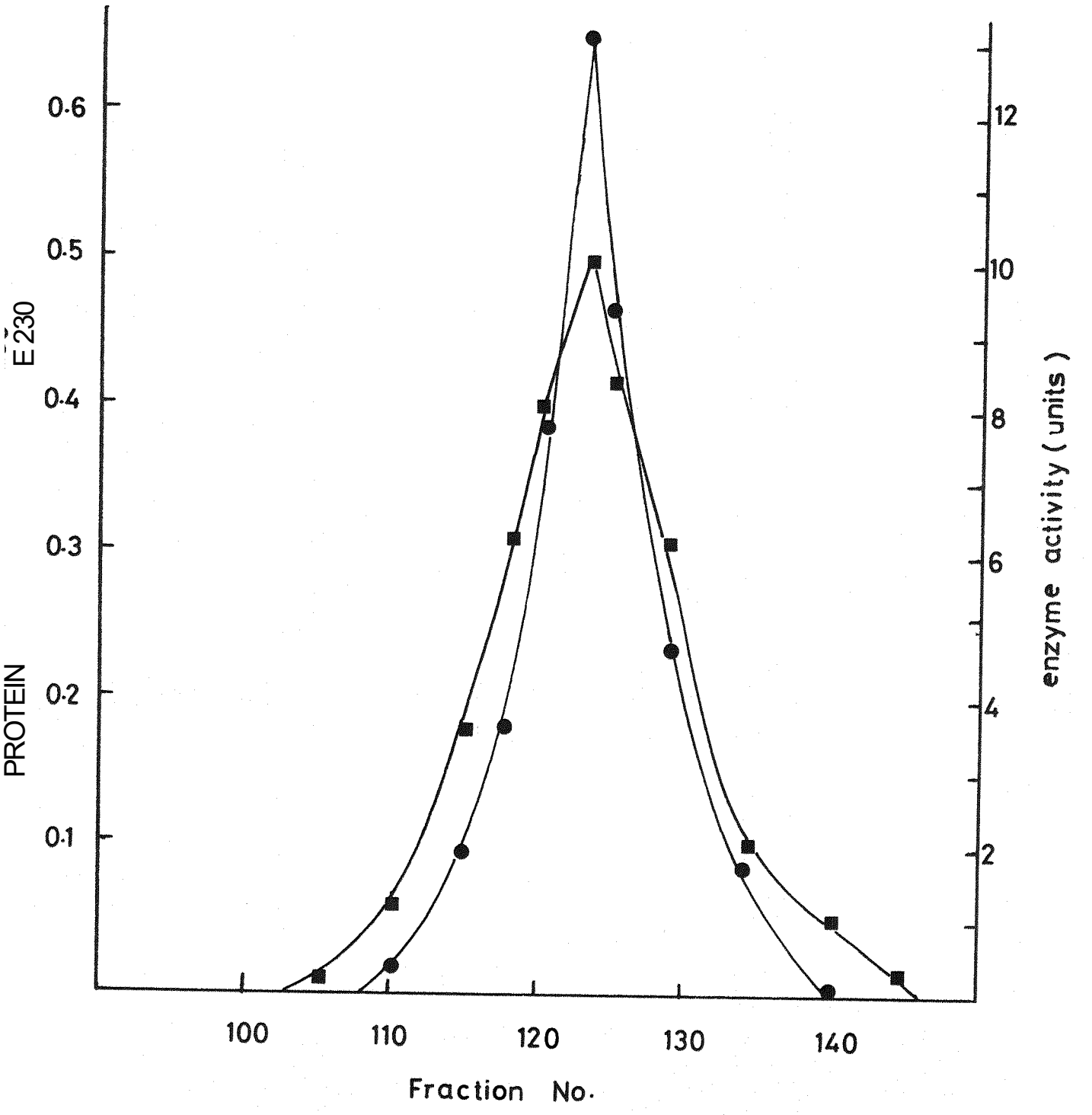


fig.18. Purification through a DEAE-cellulose column. (■) protein at E_{230}, enzyme activity (●).

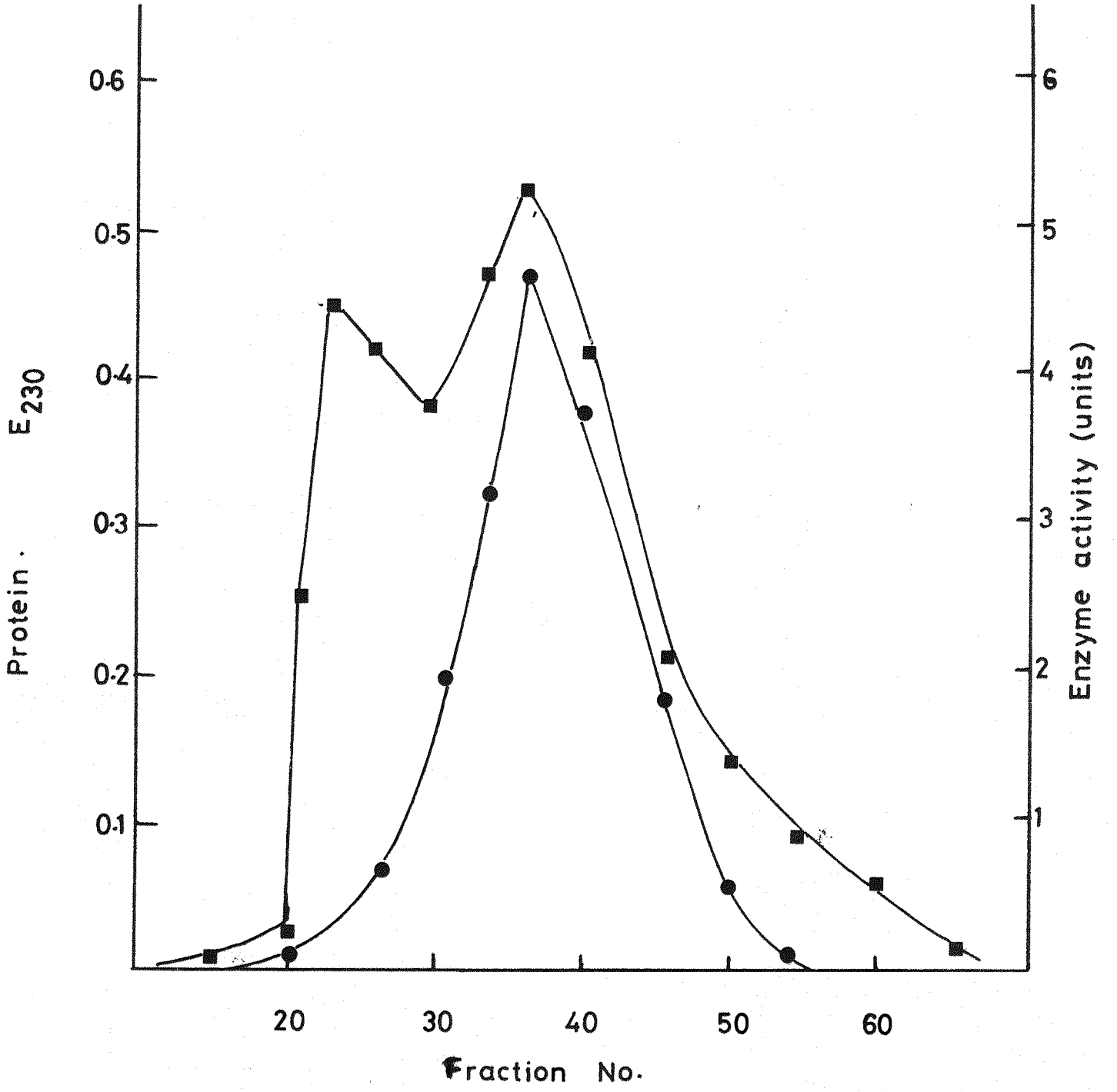


fig.19. Purification through Sephadex G-200. Fractions of 3 ml were collected and assayed for enzyme acivity(●) and protein (■) by Extiction at 230 nm.

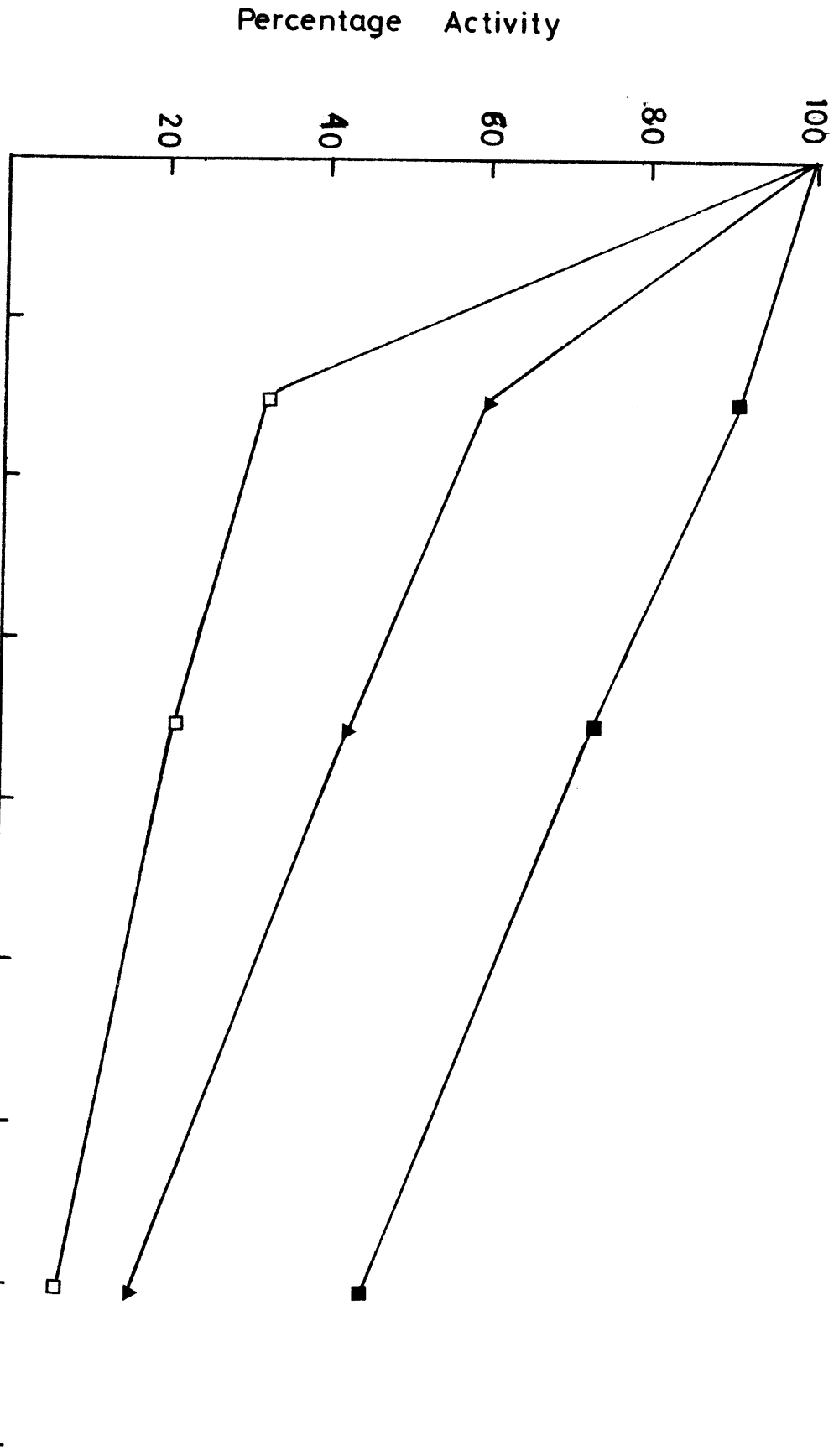


fig.20. Enzyme storage under various conditions. (■) In deep freeze (-5°C) with DTT, In fridge (4°C) with DTT (▲), in deep freeze without DTT (□)

Inactivation of N-acetylneuraminic acid lyase by bromopyruvate

Purified enzyme obtained from Cl-perfrigens was incubated at 37°C with 5mM bromopyruvate and 50 µl portions were assayed for enzyme activity at various time. The standard assay system contained 1mM dithiothreitol to inactivate bromopyruvate. The activity was plotted against time (Fig.21).

Inactivation of frog liver N-acetylneuraminic acid lyase by bromopyruvate

Enzyme obtained from frog liver was treated with bromopyruvate in the same way as above. The results are illustrated in Fig.22.

In a following experiment it was found that the optimum pH for the potassium phosphate buffer used in the assay for the enzyme activity was 7.2 (Fig.23).

Comparison of the rates of reaction of bromopyruvate and chloropyruvate with N-acetylcysteine

The experiment has been done as it was described in the methods section. The rate of reaction of chloropyruvate with a simple thiol, N-acetyl-cysteine, was slower than that of bromopyruvate. The approximate second order rate constants at 9°C were 1.65 and 10.6 $l.mol^{-1}sec^{-1}$, respectively. The increase in volume (0.2 ml) and the consumption of halogenopyruvate during the reaction were disregarded for the calculation of the rate constants assuming pseudo first order kinetics. The rate constants must therefore be regarded as approximate. In Fig.24and Fig.25 is illustrated the rate of reaction of bromopyruvate and chloropyruvate with N-acetyl-cysteine.

Rates of inactivation of N-acetylneuraminate lyase by chloropyruvate and bromopyruvate

The enzyme and the inactivator were incubated at 5°C, and at required time portions of 10 ml were pipetted into the standard assay mixture containing 1mM dithiothreitol at 37°C. The activity remaining was measured. As inactivators were used 0.5mM bromopyruvate, 0.9mM

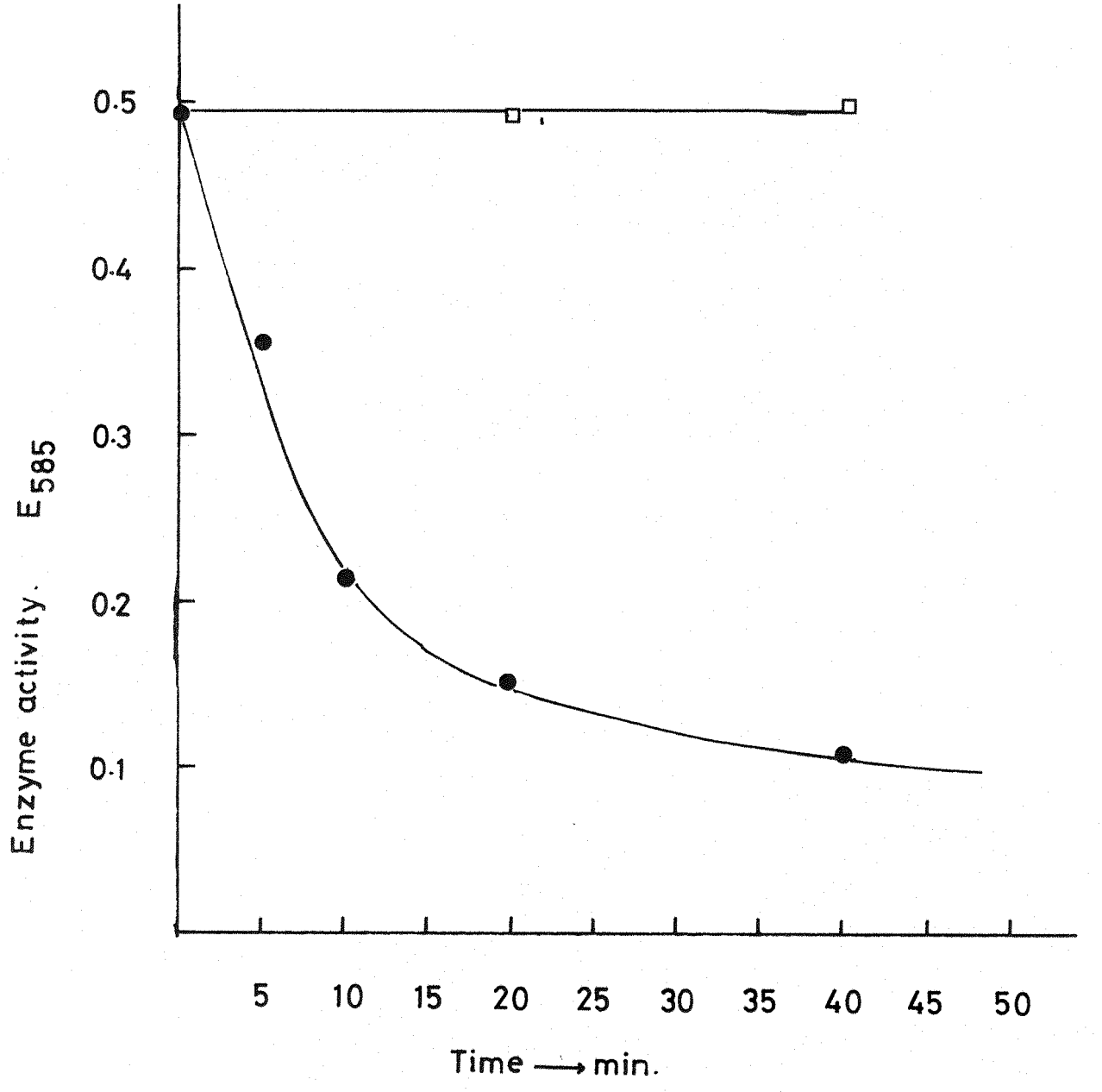


fig.21. Inactivation of Cl.perfringens N-acetylneuraminic acid lyase by bromopyruvate (●). No addition of bromopyruvate (□).

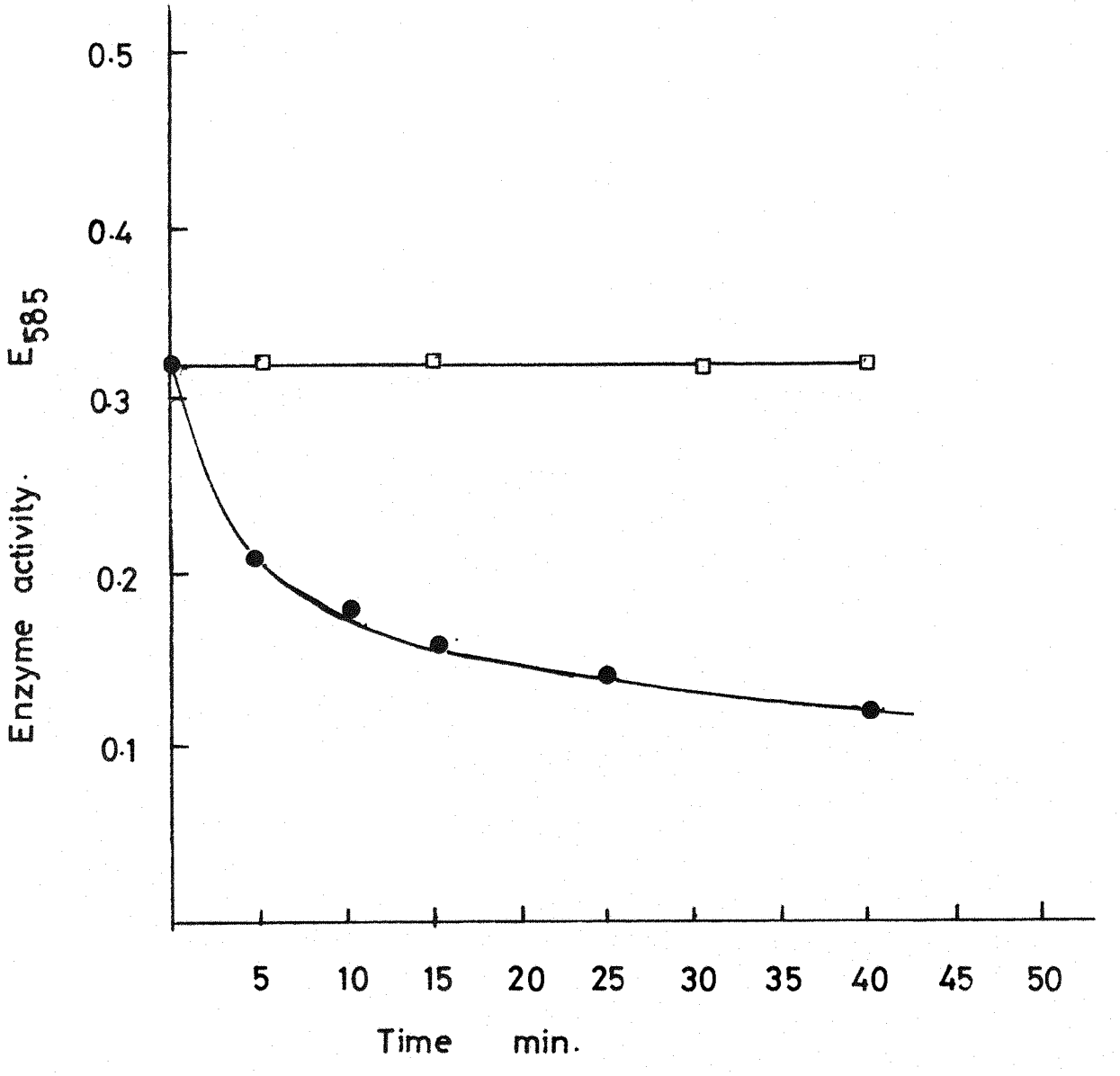


fig.22. Inactivation of frog liver N-acetylneuraminic acid lyase by bromopyruvate(●). No addition of bromopyruvate(□).

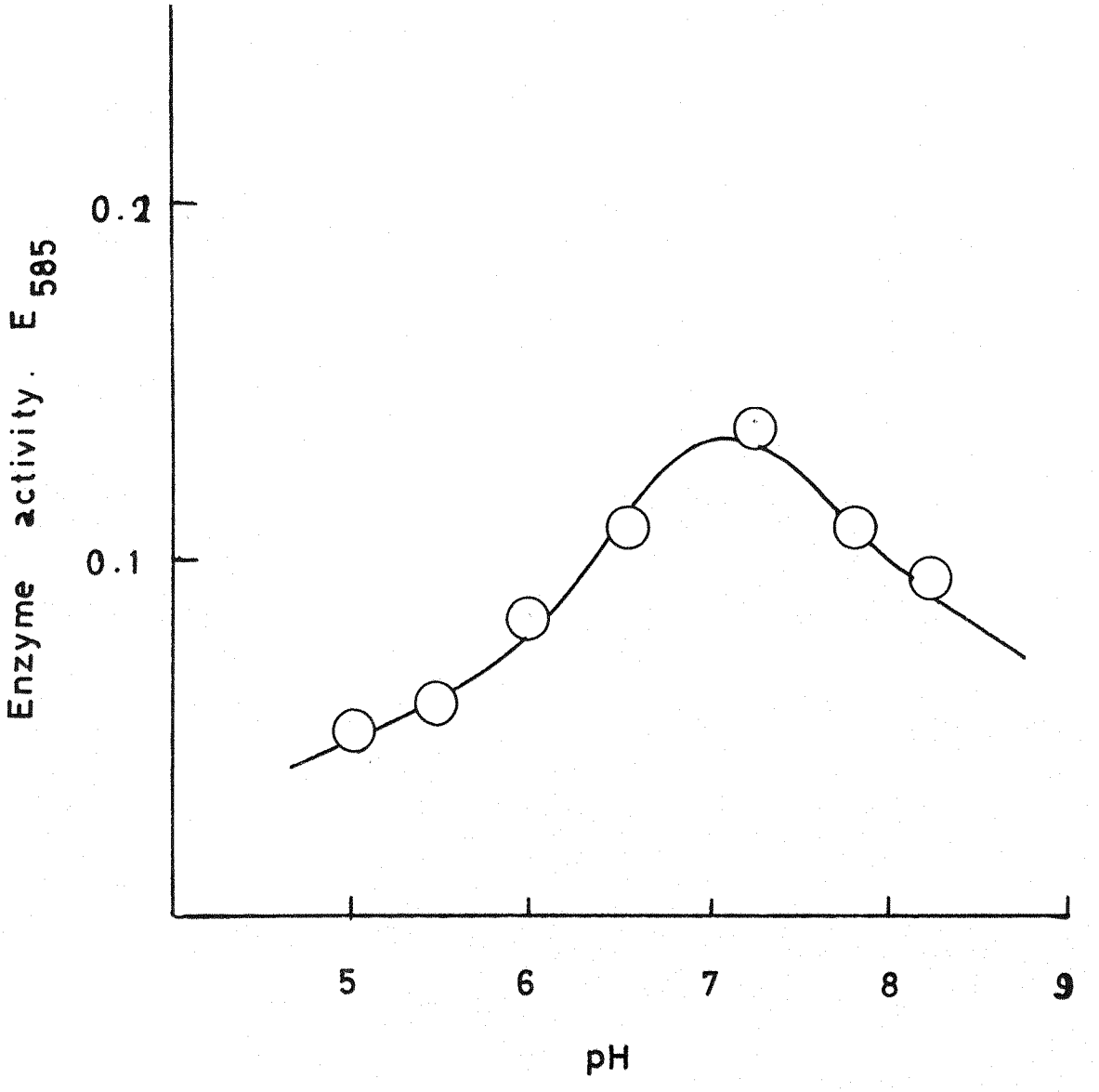


fig. 23. pH Optimum of N-acetylneuraminic acid lyase from frog liver.

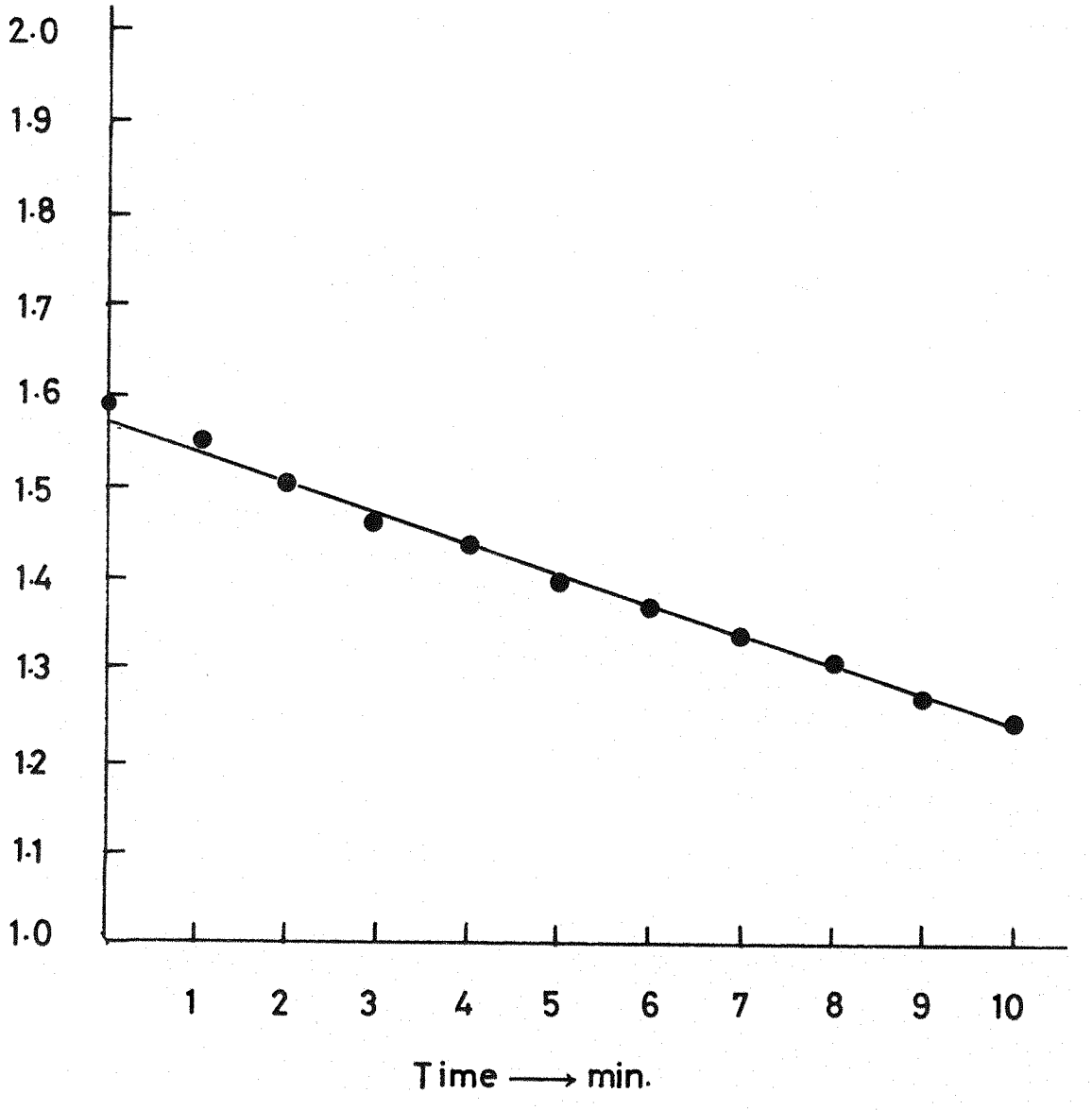


fig. 24. Reaction of N-acetylcysteine (0·2ml, 10mM) with bromopyruvate (0·1ml, 100mM) at 9°C.

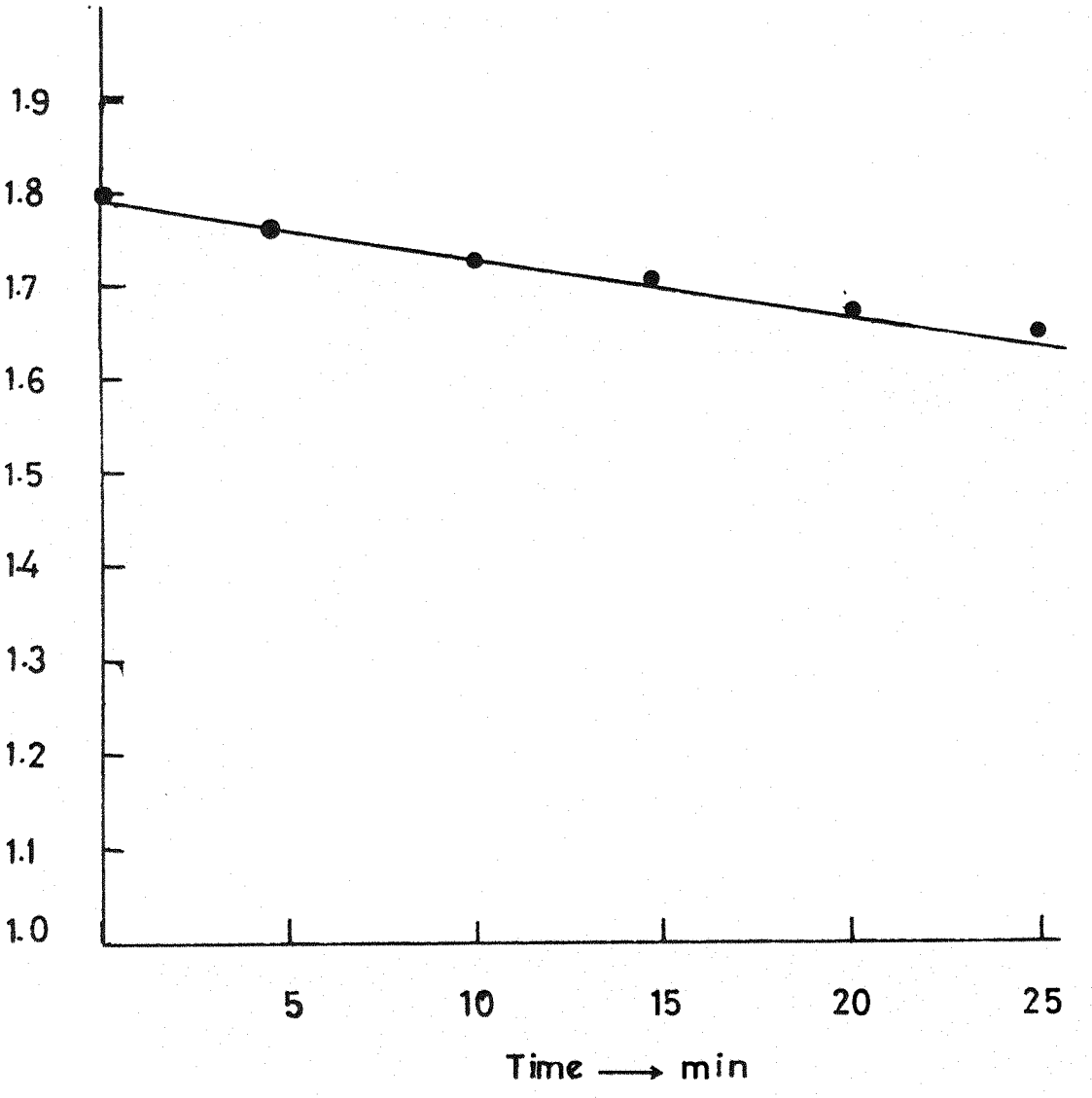


fig.25. Reaction of N-acetylcysteine (0.2 ml, 10mM) with 0.1ml of 100mM chloropyruvate at 9° C.
(Not the different time scale from fig.24)

chloropyruvate and 0.9mM chloropyruvate in the presence of 10mM pyruvate. The $\log_{10}$% activity was plotted against time (Fig. 26.)

Reaction of N-acetylneuraminic acid lyase with (^{14}C) chloropyruvate

The inactive enzyme (2.5mg of protein) obtained after the treatment with radioactive chloropyruvate (see Methods section p 42) (1.2 μmoles, 1.59 x 10^7 cpm/μmole) was chromatographed on Sephadex G-25 (Fig.27) and the radioactive fractions (5 - 9) were pooled out and freeze-dried. Then it was dissolved in 1 ml of water and two small portions of 10 μl were tested for radioactivity and protein. The measured specific activity was 2.9 x 10^5 cpm/mg protein. This corresponds to 4.5 pyruvate residues attached per mole of enzyme using a molecular weight of 2.5 x 10^5.

Reduction of the pyruvate-N-acetylneuraminic lyase complex with sodium borohydride

The N-ANA lyase-pyruvate complex was stabilized with sodium borohydride as described in the experimental sections. As shown in Fig. 28 the enzyme activity was determined before and after the treatment with sodium borohydride and compared with the control treated with sodium borohydride in the absence of pyruvate.

In another experiment a sample of the same manufacturer's batch of sodium pyruvate (1.4 μmole, 1.37 x 10^7 cpm/μmole) and same batch of enzyme (2.5 mg) as used in the previous experiments was treated with sodium borohydride (see p 42). Finally the freeze-dried protein was applied to the Sephadex G-25 column (Fig.29). The specific activity of the combined fractions containing the majority of the radioactivity was determined (2.0 x 10^5 cpm/mg protein). Assuming a molecular weight for the enzyme of 2.5 x 10^5, this corresponds to 3.7 moles of pyruvate per mole of enzyme.

Electrophoresis

Electrophoresis was on Whatman No.1 paper using a Shandon vertical electrophoresis tank and a Vokam power pack at 300v. The buffers

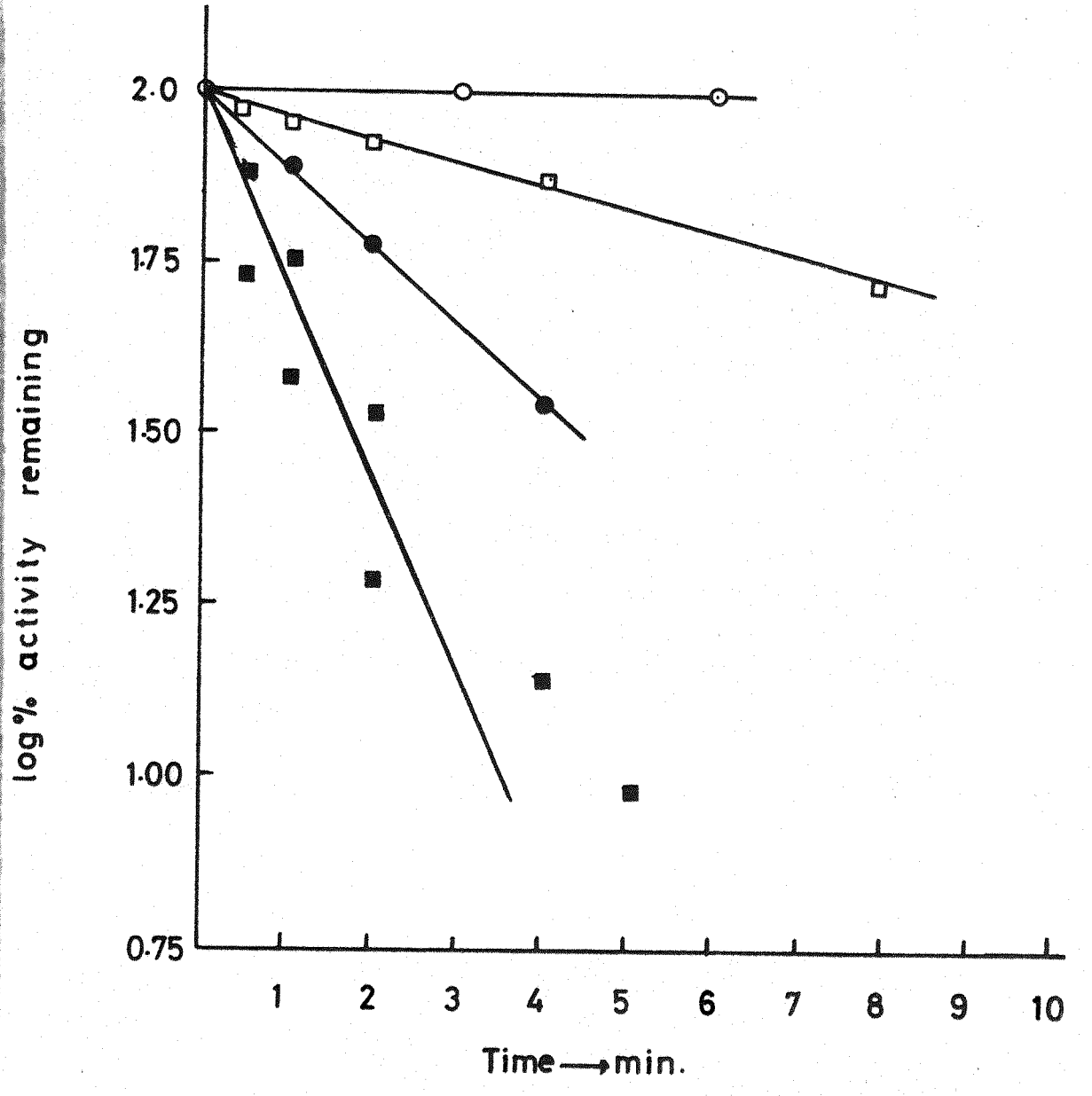


fig. 26. Rates of inactivation of N-acetylneuraminic acid lyase by chloropyruvate and bromopyruvate at 5°C. The activity remaining was measured. (○) No inactivator; (□) 0·5 mM-bromopyruvate, (■) 0·5 mM-chloropyruvate (two experiments), (●) 0·5 mM-chbropyruvate in the presence of 10 mM-pyruvate.

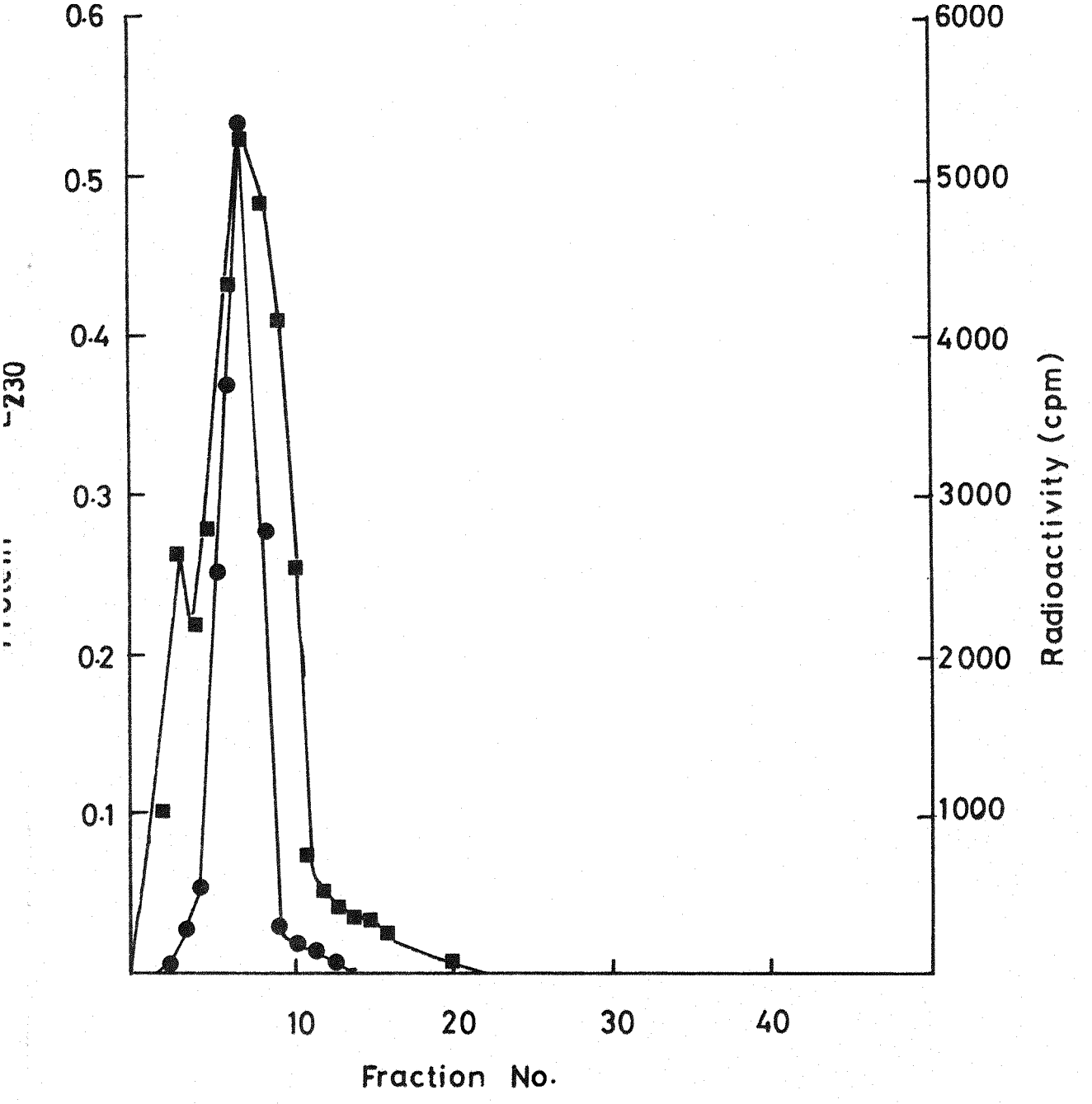


fig.27. Chromatography through Sephadex G-25 after inactivation by radioactive chloropyruvate.
(■) protein at E_{230}, (●) radioactivity.

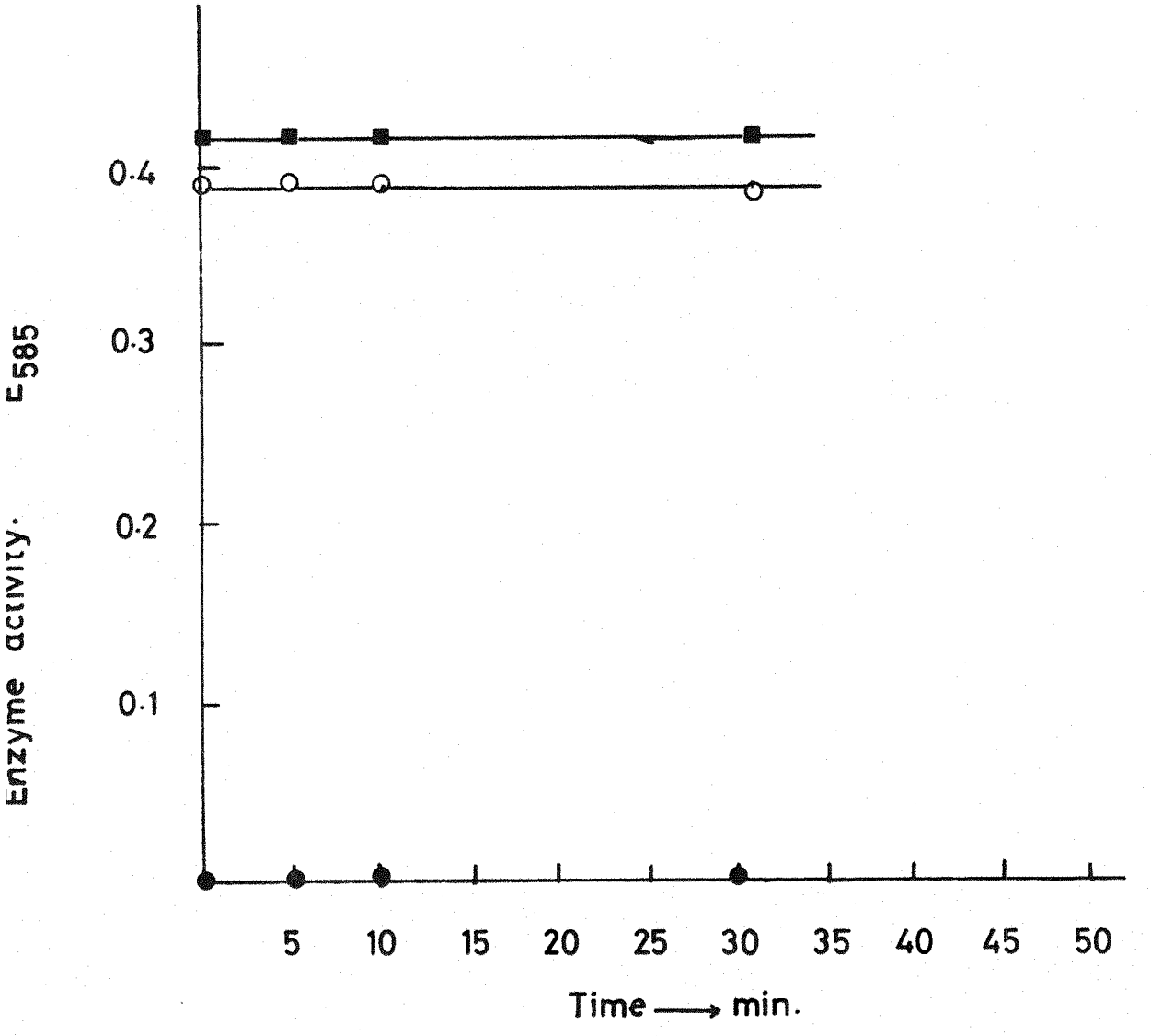


fig. 28. Inactivation of N-acetylneuraminic acid lyase with $NaBH_4$ in the presence of pyruvate. (○) pyruvate, (●) pyruvate and $NaBH_4$, (■) $NaBH_4$.

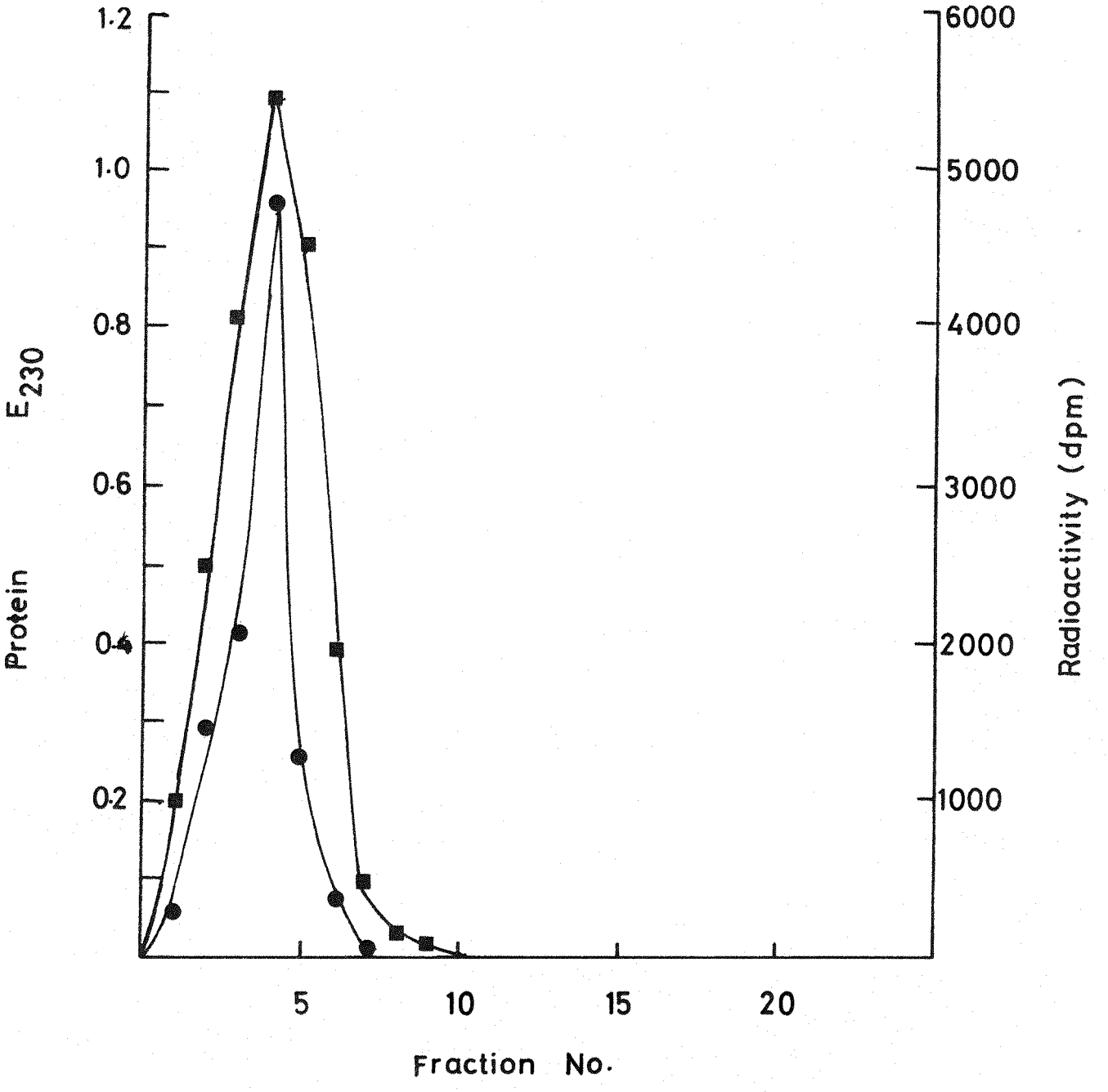


fig.29. Chromatography on a Sephadex G-25 column after inactivation by $NaBH_4$ in the presence of radioactive pyruvate, (■) protein at E_{230}, (●) radioactivity.

were 25% formic acid-acetic acid-water (13:29:25% by volume) pH 1.6; pyridine-acetic acid-water (50:2:948, by volume) pH 6.0; and sodium diethyl barbituric acid-water (10.3:1.84:1000,W/W/v) pH 8.6. In 100 min S (3-lactic acid) cysteine migrated - 3.0 cm, + 3.0 cm and + 5.1 cm towards the anode respectively (Fig.30).

Identification of the residue attacked by chloropyruvate

After borohydride reduction to stabilize the pyruvate residue, the product of (^{14}C) chloropyruvate inactivation was hydrolysed by 6M-HCl. The hydrolysate radioactive product moved identically to S - (3-lactyl)-cysteine on electrophoresis at three different pH's (Fig.30)

Molecular weight of N-acetylneuraminic acid lyase

The molecular weight of the enzyme from Clostridium perfrigens was determined using a Sephadex G-200 column as is shown in the methods section. Two slightly different experiments took place for the determination of the M.W.

a) The enzyme, which was applied on the Sephadex G-200 column together with blue dextran, cytochrome C, lactate dehydrogenase, malate dehydrogenase, catalase and urease, was the enzyme obtained after the ammonium sulphate fractionation (step 5-6 of the purification procedure). In the determination of this enzyme preparation the molecular weight was about 250,000. The elution volume was plotted against log (mol.wt.) for the mixture of the known molecular weight proteins (Fig 31.).

b) In the second experiment the enzyme used on the Sephadex G-200 column was obtained after DEAE-cellulose and Sephadex G-200 step of the purification procedure. In this second method the molecular weight was determined about 100,000. (Fig 32).

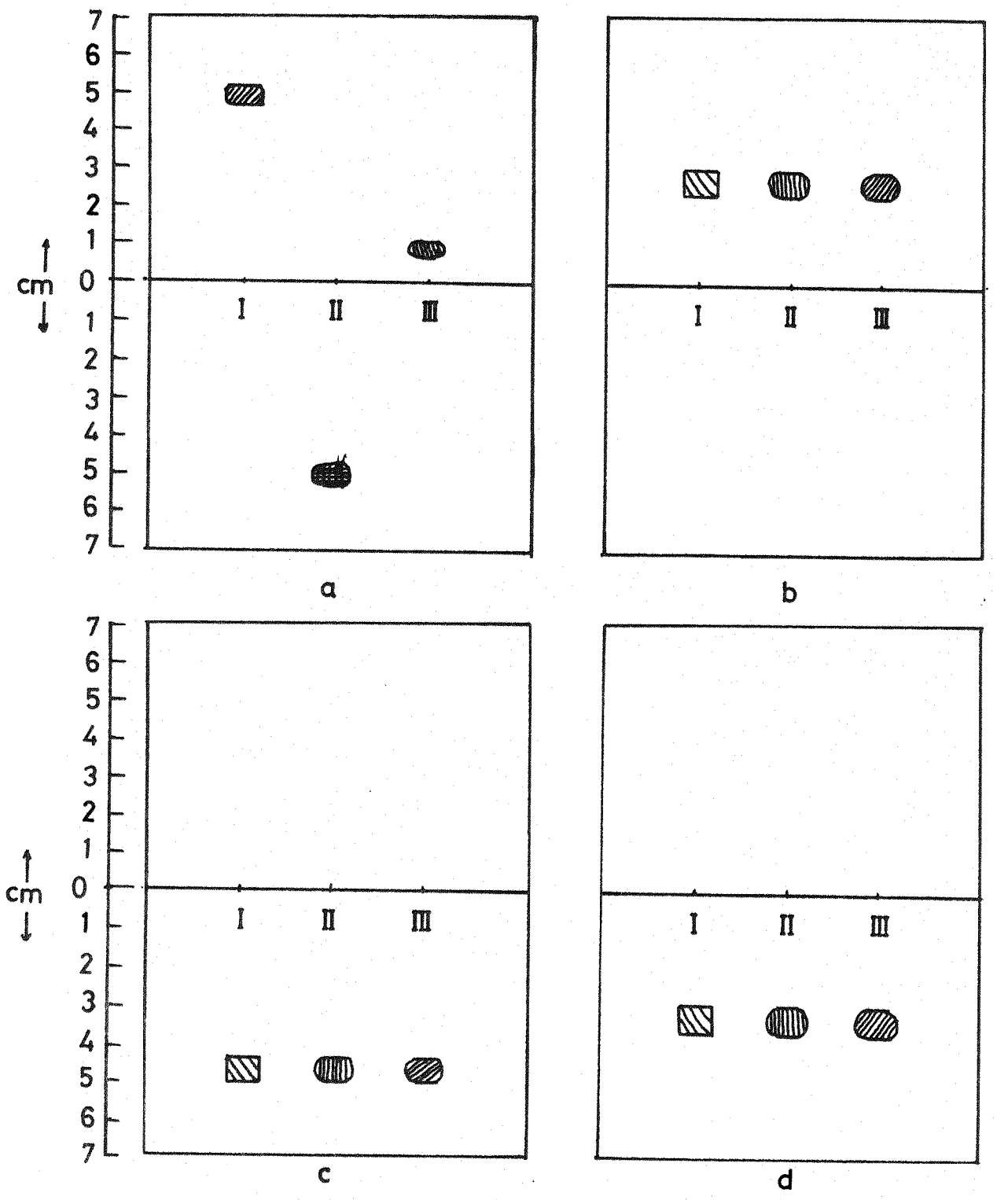


fig.30. Electrophoresis of : radioactive chloropyruvate enzyme hydrolysate (I), enzyme + S-lactyl-cysteine (II), S-lactyl-cysteine (III), buffers used :

b) formic acid: acetic acid: H_2O, pH 1.6

c) sodium diethyl barbiturate: diethyl barbiturate: H_2O, pH 8.6

d) pyridine: acetic acid: H_2O, pH 6.0

a) electrophoresis of lysine (I), glutamate (II), glysine (III), buffer as -d-

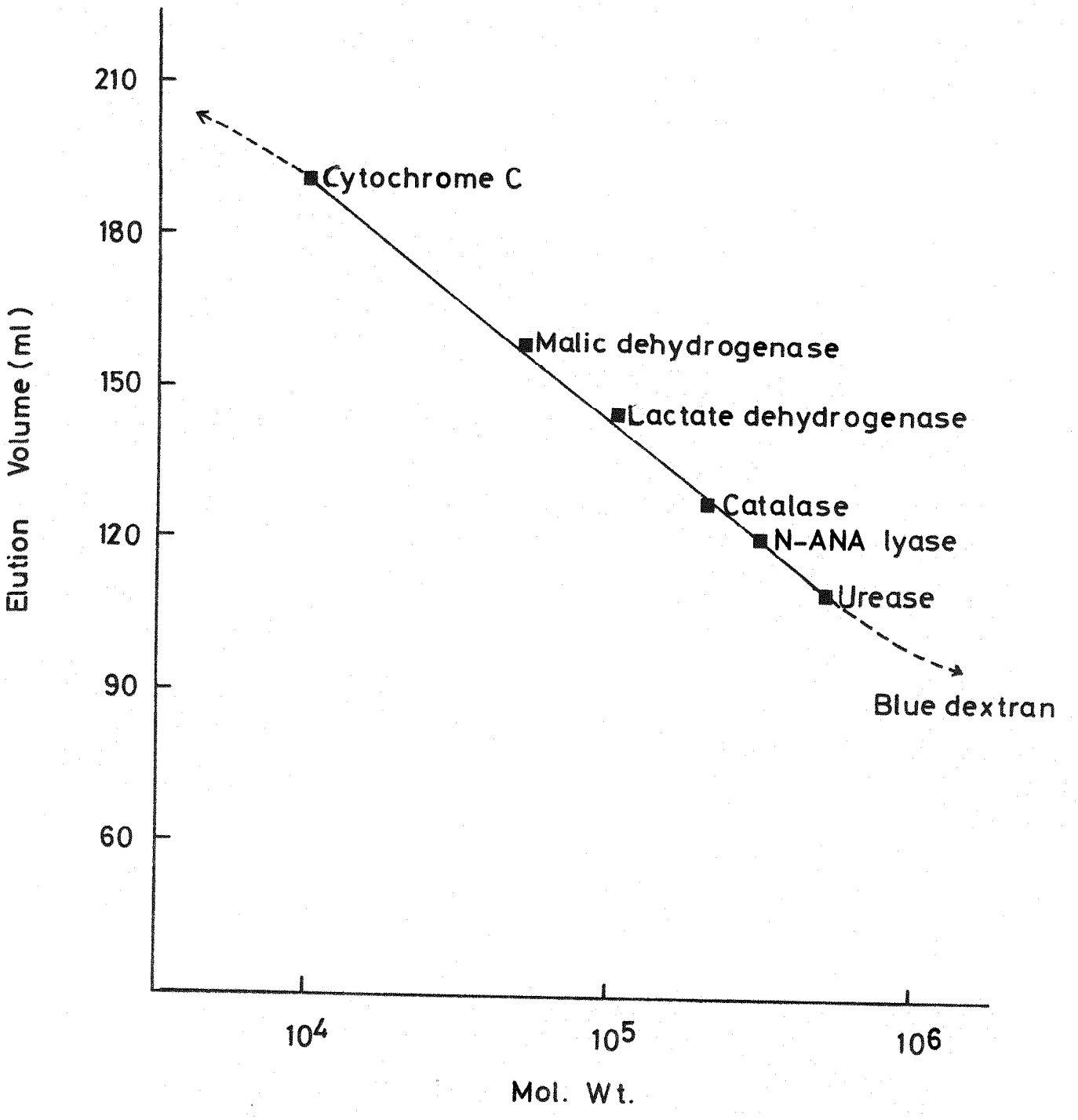


fig.31. Molecular weight determination of N-acetylneuraminic acid lyase (Barnett et al, 1971)

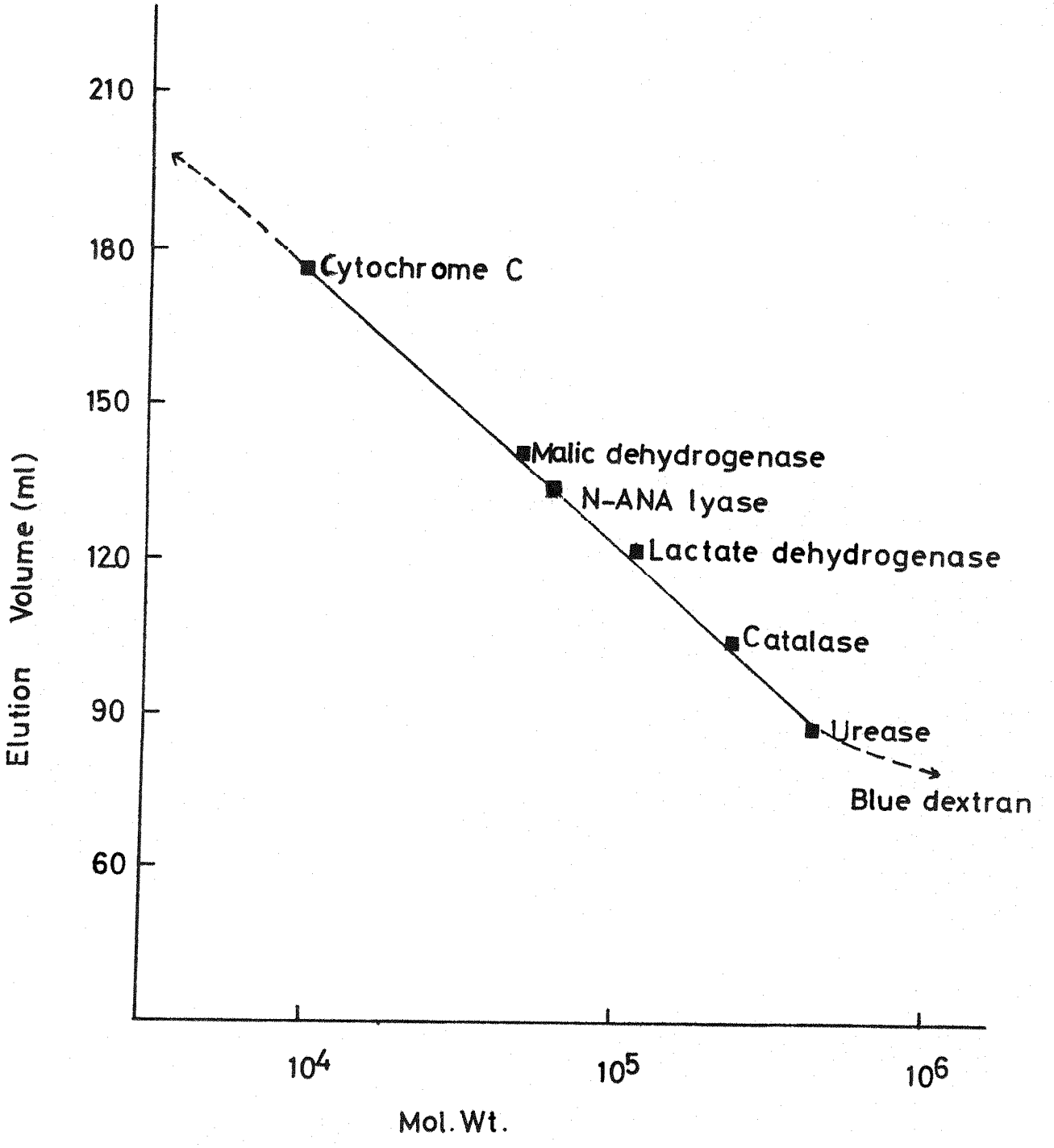


fig. 32. Molecular weight determination of N-acetylneuraminic acid lyase (Devries-Binkley, 1972)

DISCUSSION

Active site residues of N-acetylneuraminic acid lyase

Barnett et al (1971) have suggested a possible mechanism for N-acetylneuraminic acid lyase (see fig.14, p.21) In addition to the $-NH_2$ group of a lysine residue at the active site of the enzyme, which reacts with the carbonyl group of the substrate to form a Schiff's base, the mechanism requires a group 'x' close to the point of condensation able to accept the hydrogen of the alpha carbon of the carbonyl of substrate. Such a group should also act as a nucleophilic.

In an attempt to investigate the nature of this 'x' basic group, they used bromopyruvate as an analogue of pyruvate, which is a substrate for the enzyme. It was expected that the nucleophile of the enzyme would displace the bromide ion to form a stable covalent enzyme derivative. They found that bromopyruvate reacted with N-acetylneuraminic acid lyase and inactivated the enzyme. Reaction was at, or close to, the pyruvate binding site, because pyruvate gave protection to the enzyme against inactivation by bromopyruvate. However they found also that bromopyruvate was not so specific as they had hoped for the active site of the enzyme, because the ratio of bromopyruvate moles bound per active site of the enzyme (measured by the number of (^{14}C)-pyruvate residues attached by reduction of the enzyme-pyruvate Schiff's base complex) was about 5:1 which suggests that bromopyruvate reacted with any free reactive group.

To find the nature of the residue alkylated, they prepared and used radioactive bromopyruvate to inactivate the enzyme. The hydrolysate of the enzyme gave one product identical with S-(3-lactic acid)-cysteine. The sulfhydryl group of this thiol containing amino acid appeared to be the functional group of the enzyme which reacts with bromopyruvate.

This thesis describes an attempt to use chloropyruvate in the hope that it might be more specific.

Chloropyruvate, because of the stronger bond between the halogen

and the carbon atom, should be less reactive than bromopyruvate. As expected the rate of the reaction of chloropyruvate with, N-acetylcysteine, a simple thiol compound, was slower than that of bromopyruvate with the same compound (Fig 25 ,Fig 24). The approximate second order rate constant at 9°C andpH 7.2was 1.65 $mol^{-1}sec^{-1}$ for chloropyruvate and 10.6 $mol^{-1}sec^{-1}$ for bromopyruvate.

However, in contrast, chloropyruvate inactivated N-acetylneuraminic acid lyase ten times faster than bromopyruvate (Fig 26). Protection by pyruvic acid against inactivation of N-acetylneuraminic acid lyase by chloropyruvate (Fig 26) shows that the reaction of chloropyruvate with the enzyme was at, or close to, the active site.

After reduction of the enzyme-radioactive pyruvate Schiff's base complex by sodium borohydride the number of (^{14}C)-pyruvate residues bound to the enzyme was calculated. Assuming a molecular weight of about 250,000 this gave 3.7 pyruvyl residues bound to the enzyme. This suggests that there are about four active sites per mole of N-acetylneuraminic acid lyase, and confirms the results of Barnett et al (1971).

After reaction with 1mM radioactive chloropyruvate for 2 min. at 37°C the enzyme was completely inactive (under the same conditions bromopyruvate only inactivated the enzyme about 35% (Barnett et al 1971)). Calculating the specific activity of the inactive protein with that of the radioactive chloropyruvate, and assuming a molecular weight 250,000, 4.5 pyruvyl residues were found to be bound to the enzyme protein. That means that the ratio of chloropyruvate moles per active site of enzyme was about 1.1. Compared with the previous report by Barnett et al, that 5 moles of bromopyruvate bound per active site of enzyme, this shows that chloropyruvate appears, at least for this enzyme, to be much more specific than bromopyruvate.

For identification of the residue attacked by chloropyruvate, the enzyme was inactivated by radioactive chloropyruvate. After passage through Sephadex G-25 column the radioactive fractions were pooled and reduced with

sodium borohydride to stabilize the pyruvate residue. As lactate hydrolysis of the reduced protein gave only one detectable radioactive compound, which was identical with S-(3-lactic acid)-cysteine on electrophoresis at three pH values (Fig 30).

These results show that the x basic group which is at, or close to, the active site of N-acetylneuraminic acid lyase, responsible for accepting the proton lost during an aldol condensation is probably cysteine, which reacts with chloropyruvate to form an inactive enzyme complex.

Molecular weight and polymerisation of the enzyme

Using Sephadex G-200, Barnett et al (1971) have reported an apparent molecular weight of 250,000 for N-acetylneuraminic acid lyase. Devries and Binkley (1972) by using also Sephadex G-200 for the determination of the molecular weight, but using an enzyme preparation purified with a slightly different procedure, suggested an apparent molecular weight of 92,000 for N-acetylneuraminic acid lyase.

The apparent molecular weights of two different enzyme preparations were therefore determined using Sephadex G-200 column chromatography.

In the first enzyme preparation was similar to that of Barnett et al. The enzyme applied to the Sephadex G-200 column was a sample of the enzyme obtained after the ammonium sulphate fractionation step of the purification procedure and the apparent molecular weight was 250,000.

In the second preparation an enzyme preparation purified by DEAE-cellulose chromatography was used. The molecular weight was about 100,000.

These results suggest that the enzyme can polymerize, and probably exists either as the dimer or tetramer in the two forms.

The electrophoretic results of Devries and Binkley(1972), which show the presence of two separately migrating forms of active enzyme, and which have been confirmed in our laboratory (G. Rasool,Ph.D thesis 1972) support the suggestion of a polymeric enzyme.

It is obvious that the DEAE-cellulose chromatography, during the purification procedure of the enzyme, plays a remarkable role in altering the apparent molecular weight of N-acetylneuraminic acid lyase. A possible explanation for this observation may be that when the enzyme passes through the DEAE-cellulose column a small molecular weight of protein or peptide responsible for keeping the two dimers together, may be removed.

In this context, it is interesting to note that the high molecular weight is not a strict multiple of the low molecular weight, although the values found, 250,000 and 100,000, are subject to large errors due to the approximate nature of the method of molecular weight determination.

A P P E N D I X

DETERMINATION OF PEPTIDES OF RIBONUCLEASE

INTRODUCTION

A simple approach to amino acids sequence determination of a protein involves cleavage of the original protein into different groups of short peptides, which can then be sequenced and overlapped to reconstruct the original protein.

The majority of proteins exist as multi-subunit structures. The techniques to be used for the breakdown of a protein depend upon whether the chains are covalently or noncovalently linked.

In the first case chemical methods are employed. In the second case of the noncovalently linked multi chain structures the chains held together by relatively weak forces (hydrogen bonds, hydrophobic bonds, electrostatic interactions, etc.). Some of the methods for the dissociation of these proteins are:

a) change of pH (Kawahara, K., Tanford, C., 1966)(Penhoet,E., Lochman, M., Valentine, R., Rutter, W., 1967).

b) chemical modification of the charge. This method is used in cases in which multi-subunit proteins do not dissociate in a practicable pH. The classic example of this type is the use of succinylation to add negative charges (Goldtein, J., Hill, R.,J.,1963).

c) strong denaturants. Concentrated solutions of Guanidine hydrochloride, urea and sodium dodecyl sulphate denature most proteins (Temford et al, 1967).

d) reduction or oxidation of disulfide bonds. In cases in which the chains are connected by disulfide cross-links reduction with a thiol reagent such as sulfite (Wake, R.G., 1962), or oxidation with performic acid (Harris, G.K., Tigane, E., Hanes, G.S. 1961) should be considered.

Such procedures may require the accompanying use of a denaturing agent like guanidine hydrochloride or urea.

The next step is the degradation of the macromolecule into low

molecular weight peptides, which can completely characterized with respect to composition and sequence. As the structure of large proteins is examined the need for methods of selectively clearing the polypeptide chain at specific sites becomes necessary.

i) Non-enzymic cleavage

There are a lot of methods for non-enzymic cleavage of peptide bonds and some of them are less specific than the others.

The most specific and generally applicable method available is the cyanogen bromide cleavage of the peptide chain at methionyl residues (Witkop, B., 1961).

Another useful method is the hydrolysis of protein by acid under conditions which do not cleave all peptide bonds. Basically two methods are employed: (a) partial hydrolysis with concentrated or constant boiling hydrochloric acid and (b) hydrolysis in dilute acid solution.

ii) Enzymic degradation

Enzymes catalyzed cleavage of a polypeptide chain offers many distinct advantages over partial acid hydrolysis.

The exact conditions for enzymic digestion depend upon the enzyme which is used, but the following factors are important of the type of digest : (a) pH, (b) temperature, (c) enzyme to substrate ratio, and (d) time of incubation.

The protein must be prepared in one of the following ways for enzymatic digestion:

1) Heating (Foltman, B., Hartley B.S.,1967)

2) Trichloroacetic acid precipitation, which is an efficient method for removing bound amino acids and peptides (in a case when autolysis products are possible contaminats).

3) Reduction and S-Carboxymethylation. (Crestfield et al 1963)

4) Performic acid oxidation (Hirs,C.H.W., 1956)

Enzymes used for enzymic degradation, usually are:

Trypsin

Trypsin exhibits the highest degree of substrate specificity for an endopeptidase, this enzyme hydrolyzes only those bonds involving the carboxyl groups of lysyl and arginyl residues.

Chymotrypsin

This enzyme hydrolyzes perferentially peptide bonds involving the carboxyl groups of tyrosine, phenylalanine, and leucine.

Pepsin

Pepsin can hydrolyze peptide bonds formed by either the amino or carboxyl groups of phenylalanine, tyrosine, glutamic acid, cystine and cysteine. In addition to protease activity, pepsin also hydrolyzes ester bonds.

Fractionation of Peptides

The successful separation of peptide mixture resulting from either the chemical or enzymic degradation of a protein requires the application of more than one technique. Initial fractionation may be performed on a cation exchange resin such as Dowex 50, followed by chromatography of impure fractions on the anion exchanger Dowex 1 as recommended by Schroeder et al (1962).

Further fractionation of unresolved peptide mixtures may be accomplished by gelfiltration, chromatography on cellulose or dextran gels, or paper electrophoresis and chromatography . It was intended to find the sequence of peptides surrounding the active site of NANA lyase. As a preliminary to this study specific degradents of ribonuclease into peptides was undertaken.

EXPERIMENTAL

PERFORMIC ACID OXIDATION OF RIBONUCLEASE AT -10°C

Performic acid was prepared by adding 0.1 ml of 30% H_2O_2 (reagent Superoxol, Nerck) to 2.0 ml of 99% formic acid (Mathelson, Coleman and Bell,Inc.). The resulting solution was allowed to stand at room temperature (25°C) for 2 hours in a stoppered flask. In another flask 4 mg ribonuclease were dissolved completely in 1.0 ml of 99% formic acid, after which 0.2 ml of anhydrous methanol was added with stirring. The quantity of performic acid used was 12 times that required to transform all of the cystine in the ribonuclease to cysteic acid, and all of the methionine to the sulfone. The protein solution and the performic acid were transferred to the separate arms of a glass-stoppered test-tube to which a side arm had been sealed. The reactants were cooled for 30 minutes in an acetone-dry CO_2 bath maintained between -7°C and -10°C and then mixed by tipping the tube. The reaction was allowed to take place at the same temperature for 2.5 hours. The contents of the tube were rinsed with 5 ml of ice water into a flask containing 25 ml of water at 0°C. The diluted solution was frozen immediately, and the solvents were removed by lyophilization. The residue was dissolved in 4 ml of water, transferred to a smaller flask with the aid of a further 4 ml of water and again lyophilized.

The powders obtained by this procedure were stored at 4°C until required. In this method it is necessary to perform the oxidation on halide-free protein, since free halogen formed in the presence of performic acid results in the production of halotyrosines. The fact that tryptophanyl residues are concerted by this oxidizing agent to kynurenine and other degradation products particularly limits the applicability of this method. Removal of halide ion is accomplished by passing a 10% solution of protein in 0.01M acetic acid over a bed of Dowex 2 x 10 in the acetate form equilibrated with the same solvent. One ml of resin bed is used per ml

of a protein solution. The halide-free preparation is recovered by lyophilization of the filtrate.

Method of hydrolysis by Trypsin

Ribonuclease after the oxidation was suspended in 1 ml of water. Trypsin, in an amount equal to 1% of Ribonuclease by weight, was added and the solution stirred for 24 hours at room temperature. Portions of 50 ml were withdrawn after 4,8 and 24 hours, centrifuged and the supernatant was applied on a Whatman No.3 paper for the chromatography procedure.

Chromatography

Descending chromatography is performed with full-sized sheets of Whatman 3MM. The origin, guide strips and lines to indicate folding were drawn on the paper as illustrated in Fig.33 The paper was folded along lines a and b as indicated in the Fig., and the sample together with the methyl red was added in order to see the progress of the separation, was applied to the origin, which should not exceed 6mm in width

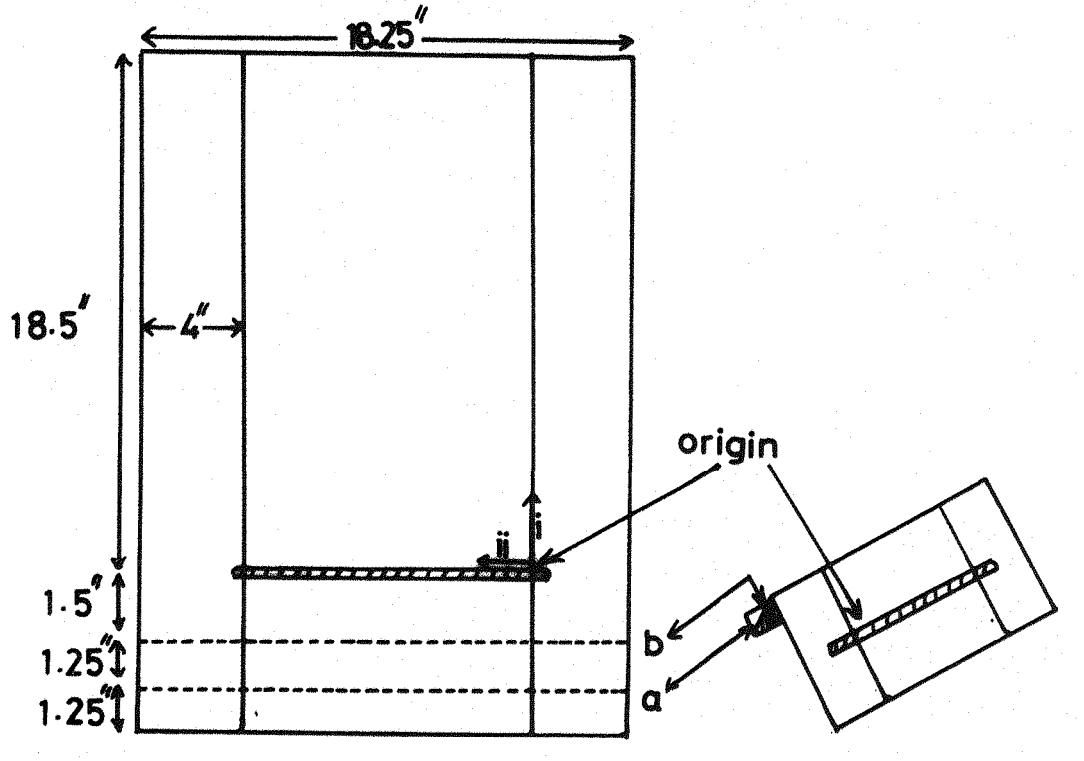


Fig. 33

The chromatogram was developed for a period of 16 to 20 h. The solvent systems which were used in direction i (Fig 33) were L-Butanol 200: glacial acetic acid 30 : H_2O:75 (for chromatography) and L.Butanol 4: glacial acetic acid 1: H_2O 5 (in the bottom of the chromatographic chamber). Then the paper was dried and rechromatographied for an additional 20 h. The solvent systems which were used this time in direction ii (Fig 33) were L-Butanol 75 pyridine 50: glacial acetic acid 15: H_2O 60 and Pyridine 7: iso-amyl alcohol 7: H_2O 6. (Ratio by volume). After the chromatography the paper was dried and the peptide zones were located with ninhydrine.

Results

The number of peptides of Ribonuclease after the treatment with trypsin, which hydrolyzes the bonds involving the carboxyl groups of lysyl and arginyl residues, is in agreement with what was expected from literature.

REFERENCES

Aldridge, W.N.

Biochem.J., 53, 62, (1953)

Andrews, P.,

Biochem.J., 96, 595, (1965)

Bachawat, H.,

C.A., 1955

Baker, J.P., and Rabin, B.R.,

Eur.J. Biochem., 11, 154, (1969)

Balls, A.K., and Aldridge, F.L.

Proc.Natl.Acad.Sci., US, 41, 190, (1955)

Balls, A.K., and Wood, H.N.,

J.Biol. Chem., 219, 245, (1956)

Balls, A.K., and Jansen, E.F.,

in F.F.Nord, ed., Advances in Enzymology, Vol.XX,p321
Interscience, New York - London 1952

Barnett, J.E.G.,

Biochem.J., 105, 42, (1967)

Barnett, J.E.G., Corina, D.L., Rasool, G.,

Biochem. J., 125, 275, (1971)

Barnett, J.E.G., Atkins, G.R.S.,

542 Meeting of the Biochemical Society,
University of Southampton, September 1973.

Barod, R.C., and Gusalus, J.C.,

J. Bacteriol., 59, 387, (1950)

Beisenherz, G., Boltze, H.J., Bücher, T., Czok, R., Garbade, K.H.,
E.Mayer-Avendt, Fleiderer, G.

Naturfarsch, N., 86, 555, (1953)

Bell, R.P., and Higginson, W.C.E.,

Proc.R.Soc., 197A, 141, (1949)

Berghauser, J., Falderbaun, I., Woenckhaus, G.,

Hoppe-Seyler's Z.Physiol.Chem., 352, 52, (1971)

Brown, G.W., Jr., Brown, W.R., Cohen, P.P.,

J.Biol.Chem., 234, 1775, (1959)

Bromgraber, E.G., Abood, L.G.,

J.Biol.Chem., 235, 1847 (1960)

Brunetti, P., Jourdian, G.W., Roseman, S.,

J. Biol.Chem., 237, 2447, (1962)

Cash, D.J., and Wilson, I.B.,

J. Biol.Chem., 241, 4290, (1966)

Chanch, B., Machly, A.E.

Methods in Enzymology, Vol 2, p.764 by Colowick, S.P., and Kaplan, N.O., New York Academic Press Inc.

Chalambor et al

C.A., 1962

Chang, G., Hsu,R.,

Biochem.Biophys.Res.Commun., 55 (3),580, (1973)

Comb, D.G., and Roseman, S.,

J.Am. Chem.Soc. 80, 497, (1958)

Comb, D.G., and Roseman, S.,

J.Biol.Chem., 235, 2529 (1960)

Crestfield, A.M., Moore, S., Stein, W.H.,

J.Biol.Chem., 238, 622 (1963)

Devries, G., Binkley, S.B.,

Arch.Bioch.Biophys., 151, (1),243, (1972)

Devries, G., Binkley, S.B.,

Arch.Bioch.Biophys., 151, (1),234, (1972)

Foltman, B., Hartley, B.S.,

Biochem.J., 104, 1064 (1967)

Gary, D.N., Kupferberg, L.L., Graff, L.H.

J. Bacteriol., 69, 478, (1959)

Gladner, J., and Laki, K.,

J.Am.Soc., 80, 1263, (1958)

Glikina, M.W., Finogenov, P.A.,

Biochimiya, 15, 457, (1950)

Goldstein, J., Hill, R.J., Konigsbreg,M.,

J.Biol.Chem., 238, 2016, (1963)

Gray, C.J.,

Enzyme catalysed reactions, Van Nostrand Reinhold Comp., London, 1971, p.34

Grazi, E., Meloche,H., Martinez,G., Wood,W.A., Horecker, B.L.,

Biochem.Biophys.Res.Commun., 10, 9 (1963)

Grazi, G., Rowley, P.T., Cheng,T., Tchola,O., Horecker,B.L.,

Biochem.Biophys.Res.Commun., 9, 38, (1962)

Hartley, B.S.,

Biochem.J., 64, 27, (1956)

Harris, G.K. Tigane, E., Hanes,G.S.,

Can.J. Biochem.Physiol., 39, 439, (1961)

Herbert,D., Gordon,H., Subrahmanyan,F., Green D.E.,

Bioch. J., 34, 1108, (1940)

Hirs, C.H.W.,

J.Biol.Chem., 219, 611, (1956)

Hoffee, P.Lai, C.Y., Pugh, E.L., Horecker, B.L.,

Proc.natl.Acad.Sci., U.S.A. 57, 107 (1967)

Hopkins, F.G., Morgan, E.J., Lutwakmann, C.,

Bioch.J., 32, 1829, (1938)

Horecker, B.L.,

J.Cell.Physiol. 54, Suppl.1,90 (1959)

Horecker, B.L., Potremoli, S., Ricci, C., Cheng,J.,

Proc.Natl.Acad.Sci.USA, 47, 1949 (1961)

Horecker, B.L., Rowley, P.T., Grazi,E., Cheng,T., Tchola, O.,

Biochem. 338, 36 (1963)

Ingram. J.M., Hochster, R.M.

Can.J. Biochem., 45, 929, (1967)

Jagganathan, V., Singh,K., Damodaran, M.,

Biochem.J., 63, 94, (1956)

Jagganathan, V., Singh, K.,

B.B.A., 15, 138, (1954)

Jansen, E.J., Jang, R., Balls, A.K., Nutting, M.,

J.Biol.Chem., 179, 189 (1949)

Jordan M., Ingram, J.M., Wood, W.A.

J.Biol.Chem., 240, 4146 (1965)

Kawahara, K., Tanford, G.,

Biochemistry, 5, 1578 (1966)

Knox, P.K., Stumpf,D.E., Green, V.H.,

Auerbach J. Bacteriol., 55, 451, (1948)

Kobes, R.D., Dekker, E.E.,

Biochem.Biophys.Res.Commun., 25, 329 (1966)

Konberg, A.,

Methods in Enzymology, Vol.1, 441, (1955) by Colowick S.P., and Kaplan, N.O., New York, Academic Press Inc.

Koshland, D.E., Jr.,

Adv. Enzymol. 22, 45, (1960)

Kuhn, R., and Tiedeman, H.,

N.Naturforsch. 86, 428 (1953)

Lebherz, H.G., and Rutter, W.J.,

Biochemistry, 8, 109 (1969)

Lowery, O.H., Rosebrough, N.J., Farra, A.L., Randall, R.J.,

J.Biol.Chem., 193, 265, (1951)

Maldonado, M.E., Kyung-Ja,O.H.Perry, A.,

J.Biol.Chem., 247, (9), 2711, (1972)

Maruasi, J., Makoto, S.,

Mem.Fac.Educ., Kumato Univ., Sect.1, Nat.Sci., 21, 97 (1972)

Meloche, H.P., Luczak, M.A., Wurster, J.M.,

J.Biol.Chem., 247, (13), 4186, (1972)

Meyerhof, O., Lohman, K., Schuster, P.,

Biochem.Z., 286, 301 (1936)

Mounter, L.A., Alexander, H.C., Tuck, K.D., Dien, L.T.H.,

J.Biol.Chem., 226, 867, (1957)

Mueller, H.E.,

C.A., Vol 55,1973 (38245)

Okarmoto, M., Morino, Y.,

J.Biol.Chem., 248,(1), 82, (1973)

Ochoa, S.,

Methods in Enzymology, Vol 1 p.735, (1955) by Colowick,S.P., and Kaplan, N.O., New York Academic Press Inc.

Peanasky, R.J., and Lardy, H.A.,

J.Biol.Chem. 233, 365, (1958)

Penhoet, E., Lochman, M., Valentine, R., Rutter, W.,

Biochemistry, 6, 2940, (1967)

Rashed, N., and Rabin, B.R.,

European J.Biochem. 5, 147, (1968)

Richards, O.C.,

Ph.D. dissertation, University of Illinois, (1960)

Rosso, R.G., Adams, E.,

Biochem.Biophys.Res.Commun. 23, 842, (1966)

Rutter, W.J.,

Federation Proc., 23, 1248 (1964)

Rutter, W.J., Ling, K.H.,

Biochem.Biophys. Acta 30, 71, (1958)

Schauer, R., Wember, M., Wirtz-Peitz, F., Ferreina Do Amaral, C.,

Hoppe-Seyler's Z.Phys.Chem.352, 1073, (1971)

Schroeder, W.A., Jones, R.I., Cormick, J., Mc.Cullag, K.,

Anal.Chem., 34, 1570 (1962)

Schoellmann, G.,

Biochemistry, 2, 252, (1963)

Sirbasky, D.A., Binkley, S.B.,

Biochem.Biophys.Acta, 206, 479 (1970)

Speck, J.C., Jr., Rowley, R.T., Horecker, B.L.,

J.Am.Chem.Soc., 85, 1012 (1963)

Stellwagen, E., Schachman, H.K.,

Biochemistry, 1, 1056 (1962)

Stumpf, R.K.,

J.Biol.Chem. 176, 223, (1948)

Su, J.C., Ling, K.H.,

Formosa Med.Assoc. 55, 18, 216 (1956)

Suttajit, M., Urban, C., McLean, R.,

J.Biol.Chem., 246 (3) 810, (1971)

Tamford, C., Kawahara, K., Laranje, S.,

J.Am.Chem.Soc. 89, 729, (1967)

Taylor, J.F., Green, A.A., Cori, G.T.,

J.Biol. Chem., 173, 591, (1948)

Topper, Y.J., Mehler, A., Bloom, B.,

Science, 126, 1287, (1957)

Vanderheiden, B.S.,

Ph.D., dissertation, University of Washington (1956)

Warburg, O., Crawehn, K.,

Z.Naturforsch, 96, 206, (1954)

Warburg, O., Christian, W.,

Biochem.Z., 314, 149 (1943)

Wake, R.G., Cecil, R.,

Biochem. J., 82, 401 (1962)

Witkop B.,

Advances in Protein Chem., 16,221 (1962)

Yasnikov, A.A., Boiko, T.S., Volkova, N.V., Mel'nichenko, I.V.,

Biokhimiya,31,969, (1966), C.A.

Zygmunt, W., Martin, T.,

J.Pharmac.Sci.53 (1964) C.A.