INVESTIGATION OF PURIFICATION PROCEDURES TO ISOLATE RAT MITOCHONDRIAL δ-AMINOLAEVULINIC ACID SYNTHETASE

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Graham R. Parslow B.Sc (hons) dip.Ed.

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University of Adelaide.

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SUMMARY

1. A Sample of rat liver mitochondrial ALV-Synthetase was purified to a specific activity of 4,684 units/ mg, the highest activity yet observed from a mammalian source.

2. The sequence of purification steps that permitted the isolation of the high specific activity enzyme noted above was developed during the work reported here. The sequence of procedures finally used to purify a mitochondrial extract included 0-50% ammonium sulphate precipitation, 5-20% (w/v) polyethylene glycol precipation, CM-Sephadex chromatography, Sephadex G-100 filtration, and electrophoresis.

3. Attempts to duplicate previously reported purifications of ALV-Synthetase by use of affinity chromatography were unsuccessful.

4. Isoelectric focusing gave no clearly useful or preparative separations of ALV-Synthetase in pH gradients.

ACKNOWLEDGEMENTS

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Former members of the Biochemistry Department, University of Adelaide to whom I am particularly indebted for their discussions and interest include Dr. M.J. Whiting, Dr. A.M. Edwards and Mr.D. Elder.

I also wish to express my appreciation of Mrs. J. Emery for the generous donation of her time to type much of this thesis.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in this, or any other University. To the best of my knowledge and belief, this thesis contains no material which has been previously published or written by any other person, except where due reference is made in the text.

GRAHAM R. PARSLOW

ABBREVIATIONS

Other abbreviations not listed here are acceptable to the Journal of Biological Chemistry without definition or are explained in the context of their appearance.

AA	aminoacetone
AIA	allylisopropylacetamide
ALV	δ-aminolaevulinic acid
ALV-s	ALC-synthetase (succinyl-CoA; glycine C-succinyltransferase, decarboxylating E.C 2.3.1.37)
BSA	bovine serum albumin (fraction V)
DDC	diethyl -1, 4-dihydro-2, 4, 6- trimethyl-pyridine-3, 5-dicarboxylate (comm. dicarb-ethoxy dihydrocollidine).
DTE	dithioerythritol (Cleland's reagent)
PBG	porphobilinogen
PLP	pyridoxal 5-phosphate
RBC	red blood cell
SA	specific activity
TAT	tyrosine amino transferase (EC 2.6.1.5)
TCA	trichloroacetic acid
TNBS	2, 4, 6-trinitrobenzene sulphonate
$t_{½}$	half life
R.spheroides	Rhodopseudomonas spheroides

CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

δ-Aminolaevulinic acid synthetase is the first and rate limiting step in the haem biosynthetic pathway (1). A satisfactory understanding of this enzyme will provide an insight into the regulation of haem synthesis in cells, a better understanding of mitochondrial biogenesis, and the potential for more enlightened treatment of the porphyria diseases. Normally in all life forms the activity of δ-aminolaevulinic synthetase is regulated to a minimum level adequate to meet the metabolic requirements for haem pathway products. Elevation of δ-aminolaevulinic acid synthetase and overproduction of porphyria pathway intermediates is associated with manifestations of the porphyric diseases. It is this group of metabolic disorders that provides a continued practical interest in the regulation of δ-aminolaevulinic acid synthetase.

The inherited porphyrias are usually inherited according to distributions for an autosomal dominant gene (2), because excess enzyme activity rather than a defective enzyme is involved. George III, *the mad king*, has been diagnosed retrospectively to have suffered from acute intermittent porphyria which greatly affected his duties of state. History however no longer seems bent on maligning him, but on admiring his fortitude in the face of the torments his disease and physicians caused him (3, 4).

A further porphyria disease, congenital erythropoietic porphyria, has the unpleasant symptoms (among others) of red staining of the teeth and urine and cutaneous photosensitivity with severe scarring and often

mutilation of fingers and ears. Such physical deformaties may have made sufferers in the past avoid human company, venturing out largely at night to avoid the intolerable light of day and thereby generating the legends about werewolves that have come down to us (5). Erythropoietic porphyria is also a potentially serious economic problem in cattle since one particular stud bull in the U.S.A. was found to be a carrier of porphyria after siring some 100,000 offspring (6).

A severe limitation to progress in understanding the cellular regulation of δ-aminolaevulinic acid synthetase, and thereby porphyria, in mammals has been the failure of efforts to obtain homogeneous isolated enzyme. Progress towards that achievement is reported in later chapters here, although with the exception of cation exchange chromatography, no techniques investigated were adequately reproducible to form the basis of a routine purification procedure. The failure of attempts to repeat published methods of PLP cofactor dependant affinity chromatography was particularly disappointing.

This chapter contains a summary of the chemistry of the ALV-synthetase reaction, and the reported properties of ALV-synthetase derived from various sources. A section on the use of drugs to create porphyric conditions in experimental animals is followed by a brief statement on the inadequacy of presently available data to confirm any specific molecular mechanism involved in the physiological regulation of haem metabolism in mammals.

1.2. THE CATALYTIC ACTIVITY OF ALV-SYNTHETASE

ALV-synthetase (EC 2.3.1.37) requires glycine and succinyl-CoA as substrates and utilizes pyridoxal 5'-phosphate (PLP) as a cofactor (7, 8). Although PLP requiring enzymes are generally subject to continued speculation regarding mechanism of action (9), the formation of a Schiffs base between glycine and PLP (shown in Fig. 1) seems well established (7): the subsequent rearrangements during decarboxylation and succinyl-CoA linkage are less certain. Separate cytosol and mitochondrial forms of ALV-synthetase have now been identified in eukaryotes.

The enzyme partially purified from rat cytosol has -SH groups at the active centre which bind the PLP cofactor and may also participate in divalent cation binding (7): decarboxylation of the glycyl carboxyl apparently terminates the enzymic reaction and releases the product, ALV. Mitochondrial rat enzyme also has -SH groups involved in the catalytic activity and preliminary kinetic studies suggest a similar mechanism to that of the cytosol enzyme (10). Purified ALV-synthetase from the bacterium R. spheroides also apparently has decarboxylation as the final step in the enzymic mechanism (11).

1.3. THE ISOLATION OF ALV-SYNTHETASE

ALV-synthetase activity in the livers of experimental animals is usually low, but elevations of the order of tenfold or greater can be produced by the

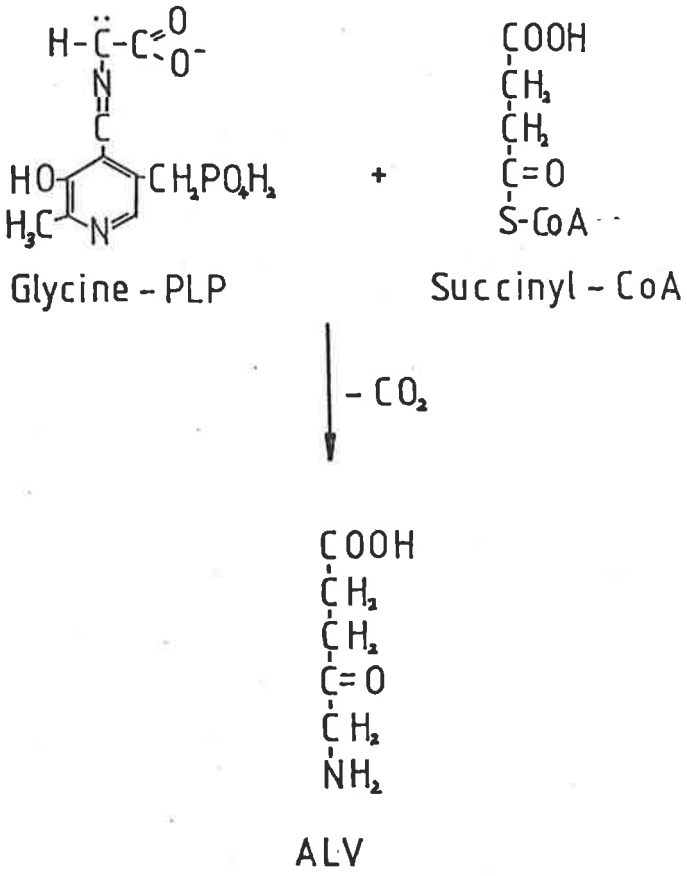


Fig. 1. The reaction catalysed by ALV-synthetase

Glycine forms a Schiffs base with pyridoxal 5' — phosphate cofactor at the active site. Various unstable intermediates have been proposed to achieve decarboxylation and condensation with succinyl-Coa to yield δ-aminolaevulinic acid.

administration of porphyrinogenic drugs. By drug induction of high levels of ALV-synthetase in eukaryote system it has been practical to isolate the enzyme in various degrees of purification depending on the source. The enzyme from chicken liver has now been reported purified to electrophoretic homogeneity, but this had not been achieved when the work in this thesis was in progress. A comparison of the properties of the homogeneous chick enzyme with eukaryote preparations has been made in Table 1. This table should be referred to for extension of the information presented below.

Enzyme activity has been detected in spleen (13), marrow (14), brain (15, 16), and heart (12, 15), although it seems that only the kidney and liver enzymes are induced by the porphyrinogenic drugs (12, 15, 17). The attention of researchers has nevertheless been almost exclusively directed towards hepatic ALV-synthetase.

(a) Mammals

(i) Foetal mitochondrial-form

Foetal rat liver has a high constitutive ALV-synthetase activity, about tenfold the adult level, which declines shortly after birth (24). During this constitutive phase no increase is produced by chemicals which induce in the adult (25). Woods and Murthy (19) have achieved a partial purification of the foetal enzyme (see Table 1). They found that the foetal enzyme became less stable to storage with higher purification and 50%

Table 1. ALV-synthetase purifications reported from mammalian and avian sources.

The order of purification steps and use of centrifugal methods have not been indicated.

Source	Rat liver mitochondria Kaplan (18), 1971.	Foetal rat liver mitochondria Woods & Murthy (19), 1975.	Rabbit reticulocytes; electrophoretically homogeneous. Aoki *et al.*(20), 1971.	Rat liver mitochondria Whiting & Elliott (21), 1972.	Rat liver cytosol. Scholnick *et al.*(22), 1971.	Chicken liver mitochondria; electrophoretically homogeneous. Whiting & Granick (23), 1976.
Specific activity n.moles ALV/mg/30min	19.2	92	156	530	540	10,250
molecular weight	>500,000	47,000	200,000	77,000	300,000	87,000
isolectric point			5.9			7.0
Km succinyl-CoA(mM)			0.06	0.2	0.07	0.03
Km glycine (mM)		17	10	19	10	2
Ki haemin (μM)	50	-	~15	10	20	35
Inhibitors		Na^{+} Pb^{++} Co^{++}	CoA	Pb^{++} NEM PHMB	PCMB, NEM Co^{++} Mn^{++}	PCMB, NEM Pb^{++}
Activators		haemin cyt.c	iodo-acet amide	CoA, ATP	Na^{+}	Mg^{++} Ca^{++}
% saturation amm.SO_4 to precipitate			35	50	30	50
			Fold of Purification			
Sephadex gels	0	3.6	3.0	2.1	2.0	3.8
CM-ion exchange	0					
DEAE-ion exchange	0					
isolectic focussing			9.6			2.4
hydroxyl apatite	1.4					1.1
affinity chromatography		5.1		11.5		4.2
detergent treatment		1.6				
calcium phosphate	2.3				2.7	
electrophoresis	0					
acid precipitation	0					

Table 1

of activity from their most purified sample was lost after 7 days at -20°C.

Anomalous properties of the enzyme reported were a 1.5 fold stimulation by 200 μM haemin and a similar activation by cytochrome C.

(ii) Adult mitochondrial form

Early attempts to isolate ALV-synthetase from experimental animals were thwarted by the instability of activity that was obtained (2). The first report of stable mitochondrial enzyme being isolated in a form that could be usefully studied was made by Kaplan in 1971 (18). He disrupted mitochondria by deoxycholate treatment and found the ALV-synthetase activity in a high molecular weight protein aggregate. He was unable therefore to obtain useful purification by ion exchange chromatography, heat treatment, acetone fractionation, acid precipitation, ammonium sulphate precipitation, gravimetric methods or gel filtration. Calcium phosphate and hydroxyl-apatite gels were useful and the properties of the rat liver enzyme after these fractionation procedures are presented in Table 1.

During work in this laboratory, Whiting discovered that the aggregate complex obtained from disrupted guinea pig mitochondria could be reduced to a molecular weight of 77,000 by treatment with 0.8 M NaCl combined with 1 mM DTE (21). Rats were found to be more highly induced by the porphyrinogenic drug DDC than guinea pigs to provide a source of ALV-synthetase for purification, and the conditions that solublized the

guinea pig enzyme also liberated a molecular weight 77,000 form from rat mitochondria (21). The solublized rat enzyme was purified 40 fold from mitochondria by gel filtration, ammonium sulphate fractionation, and affinity chromatography (Table 1). Following this report by Whiting and Elliott the greater part of the experimental work presented here was an attempt to repeat and extend the purification of mammalian (rat) ALV-synthetase.

ALV-synthetase in mitochondria has been established to be either a matrix enzyme or loosely bound to the inner mitochondrial membrane (21, 28, 29) according to marker enzyme distribution studies following disruption.

(iii) Adult cytosol form

Granick and Urata (1) reported in 1963 that ALV-synthetase was undetectable in the cytosol of guinea pigs and that only a mitochondrial form could be demonstrated. A number of reports of a cytosol form in liver and one report for kidney (15) have since appeared, although some authors have attributed the cytosol location to mitochondrial contamination (29). However the differences in physical properties between soluble fraction and mitochondria associated enzymes observed in work conducted here (not presented), and by others who have reported a soluble enzyme, leaves little doubt that a cytosol form does exist. Patton and Beattie (29) could not detect a cytosol form in Sprague Dawley rats, but Scholnick *et al.*(22) have reported a partial purification from this strain so that genetic

difference is unlikely to account for failure to detect a cytosol form. The substrate succinyl CoA is not generated in the cytosol, only in mitochondria, so the enzyme is not considered to be related to cellular porphyrin synthesis (22). Cytosol ALV-synthetase is generally thought to be a transitional form awaiting transport to a mitochondrial location.

Kikuchi's group reported a cytosol enzyme in rats in 1969 (31) and a partial purification of 200 fold was reported by Marver's group in 1972 (22): see Table 1. The cytosol form is much larger then the mitochondrial form and has been suggested to comprise the mitochondrial form with additional peptide sequences (21).

Evidence that the cytosol form is in transit to mitochondria was initially gathered from the time course of change in subcellular activities following administration of porphyrinogenic drugs. Beattie and Stuchell (32) reported that AIA caused cytosol activity to reach maximum levels after 1 hour, total activity to peak at 1-2 hours and mitochondrial activity to peak at 4 hours. This time course for induction in Sprague Dawley rats is more rapid than any other workers have reported (e.g. 33) and an increase of cytosol form before mitochondrial appearance during induction is not confirmed by results obtained by Edwards after AIA injection (34, see Fig 5-3). The finding that an antibody to the mitochondrial rat enzyme is only partially cross reactive with the cytosol form (21) also dictates caution in presuming the cytosol form to be a precursor of the mitochondrial form. If a precursor relationship

is established it will be interesting to know how mitochondrial protein synthesis is involved since the inhibitor chloramphenicol has been reported both to inhibit acquisition of enzyme in mitochondria by 50-60% while cytosol form increased as in controls (32) and to have had no effect on the course of AIA induction (31).

The first direct physical evidence relating the cytosol form to the mitochondrial form has been presented recently by Kikuchi *et al.*(35). He has found that rat cytosol enzyme has a molecular weight of 170,000 (or approximate multiples thereof) and papain or snake venom treatment for 10 minutes releases a molecular weight 110,000 form. Kikuchi has determined a molecular weight of 110,000 for the enzyme he obtained from mitochondria and finds no reduction in size of this enzyme following the peptidase treatment which reduced the size of the cytosol form. Significantly the saturation of ammonium sulphate at which the cytosol enzyme precipitated (0-35%) was changed by papain treatment to 35-50%, the range in which the mitochondrial form precipitates.

(b) Chickens

(i) Mitochondrial form

Whiting and Granick (23) reported purification to homogeneity of a form with molecular weight 87,000 and other properties are presented in Table 1. This work was reported after completion of the work in this thesis. The kinetic parameters for the chicken enzyme appear to be significantly different from those

measured for rat enzyme although an antibody to the chicken enzyme shows cross reactivity to rat mitochondrial enzyme (Whiting, unpublished observation). A second form of the enzyme may be present in small amounts (23) and doubt has now arisen concerning the size of the principal native enzyme (36).

(ii) Cytosol form

Whiting in this laboratory has identified a cytosol enzyme of molecular weight 70,000 and observed synthesis of this form in a cell free extract (37). Mitochondria added to the *in vitro* synthesizing system did not incorporate a significant amount of the newly synthesized ALV-synthetase. It was still considered however that *in vivo* the 70,000 molecular weight form was synthesized on cytoplasmic ribosomes then rapidly incorporated into mitochondria where its molecular weight was reported to be 49,000 (37). Clarification of the physiological significance and cellular location of other sizes of chick enzymes is continuing in this laboratory.

Ohashi and Kikuchi (38) have reported a molecular weight 300,000 form in cock liver which may dimerize to 600,000.

(c) Micro-organisms

(i) Rhodopseudomonas spheroides

Fanica-Gaigner and Clement-Metral have obtained homogeneous preparations of two forms of ALV-synthetase from the photosynthetic bacterium R. spheroides (39, 40). The two isozymes have identical molecular weight (105,000), the same ATP

inhibition, the same PLP content and are both totally inhibited by 100 μM haemin. One form has one titratable -SH group and a pI (isoelectric point) of 5.1, the other has 7 -SH groups and a pI of 6. It has been suggested that one form is located in the chromophores and would be involved in chlorophyll production for photometabolism, the other form in the cytoplasm would be involved in dark aerobic metabolism (39).

1.4. EXPERIMENTAL PORPHYRIA PRODUCED BY DRUG INDUCED ELEVATION OF ALV-SYNTHETASE ACTIVITY.

The attempted purification of ALV-synthetase reported here used liver from rats treated with the porphyrinogenic compound DDC. The time course of DDC induction of ALV-synthetase from various laboratories shows that maximum enzyme activity can be achieved from 12 hours (30) to 3 days (41) after administration. Results presented in Chapter 3 here represent an investigation into the conditions best suited to achieve maximum DDC induction of ALV-synthetase using the strain of rats routinely supplied to this laboratory. It was found that a single DDC dose induced the activity to maximum levels after 12 - 24 hours and that the activity routinely achieved was comparable to and occasionally exceeded, the activities reported by other investigators. Optimal induction by DDC here represented a 10 - 15 fold increase over the basal level.

Most investigators studying experimental porphyria have used allylisopropylacetamide (AIA) and DDC

as inducer drugs because these have been found to be most active in experimental animals. The inducer 2,3,7,8- tetrachlorodibenzo-p-dioxin is the most potent inducer of ALV-synthetase in guinea pigs (42), but the exceptionally low LD_{50} of 1 µg/Kg body weight does not commend it for general experimentation.

The first experimental porphyria was produced by Stokvis in the last century by administration of sulphonal to rabbits and dogs (43). Sulphonal had been observed to exacerbate clinical porphyria, and other drugs including barbiturates have similarly been established to be contraindicated in porphyria (2), many of them proving to be active inducers of ALV-synthetase in experimental systems (44, 45). Allylisopropylcarbamide (Sedormid) is an anaesthetic with inducing activity, but the active analogue AIA was found by Goldberg and Rimington to be less hypnotic (46). Solomon and Figge (47) described the porphyrinogenic activity of DDC in 1959. A considerable impetus to investigate experimental porphyria has also been provided by the chance observation in Turkey during the late 1950s of an epidemic of cutaneous porphyria (47). This was attributed to the fungicide hexachlorobenzene which was added to seed wheat, but diverted to bread making and consequently affected many people, particularly children.

The pattern of haem metabolism in experimentally induced porphyria is dependant on the drug used and may be related in part to various categories of the human disease. AIA and Sedormid produce a pattern of excretion

suggestive of acute intermittent porphyria; DDC and griseofulvin produce a pattern suggesting variegate porphyria (or erythropoietic porphyria in mice); and hexachlorobenzene induces conditions comparable to porphyria cutanea tarda (48, 49). In addition to haem pathway related changes, various non-specific effects including hepatomegeally and increased fatty acid synthesis may result from extended exposure to porphyrinogenic drugs (50, 51). Griseofulvin as well as possessing porphyria inducing properties is an antimitotic drug which promotes aggregation of microtubule protein (52). Extensive general ultrastructural damage is also noted in clinical porphyria (53) and is produced in chronic experimental porphyria (49).

(i) Transcriptional promotion by porphyrinogenic drugs

DDC promotion of an increased rate of transcription in rat liver has been demonstrated by Nawata and Kato (41). Wistar King strain rats were dosed with DDC daily over 7 days with continued fasting. A peak of ALV-synthetase induction was obtained after 3 days and fell markedly between days 3 to 7. Pulse labelling with ^{32}Pi indicated a 2-fold elevation of new RNA synthesis after 24 hours dosing which returned to control levels by day 2 and decreased slightly on subsequent days. Because RNA synthesis was elevated preceding maximum ALV-synthetase activity Nawata and Kato considered that a transcriptional increase was fundamental to the mechanism of DDC induction. The increase in transcription is unlikely to be specific for

ALV-synthetase mRNA however since Del Favero *et al.* (54) observe in mice that DDC, while causing a loss of single ribosomes, causes a wide range of polyribosome sizes to appear. As well as suggesting the possibility of direct drug intervention in translation this result also indicates that a unique size of RNA is not synthesized in response to DDC (i.e. the transcriptional stimulation is not specific).

Sardana *et al.* (55) have shown that AIA can elevate RNA synthesis by 3-fold, 1 hour after injection to rats. The AIA stimulation of transcription is completely prevented by haemin (55) implying a certain degree of specificity for products of the haem pathway. However in the summary of observations of haemin effects on AIA induction Sweeny *et al.* (56) conclude that the role of haemin in suppressing induction phenomena reflects a regulatory role not related to a role in haemoprotein synthesis. Haem also represses the flavoprotein NADPH cytochrome c reductase and prevents increases in microsomal protein and phospholipid following porphyrinogenic drugs (57). The type of RNA synthesis stimulated by AIA seems to be almost exclusively mRNA (as judged by the content of polyAdenylic acid), but a variety of mRNA types are synthesized so that AIA is not apparently highly specific for stimulating ALV-synthetase mRNA transcription (58). A finding that isotopically labelled AIA did not become associated with isolated nuclei, but was distributed between cytosol and microsomes suggests an indirect stimulation of transcription by AIA (27) rather than the direct nuclear involvement observed for

transcription-inducing steroids (59).

1.5. REGULATION OF THE HAEM BIOSYNTHETIC PATHWAY

Haem synthesis *in vivo* requires the action of seven distinct enzymes on ALV by the pathway summarized in Fig. 2. The activities of the pathway enzymes are usually greater than required to metabolize the ALV produced in normal cells, but may become limiting during porphyria. Various forms of clinical porphyria are characterized by the pattern of excretion of pathway intermediates as determined by inherited differences in the constitutive activity of the pathway enzymes (47, 62). Purification and study of the porphyrin metabolising enzymes of the pathway also remains of current interest (63, 64, 65), particularly in regard to the mechanism of enzyme action.

The activity of the second enzyme, ALV-dehydratase apparently can be genetically controlled in rats since it decreases in iron deficiency (66), but this is not observed in man (67). Selective chemical inhibition of the dehydratase by aminotriazole can be used to experimentally prevent haem synthesis (68, 69) and variations in the oxidation-reduction potential in cells might also modify the activity of the enzyme (8). Uroporphyrinogen cosythetase is limiting in congenital erythropoietic porphyria and may represent the inherited lesion in this disease (6). A similar role of pathway limitation imposed at the final enzymic step, iron insertion into the porphyrin moiety by ferrochelatase,

Fig.2. The haem biosynthetic pathway

The series of reactions shown here were established by Shemin and Coworkers (60, 61). The enzymes in the pathway are:

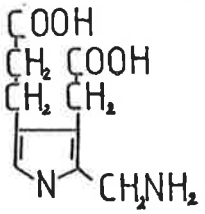
1. ALV-synthetase
2. ALV-dehydratase (PBG-synthetase)
3. PBG-deaminase
4. Uroporphyrinogen cosynthetase
5. Coproporphyrinogenase
6. Protoporphyrinogen oxidase
7. Ferrochelatase

Succinyl Co-A + Glycine

1

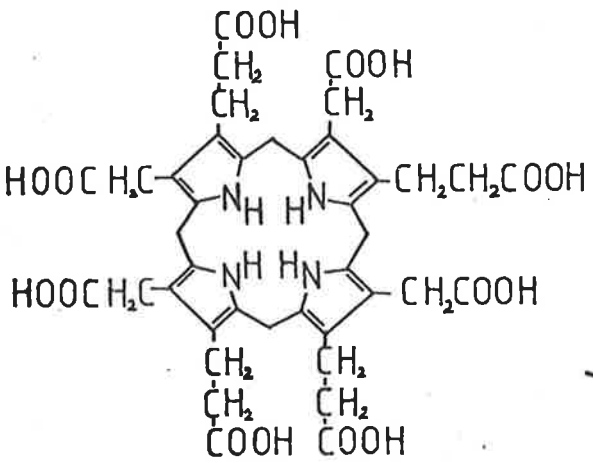
δ-Aminolaevulinic acid : ALV

2



Porphobilinogen
PBG

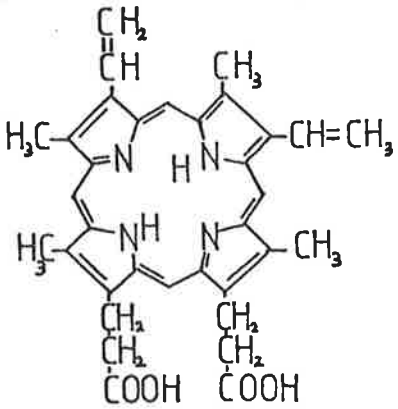
3 ; 4



Uroporphyrinogen III

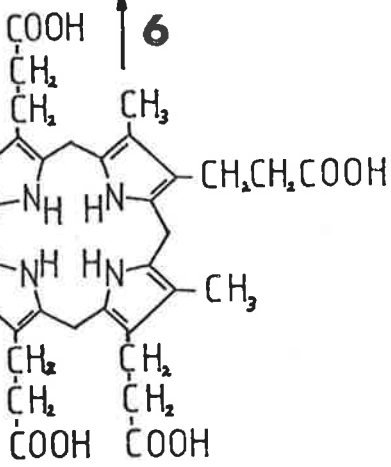
Haem

Fe^{++} 7



Protoporphyrin IX

6



5

Coproporphyrinogen III

CYTOSOL

MITOCHONDRION

has been advanced to explain clinical and experimental porphyria (70-73), but remains of uncertain merit. The involvement of elevated ALV-synthetase activity in clinical porphyria is well established however (74,75,149-151) but whether the fault is a simple enzyme over-production, a modification of the regulatory mechanism, or a combination of these, is not known.

The proposal that an aporepressor in combination with haem prevents the transcription of ALV-synthetase mRNA put forward by Granick in 1966 (26) has been of seminal value in organizing the theoretical consideration of ALV-synthetase regulation. According to this scheme the end product of the porphyrin pathway haem can directly inhibit production of ALV-synthetase by repression, and porphyrinogenic drugs were envisaged to compete with haem to yield an inactive drug-bound repressor.

It is difficult however to visualize how the various known inducer drugs (26, 48, 76) could compete with or prevent haem binding to a hypothetical aporepressor. Although groups of inducers have been characterized according to chemical structure, lipophilicity and steric considerations no consistent pattern emerges from analysis of the known inducers or enables any reliable prediction of biological activity within a series of chemicals (26, 43, 44, 73, 76, 77). A proposed model of ALV-synthetase regulation in which the level of apocytochrome P-450 determined the induction of ALV-synthetase via a positive control mechanism (27) overcame many of the objections inherent in the haem

repressor proposal, but has also proved to be in conflict with more recent data (78-81). The presence of a regulatory haem pool is itself not supported by direct measurement, but only by indirect indications (82, 83).

Much of the uncertainty in the interpretation of experiments on haem pathway regulation is compounded by the fact that the measurement of ALV-synthetase activity in samples is not necessarily indicative of the amount of enzyme protein present. Patton and Beattie (29) have even suggested that results from their studies of experimental porphyria are best explained by activation of ALV-synthetase rather than an increased amount of enzyme protein. Various physiological ALV-synthetase inhibitors of indeterminate nature have also been reported (84, 100) and the proximity of ALV-synthetase and ferrochelatase in mitochondria supports the suggestion that end product inhibition by haem is physiologically relevant (21). Although the work of Whiting and Granick (85) has established that an elevation of enzyme protein is involved in experimental porphyria of avian systems a number of differences between avian and mammalian systems have been characterized (77, 86).

The purification of hepatic mammalian ALV-synthetase therefore remains of considerable interest, in particular because a specific antibody to the enzyme will allow the relative contributions of drug activation and induction to be directly investigated. Establishment of the relationship between cytosol and mitochondrial forms would also be facilitated.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Methods not described in this chapter are given where relevant by inclusion with the results presented.

2.1. MATERIALS

(a) Chemicals

DDC was obtained from Eastman Kodak and AIA was a generous gift from Hoffman La Roche. Nembutal (pentobarbitone sodium 60 mg/ml) for injection was from Abbott Laboratories, Sydney, heparin from C.S.L., and sucrose from C.S.R. Co., Sydney. Casein hydrolysate and trypsin were from British Drug Houses Ltd. Other commercial organic and biological substances were obtained from Sigma Chemical Co.

Ammonium sulphate (enzyme grade) was from Mann Research Laboratories. Other inorganic reagents were obtained from British Drug Houses Ltd., Ajax Chemicals Ltd., Sydney, and May & Baker Ltd.

(b) Chromatographic Materials

Sephadex (all grades), Sepharose 4B, DEAE- and CM- Sephadex were from Pharmacia Sweden. AE- cellulose and phospho-cellulose were from Whatman.

(c) Buffers

PEST buffer for the preparation of mitochondria contained 0.1 mM PLP, 0.1 mM EDTA, 0.25 M sucrose and 5 mM tris-HCl pH 7.4.

Buffers for chromatography, represented by abbreviations of their components, contained 20 mM tris-HCl pH 7.4 (T), 1 mM DTE (D), 0.1 mM EDTA (E), 0.1 mM PLP (P), and 0.5 M NaCl (N).

2.2. ENZYME ASSAYS

(a) ALV-synthetase and AA-synthetase

The colourimetric assay for ALV-synthetase used throughout the work described here estimates the production of δ-amino laevulinic acid during a 20 minute incubation at 37°C and is based on the method of Irving and Elliott (200).

The following amounts of reagents (μ moles) in a final volume of 1 ml were used: tris-HCl pH 7.4, 50; glycine 100; potassium succinate 10; magnesium chloride 20; Co-A 0.3; glutathione 10; ATP 15; PLP 1; EDTA 5; and succinyl-CoA synthetase 2 units. Final pH was adjusted to 7.4 with NaOH. Added samples of homogenate or mitochondria were frozen quickly in a dry ice-ethanol mixture, stored at -15°C as required, and thawed at 37°C before assay. Enzymatic activity was terminated with 2 ml of 0.225 M TCA then tubes were centrifuged. Protein free supertant (2.5 ml) was mixed with 0.75 ml of 1 M sodium acetate and 0.1 acetyl acetone, and heated at 100°C for 15 minutes. This procedure results in the condensation of acetyl acetone and ALV to form a pyrrole (101). After allowing time to cool 1 ml of pyrrole solution was mixed with 1 ml of modified Ehrlich's reagent (102) made from 4-dimethyl amino benzaldehyde (1 g), mecuric chloride

(0.07g) glacial acetic acid (42 ml), 70% perchloric acid (8 ml) and water to 100 ml. The red pyrrole reaction colour is fully developed after 15 minutes and stable for approximately 30 minutes (201). Optical density at 552 nm was measured in a cell of 1 cm optical length.

AA-synthetase present in cell homogenates also gives a coloured pyrrole product using the above procedure and allowance for the amino acetone pyrrole product was made by selective solvent extraction using ether. The 2 ml of pyrrole solution remaining, after 1 ml was mixed with Ehrlich's reagent, was neutralized to pH 7-7.5 with 0.05 ml of Na_2HPO_4 (0.5 M). This solution was mechanically shaken with 5 ml of equilibrated ether for 30 seconds and 1 ml of the aqueous phase was withdrawn and mixed with 1 ml of Ehrlich's reagent; the optical density at 552 nm was then read as before. The equilibrated ether was prepared by shaking equal volumes of peroxide-free ether with an aqueous solution containing (in m moles) TCA 7.7; sodium acetate 15; NaOH 0.68; and Na_2HPO_4 1.1.

The ALV and AA formed during incubation were calculated by the following formulae:

$$\{ALA\} = 10.7 \ (20\beta - \alpha) \quad \text{n moles}$$

$$\{AA\} = 188 \ (0.753\alpha - \beta) \quad \text{n moles}$$

where α = combined pyrrole optical density at 552 nm

β = ether extracted optical density at 552 nm.

ALV solutions of concentration established by spectral analysis were pyrrolised using the assay conditions described above. The ALV-pyrrole gave a molar

extinction coefficient of 5.74 x 10^{-4} and 25% of the pyrrole was ether extracted. The above formulae result when the extinction coefficient and ether extraction percentage for AA-pyrrole given by Granick (87) are assumed. When mitochondrial extracts of high ALV-synthetase activity were assayed the contribution of AA-pyrrole was neglected and the formula {ALV} = 150α n moles was used.

One unit of ALV-synthetase activity is defined as the amount of enzyme catalysing the formation of 1 n mole of ALV in 30 minutes. ALV formation was found to be non-linear beyond 20 minutes incubation using the above procedure, therefore results obtained using a 20 minute incubation period have been multiplied by 1.5. Results obtained during this work defining the period of linear ALV-synthetase production during assay, the effect of protein concentration and subcellular source of sample have not been given here because Edwards in this laboratory obtained essentially identical results and these have been described previously (34).

(b) TAT assay

Tyrosine amino transferase activity was estimated by the colourimetric procedure of Rosen *et al.*(103). Incubation was allowed to proceed for 10 minutes at 37°C. *A unit of TAT activity is defined as 1 n mole of tyrosine transaminated per minute.* Calibration of the Rosen *et al.* assay procedure led to the following formula:

1 unit of TAT = 106 x ΔOD_{850}

ΔOD_{850} is the change in optical density at 850 nm in the

colour reagent following a 10 minute incubation.

2.3. DDC DOSING AND TREATMENT OF ANIMALS

Albino Wistar rats (200-300 g) were obtained from the Central Animal House, Waite Institute, Adelaide, unless another source is specified. Animals were maintained under natural light cycles and sacrificed under Nembutal anaesthesia, or for perfusion experiments under ether anaesthesia. Unless otherwise indicated animals were fasted 48 hours before sacrifice, but permitted water *ad libitum*.

When DDC was administered rats were lightly anaesthetised with ether and 0.5 g of DDC given intra gastrically as an aqueous suspension using a stomach tube. The suspension was prepared as 10 g DDC, 0.2 g tragacanth mucilage and water to 50 ml all ground in a mortar and pestle. Each rat received 2.5 ml of suspension.

2.4. PREPARATION OF MITOCHONDRIAL ALV-SYNTHETASE

The following procedures were employed to obtain samples of mitochondrial ALV-synthetase for use in the studies of various further purification techniques as reported in detail elsewhere.

(a) Preparation of porphyric mitochondria

Animals fasted 48 hours received DDC for the last 24 hours before injection with 0.5 ml Nembutal

anaesthetic (i.p.). Livers were removed and immediately placed in PEST buffer solution chilled over ice. The livers were washed with PEST buffer until most free blood was removed. Although livers were collected as rapidly as possible some delay in further processing was usual, but it was established that no loss of ALV-synthetase activity occurred when intact livers were left 20 minutes at $0^{o}C$ in PEST buffer. After mincing with scissors, livers were disrupted as a 10% w/v suspension in PEST buffer using a glass-teflon motor driven homogenizer. Mitochondria were isolated and washed in PEST buffer essentially by the centrifugal method of Schneider and Hogeboom (204). The final pellet of washed mitochondria was suspended in a minimal volume of TDEP buffer and freeze dried in a flask protected against the entry of light. A good yield of freeze dried mitochondria was approximately 10% of liver wet weight.

(b) Extraction of soluble ALV-synthetase from mitochondria

Freeze dried mitochondria were resuspended in cold water, to equal the suspension volume prior to freeze drying, pH was corrected to 7.4 if necessary then mitochondrial debris was removed by centrifugation at 105,000 x g for 1 hour. This procedure released an aggregate high molecular weight form of the enzyme which was solublized by the method of Whiting and Elliott (21), i.e. solid NaCl was added to 0.8 M and DTE to 0.001 M. Samples after this treatment have been referred to as *solublized* throughout the text here.

No increase in yield of ALV-synthetase activity from mitochondria was achieved if 0.8 M NaCl, 0.001 M DTE solution was used to disrupt mitochondria instead of water. More total protein was liberated by this procedure and the mitochondrial debris pelleted less satisfactorily after centrifugation. Water extraction of mitochondria was therefore retained as standard procedure.

(c) Ammonium sulphate precipitation of ALV-synthetase

Ammonium sulphate precipitation of solublized mitochondrial enzyme was used to concentrate the protein and to remove haemoglobin present due to RBC contamination of mitochondria. A 0-50% ammonium sulphate saturation precipitate was recovered by centrifugation at 25,000 x g for 20 minutes. Approximately 70% of protein was pelleted and recovery of ALV-synthetase activity was in the range 50-100%. Precipitates were resuspended in a minimum volume of TDEP buffer. The red colouration of contaminant haemoglobin remained in the supernatant thereby reducing the possibility of complications due to dissociated haem that may have inhibited enzyme activity (Ki haemin = 10 μM, 21). Using narrower ranges of ammonium sulphate saturation gave no useful improvement in ALV-synthetase purification.

2.5. PROTEIN DETERMINATIONS

Estimations of protein were made using the Lowry method (105) for samples of partially purified enzyme, and the biuret method (106, 107) was used for samples of cell homogenates or mitochondria. Reference solutions of BSA were used as calibration standards.

When very small amounts of protein were estimated the procedure of Schaffner and Weissman (108) was adopted. This method was calibrated to give an optical density (1 cm) increase at 630 nm of 0.048 per µg of protein.

2.6. PREPARATION OF SUCCINYL-CoA SYNTHETASE

Succinate: Co-A ligase (ADP); E.C. 6.2.1.5 was partially purified from E. coli (Crooke's strain) by a method based on that of Hildebrand and Spector (109). Bacteria were disrupted by sonication and the extract treated with streptomycin sulphate and ammonium sulphate (209), before dialysis against 0.05 M tris-HCl, 0.05 M KCl pH 7.2 overnight. The enzyme solution was clarified by centrifugation at 10,000 x g for 10 minutes and dispersed into small vials for storage at -15°C. The enzyme with a specific activity of approximately 15 µ moles of succino-hydroxamic acid synthesized/30 minutes/ mg protein when assayed by the method of Kaufman (110) was stable for many months.

2.7. CALCULATIONS

(a) Enzyme specific activity

Units of enzyme activity have been defined in section 2.2. Specific activity was calculated as units of activity per mg protein. Reference to protein as the basis for estimation is otherwise deleted here for textual convenience.

(b) Fold induction

The fold induction of enzyme levels, when a change in activity has been observed during the course of an experiment, has been calculated as the ratio of specific activity achieved (maximum unless otherwise indicated) to the initial specific activity.

(c) Half Life

Half lives were calculated for exponential decay using the formula:

$$t_{\frac{1}{2}} = \frac{0.301 \times T}{2 - \log A}$$

where T = time of decay

A = % activity remaining at time T

(d) Statistical analyses

Means, standard deviations and correlation coefficients were calculated by computer programmes.

2.8. TNBS STAINING FOR AMINO OR CARBOXYL GROUPS

Sepharose or cellulose material was suspended in 1 ml of saturated sodium borate. Three drops of 3% (w/w) 2,4,6-trinitrobenzene sulphonate were added and colour allowed to develop for 2 hours at room temperature. Unmodified material stained light yellow, free aliphatic amine groups stained deep red, and carboxyl groups stained vivid yellow. A matrix with both free $-NH_2$ and -COOH groups stained orange.

2.9. HANDLING OF ALV-SYNTHETASE DURING AFFINITY CHROMATOGRAPHY

Material applied to affinity columns was apoprotein of ALV-synthetase deprived of the cofactor PLP by Sephadex G-25 filtration using TD or TDE buffer. The occasionally low yields of enzyme activity noted may have been due in part to removal of the PLP cofactor from the enzyme since PLP-deprived mitochondrial enzyme in TDE buffer showed a half life of 9.6 hours at $4^{o}C$ under ambient lighting. Higher light intensity decreased this half life, although PLP afforded protection, so care was taken to minimize exposure of apoprotein to light.

PLP to a concentration of 0.1 mM was routinely added, as soon as practicable, to fractions that had been deprived of this cofactor during affinity chromatographic procedures. Enzyme samples were usually

deprived of PLP for 2 - 4 hours during Sepharose based affinity column experiments. Katanuma (125) suggests that loss of PLP cofactor from protein is the rate limiting step in (proteolytic) degradation of ALV-synthetase and that conformational changes in the protein could be expected upon deprivation of PLP.

2.10. CONSTRUCTION AND USE OF A SMALL SCALE APPARATUS FOR ISOELECTRIC FOCUSING IN SUCROSE DENSITY GRADIENTS

A small scale apparatus to establish isoelectric points, and investigate isoelectric focusing as a small scale preparative step, was constructed using a semi-permeable membrane to separate the lower electrolyte from the focusing gradient (139). This apparatus, shown in Fig. 2-1, uses the lower electrolyte reservoir as a temperature stabilizing jacket. The power dissipation was not allowed to exceed 8 watts in the focusing tube as the voltage was increased from an initial value of 200-250 volts to a maximum of 475 volts during the focusing.

Crude haemoglobin samples, obtained by haemolysis of rat erythrocytes, were also incorporated into some focusing gradients to provide coloured indicator molecules. The appearance of several sharply focused haemoglobin bands indicated stability of the gradient against disruption by thermal convection and that adequate focusing of protein was achieved.

Fractions of the gradient were collected by piercing the semipermeable membrane with an insert containing an 18 G syringe needle connected to 0.6 mm (i.d.) polythene tubing, connected in turn to a Gilson drop

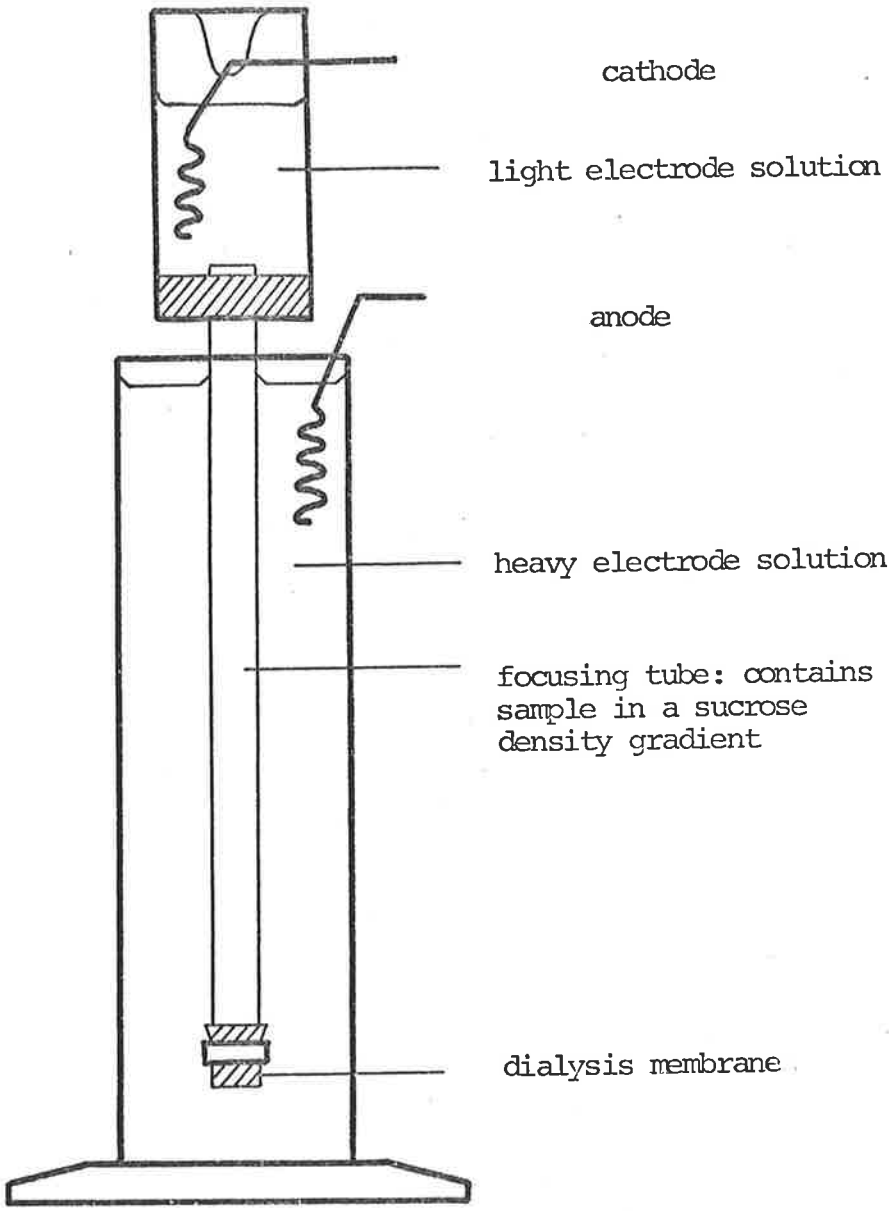


Fig.2-1. Small scale isoelectric focusing apparatus

Focusing tube: 18 cm x 0.5 cm^2
Light electrode solution: 0.2% NaOH, 0.4% ethanolamine
Heavy electrode solution: 40% sucrose containing 1% H_2SO_4 or 0.2% H_3PO_4
Focusing gradient: 10 - 40% sucrose, 1 mM DTE, 2% carrier ampholytes
Electrode connections: platinum

(concentrations, % w/v)

counting microfractionator. After a preliminary experiment in which RBC lysate and BSA protein were focused, the gradient was collected through a UVICORD flow cell (LKB Company). The absorbance at 280 nm of the haemoglobin and BSA peaks registered poorly and the separation of haemoglobin colour in the collected fractions was also poor due to mixing caused by passage through the flow cell. The absorbance at 280 nm was therefore not monitored during the experiments reported here.

When gradient eluant was passed directly to the collecting tubes the red colouration of focused haemoglobin bands could be collected without visible cross-contamination between fractions, indicating that the collection procedure was not responsible for any significant loss in column resolution. Further to this point a sample of ALV-synthetase from chicken embryo liver, purified by the first five steps in the sequence of Whiting and Granick (23) was subjected to small scale isoelectric focusing for 5 hours and resolution was at least as good as that achieved on the large scale equipment used by Whiting and Granick at this stage of their purification sequence. The recovery of the chicken enzyme from the small scale apparatus was also quantitative, whereas a recovery of 75% was reported after a 27 hour focusing period on a large scale gradient (23).

Gradients of 10 - 40% (w/v) sucrose containing 2% (w/v) carrier ampholine were formed either

mechanically or by overlayering of aliquots of decreasing densities (13 layers), with enzyme sample added to the light gradient component before mixing. The overlayering procedure avoided frothing caused by the mixing motor in mechanical gradient formers and allowed all of a sample to be applied without loss; overlayering is therefore the procedure of preference.

An ampholine concentration of 2% (w/v) was satisfactory for the levels of protein reported here ($\leq$4 mg) although higher amounts of protein or decreased ampholine concentration gave rise to gross precipitation during focusing.

pH of fractions was measured at the running temperature (4^{o}C) using a small glass electrode connected to an expanded scale Radiometer pH-25 meter. After pH measurement an aliquot of 0.5 M tris-HCl, 1 mM PLP pH 7.4 (1 - 3 volumes) was added to each fraction, the volume depending on the dilution required for convenient ALV-synthetase assay.

When wide pH range (3 - 10) ampholine was used better linearity of the pH gradient was noted when 1% (w/v) H_2SO_4, rather than 0.2% (w/v) H_3PO_4, was used as the anode electrolyte.

2.11. ELECTROPHORETIC MATERIALS AND METHODS

(a) Solutions

Solution A (pH 8.8)	1 N HCl	48 ml
	TEMED	0.23 ml
	tris	36.3 ml
Solution C	acrylamide	28 g
	bis acrylamide	0.735g
	Made to 100 ml with water	

Solutions are denoted A and C after Davis (145).

Gel Mixture	Solution A	9.0 ml
	Solution C	9.6 ml
	20 mM DTE	18.0 ml
	water	34.8 ml
Electrolyte	tris	0.606 g
	glycine	2.85 g
	water - to 1 l.	

Anode electrolyte was made 0.1 mM PLP after gel pre-electrophoresis. More PLP was added if the electrolyte reservoir was depleted of yellow colouration during a run.

(b) Gel polymerization

Gel mixture was degassed by vacuum then 0.6 ml of 10% ammonium persulphate added to initiate polymerization. The gel meniscus was overlayered with t-butanol (or water for gels containing Triton X-100). Gels were set at room temperature when glass apparatus was used, but were set at 37° C to promote better adhesion when perspex tubes were used.

(c) Apparatus

(i) Tube gels

Perspex tubes of 6 mm i.d. were routinely used. Gels including Triton X-100 would not adhere to perspex and glass tubes were used for these gels.

(ii) Preparative slab gels

Gels were set between glass plates separated by 3mm wide perspex side strips. Resultant gels were 13.2 cm wide and 12 - 13 cm in height.

(d) Sample preparation

Samples were desalted by Sephadex G-25 filtration in TDEP buffer either through a column or by the non-diluting method of Neal and Florini (148). Samples were concentrated by freeze drying if required, made to 20% (w/v) sucrose, and applied directly to the top of the gels.

(e) Running conditions

Tube gels routinely run 3 hours at 3 mA/gel and slab gels run 8 hours at 20 mA. Gels run at 4^{o}C.

(f) Sectioning gels

(i) Tube gels

Gels were released from tubes by rimming then mounted on damp filter paper on the stage of a gel microtome. The gel was frozen with powdered dry ice and successive 1 mm slices transferred to 0.5 ml aliquots of TDEP buffer. Gel slices were stood in buffer at 4^{o}C for a minimum of 2 hours before ALV-synthetase assay with

the gel slices still present. Gel slices were removed from assay tubes by centrifugation after termination of the assay period.

(iii) Slab gels

Electrophoresed gel on a glass plate was placed over a template and the gel cut to measurement with a scalpel. A central 1 cm wide strip of gel was removed for protein staining. The remaining gel was cut into successive 2.5 mm sections which were transferred to a plastic surface and frozen with powdered dry ice. A frozen gel strip was manually cut into slices of approximately 1 mm width and these slices transferred to 10 ml TDEP buffer.

After at least 2 hours to allow diffusion from the gel slices the buffer was neutralised with constant mixing to pH 7.4 with 0.5 N HCl. Gel debris was removed by centrifuging through 2.5 cm Whatman GF/C filters held in a centrifuge tube insert.

(g) Staining for protein

Gels were stained with 1% amido black in 7% acetic acid and destained in 7% acetic acid, 20% ethanol.

CHAPTER 3

DRUG INDUCED ALV-SYNTHETASE ACTIVITY IN INTACT RATS

3.1. INTRODUCTION

The work described in this chapter was undertaken largely to determine the optimum conditions under which DDC could induce hepatic ALV-synthetase. This information was particularly relevant to the treatment of rats used as a source of enzyme for the purification studies described in later chapters.

3.2. RESULTS

(a) Drug dosing procedure and animal response

The method of dosing rats with a DDC-mucilage suspension using ether anaesthesia, as described in the methods, was jointly developed for this work with Dr. M.J. Whiting. The simplicity and quantitative accuracy of drug administration achieved using this approach was not inherent in other methods that have been described (1, 30, 87). A mean dose of 2 g DDC/Kg was routinely administered and after 24 hours much of the chemical was seen to remain packed in the stomach indicating slow clearance from the alimentary system. Records of weight and sex were kept, but as previously found by Edwards (34) in this laboratory differences in response to porphyrinogenic drugs could not be related to sex or weight. Nevertheless for consistency males were used where possible.

The low aqueous solubility of DDC, determined during this work as 6-10 µg/ml at room temperature, has doubtless inclined other researchers of porphyrin synthesis

to prefer the more soluble chemical AIA as an inducing agent since the use of AIA is more commonly reported. No gross change in behaviour or appearance of rats followed dosing with DDC unlike AIA (175 mg/Kg s.c. in saline) which produced torpor and poor motor co-ordination ascribable to the hypnotic effect of this barbiturate type drug (26, 43). Urine samples from 24 hour DDC dosed rats were usually clear, but darkened upon standing indicating photo-polymerization of PBG; Ehrlich's reagent gave a positive test for PBG which is indicative of a porphyric state (88).

(b) Fasting increases and pyruvate represses DDC induction of ALV-synthetase

Table 3-1 shows that 48 hour fasting, relative to 24 hour fasting can lead to a doubled potency of DDC as an inducer of ALV-synthetase over a 5 hour period. A mean calculated DDC induction of 4.7 fold for 48 hour fasted rats was reduced to 1.9 fold by simultaneous administration of the gluconeogenic substrate pyruvate (89). Pyruvate would be expected to elicit the established glucose repression phenomenon observed for ALV-synthetase (90, 91). TAT levels however did not show systematic variation due to DDC and pyruvate dosing.

The glucose repression of ALV-synthetase by high carbohydrate observed in experimental systems has formed the basis for the generally useful application of dietary management to the control of clinical porphyria (2). In yeast direct glucose inhibition of the haem pathway enzyme protoporphyrinogen IX oxidase has been reported (92), but

Drug treatment 5h.	Prior fasting period 24h.	48h.
	ALV-synthetase (units/mg)	
DDC	1.49± .20	3.41± .84
DDC + pyruvate	.93± .38	1.35± .50
	TAT (units/mg)	
DDC	1.86± .81	6.28± 1.76
DDC + pyruvate	7.77±2.73	5.83± 2.12

Table 3-1.

Effect of pyruvate administered to rats simultaneously treated with DDC.

Drug doses were 1g DDC/Kg administered by stomach tube as a 10% suspension in 0.2% tragacanth mucilage. Pyruvic acid, neutralized to pH7 with NaOH was included in the mixture at 3.15g (36m moles)/Kg as indicated. Rats were from the same batch and each result represents the average of determinations from 3 animals. Liver homogenates were assayed for both ALV-synthetase and TAT activity. From different batches of rats the basal S.A. of ALV-synthetase was determined as 0.72±.45 (6 expt.s) and TAT as 3.30± 1.82 (12 expt.s) after 48h fasting

the mechanism of the glucose repression phenomenon in multicellular organisms remains unkown (93).

(c) Plasma concentrations of DDC

It is likely that reasonably steady plasma levels in the range 2-7 µg DDC/ml are maintained from 0.5 -18 hours after gastric dosing of rats at a mean dose of 2 g/Kg (Table 3-2). No correlation between achieved ALV-synthetase activity and plasma DDC level was demonstrated.

The persistence of DDC in the circulation noted here after alimentary administration was not seen by Gross and Hutton (30) when DDC was given intraperitoneally to mice. In the mouse study mean plasma DDC levels of 11 µg/ml 0.5 hours after i.p. injection were reported and decreased to 4 µg/ml after 3 hours, although ALV-synthetase induction continued over 12 hours. Gross and Hutton used several strains of mice and concluded that inherited genetic differences were fundamental in determining the achieved ALV-synthetase activity rather than plasma DDC levels.

(d) Partition of ALV-synthetase between cytoplasm and mitochondria

DDC was observed to cause a displacement of ALV-synthetase into mitochondria. It was determined that ALV-synthetase in uninduced liver was 48% mitochondrial, but increased to a maximum of 77% at 5 hours after DDC dosing (Table 3-2). The mitochondrial proportion decreased slightly after 5 hours treatment and the distribution after 24 hours DDC administration, determined

Number of animals	Time after DDC dose h	Plasma DDC µg/ml	ALV-synthetase		
			homogenate units/mg	mitochondria	
				mit.units/mg / hom.units/mg	% Total
1	0	0	-	-	-
2	0.5	3.1± 1.4	-	-	-
3	1	7.0± 1.8	-	-	-
2	0	0	.60± .02	-	-
2	0.25	1.0± .2	.62± .20	-	-
2	0.5	2.0± 1.1	1.58± .15	-	-
2	1	1.5± .5	1.28± .23	-	-
2	3	22.7± 1.9	2.68± .15	-	-
1	0	0	.56	1.92± .01	48
2	3	1.8± 1.2	1.43± .08	2.74± .06	69
2	4	4.8± 1.8	2.03± .38	2.90± .01	73
2	5	3.0± .6	5.85± .45	3.07± .10	77
2	6	6.3± .3	5.48± .53	2.81± .01	70
3	12	5.8± 2.7	8.90± .53	-	-
2	18	5.8± 2.5	10.25± .25	-	-

Table 3-2,

Plasma DDC concentrations, ALV-synthetase activity and distribution, after DDC dosing.

Rats fasted 2 days were dosed with DDC by stomach tube as described in the methods. Rats were ether anaesthetised, given heparin via the saphenous vein, then bled from the vena cava after laparotomy. Blood was centrifuged to obtain plasma and DDC was estimated by the method of Gross and Hutton (92). Livers were homogenized in · PEST buffer and mitochondria prepared by differential centrifugation as described in the methods. Data not indicated was not measured. Four batches of rats were used and the results have been separated accordingly.

during work described in later chapters, indicated that 65-72% of activity is mitochondrial.

Edwards (34) using rats from the same source as those studied here showed that AIA administration increased the mitochondrial component of total ALV-synthetase activity from 45% to 65% in 3-4 hours, but by 8 hours the original distribution prevailed and was subsequently maintained for the following 16 hours. A simple interpretation of this difference is that DDC increases the capacity of mitochondria to accept ALV-synthetase molecules, perhaps by direct hydrophobic interaction with mitochondrial membranes. Another explanation is suggested by the reported effect of haem on cellular distribution.

Kikuchi's group have reported that haem administration causes accumulation of cytosol activity (94, 95). In the rats used by these workers 74% of ALV-synthetase activity was mitochondrial 4 hours after AIA treatment, but this was reduced to 13% mitochondrial activity with little decrease in total activity when haem was given 2 hours prior to sacrifice (94). The data suggests that haem prevents the hypothesized conversion of cytosol to mitochondrial form and insertion into mitochondria (21). DDC treatment might therefore be expected to produce a higher proportion of mitochondrial ALV-synthetase than AIA at later times after drug treatment due to the intracellular haem levels: lower haem levels are expected after DDC due to direct inhibition of the terminal haem pathway enzyme ferrochelatase (96), an effect not produced by AIA.

(e) Induction of ALV-synthetase by drug combination

Although AIA and DDC have been hypothesized to induce by different molecular mechanisms that predict a synergistic (or at least additive) result from induction by drug combination, such interaction was not observed here (Fig. 3-1). It seems that AIA may be a more effective inducer over a short period (3 hours), DDC a better inducer at longer times (12 hours) and the combination more effective between these times (8 hours). The combined AIA + DDC dosing induction after 12 or 18 hours was not significantly different from the induction by DDC alone, supporting the possibility that an upper limit to ALV-synthetase synthesis is set by the liver (e.g. by the incorporation of rare codons in the genome).

A number of reports suggest that drug combination in chick embryo liver leads to much increased ALV-synthetase levels (96, 97, 98) when optimum concentrations of inducing chemicals are combined. This was not demonstrated here for rats, nor was it demonstrated by Edwards and Elliott (77) in rat liver cell suspensions for combinations of AIA and steroids which (by analogy with chick liver) should have induced at separate sites. Even in chicks the existence of multiple sites of drug action has now been placed in doubt by recent work (99, 111).

However it has been reported by De Matteis (112) that rats given low doses of DDC develop a condition resembling latent porphyria since they become sensitive to barbiturates and other drugs which can exacerbate porphyria. In such sensitized rats AIA, lindane and other

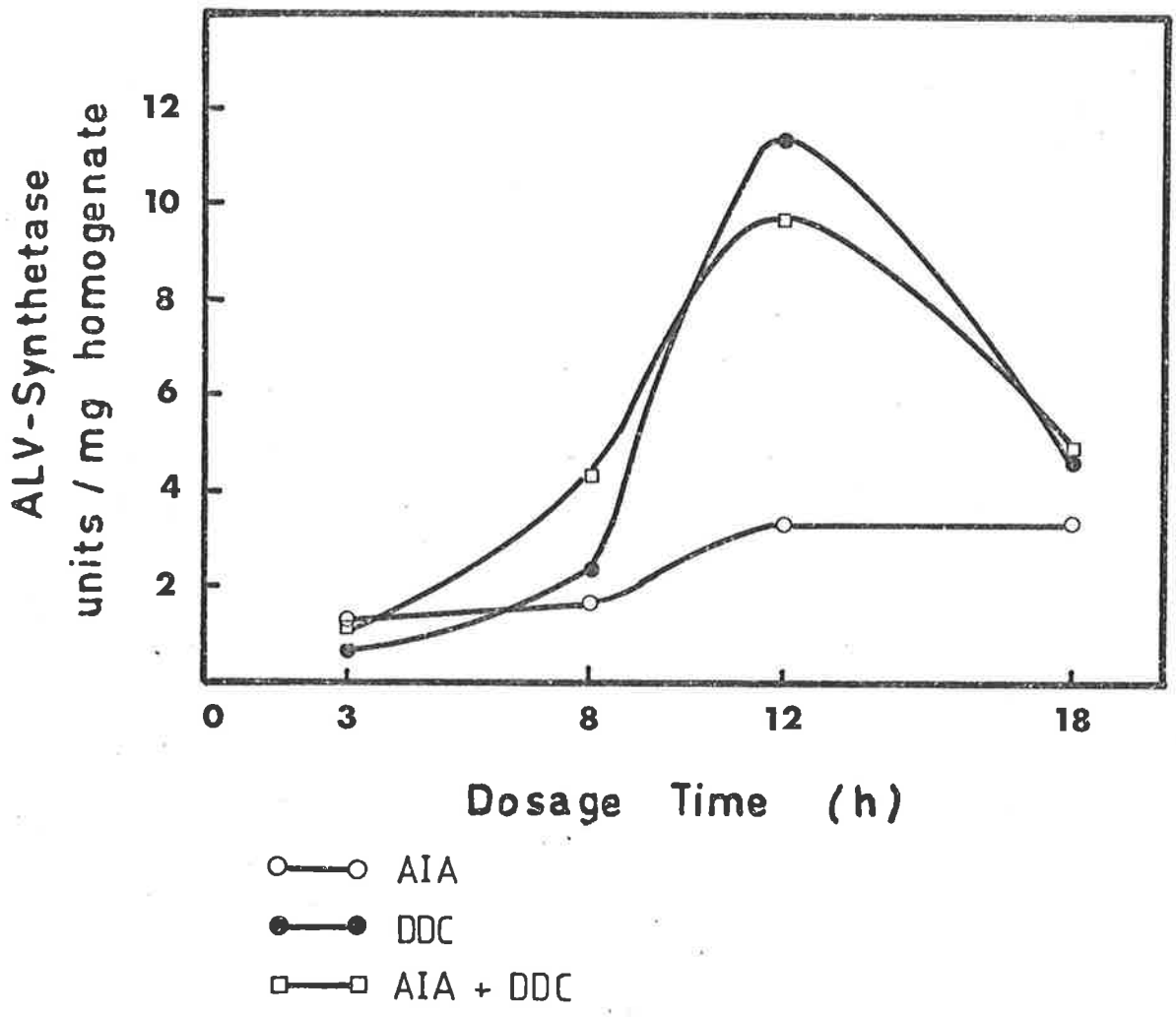
Fig. 3-1.

Induction of ALV-synthetase by drug combination.

Seven rats of the same batch (200-300 g) were used for a given induction time, although each dosage time investigated was studied on a separate day. For each dosage time studied 2 rats received 0.3 g DDC by stomach tube, 2 rats received 40 mg A1A in 2 ml 0.9% saline s.c., and 3 rats were dosed with both A1A and DDC. Rats were fasted 2 days at time of sacrifice.

The mean ALV-synthetase activity in units/mg liver protein and the standard errors are given below.

Induction time(h)	DDC	A1A	A1A + DDC
3	0.59± .06	1.28± .15	1.14± .21
8	2.40± .15	1.73± .53	4.35± .30
12	11.25±1.50	3.30± .15	9.75±2.40
18	4.65± .10	3.30±1.50	4.95± .30


ALV-Synthetase
units / mg homogenate
12
10
8
6
4
2
0
3
8
12
18
Dosage Time (h)
AIA
DDC
AIA + DDC

various drugs all potentiated the DDC induction; the generality of the phenomenon therefore provides little support for distinct molecular sites for inducer action unless DDC has a unique site of action. A comparable sensitizing has also been reported after administration of estradiolcyclopentyl propionate to rats (90), an observation arguing against a unique DDC site.

(f) Time course of DDC induction

The rise in ALV-synthetase follows a sigmoidal increase to 12 hours after DDC dosing (Fig. 3-2). Highly variable levels determined at 12-24 hours (cf. Fig.s 3-1, 3-2 and Table 3-3) suggest that the ALV-synthetase level plateaus with possible oscillations about a mean value followed by decline after 20-24 hours. Oscillations might also occur during the initial 12 hours of DDC induction (see Fig. 3-2 (ii)) which could explain the large variation in ALV-synthetase level after 3 hours (Fig. 3-2 (i)).

Except for the specific investigation of diurnal variation discussed below rats were routinely sacrificed in the morning limiting the possibility that a circadian rhythm in ALV-synthetase inducibility contributed to variation in measured activities. It is possible however that haem synthesized in the cell, in excess of that readily bound to functional target molecules, would repress ALV-synthetase synthesis: an increased wave of enzyme synthesis would then follow as the free haem concentration dropped. Such an explanation has been advanced to explain oscillations in endogenous or AIA induced ALV-synthetase

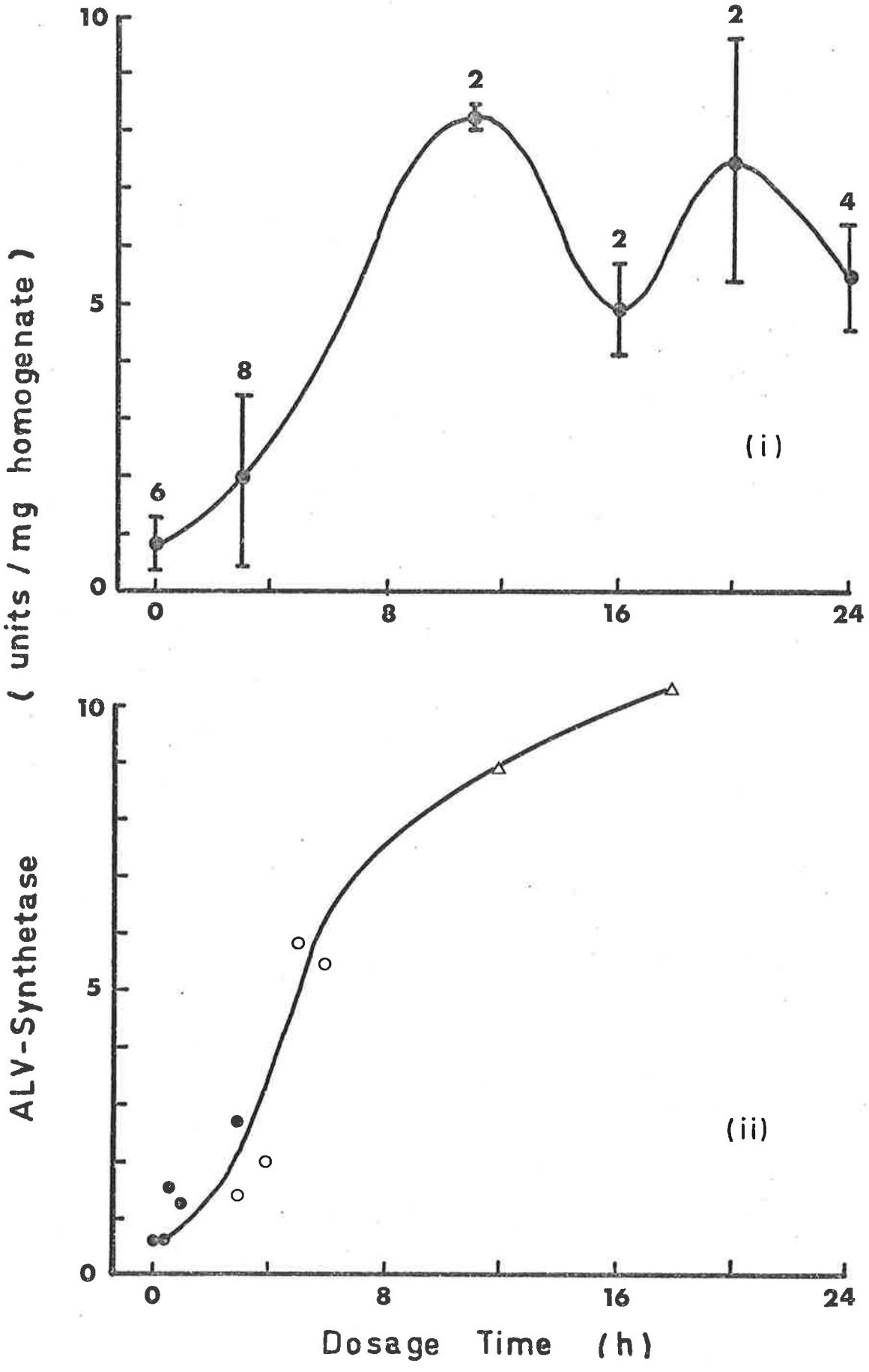
Fig. 3-2.

Time course of DDC induction of ALV-synthetase activity.

Albino Wistar rats were dosed with 2 g DDC/kg as described in Chapter 2, and ALV-synthetase activity estimated in liver homogenates as units/mg liver protein. Rats were fasted 2 days at time of sacrifice.

i) The number of animals used for each time of DDC dosage is indicated above the error bars. Animals were not treated in batches, but dosed on separate days as part of an investigation of artificial liver perfusion. The perfusion results are not otherwise presented here.

ii) This figure was assembled from the data of Table 3-2. The three separate batches of rats involved have been distinguished by different datum characters.


10
5
0
2
2
2
4
8
6
(i)
0
8
16
24
(units / mg homogenate)
ALV-Synthetase
10
5
0
(ii)
0
8
16
24
Dosage Time (h)

when rats are given bovine haemin (114) or estradiol (33). Some published time course studies also show evidence of oscillations in ALV-synthetase after phenobarbital or AIA treatment (115, 34); others for AIA, 3,4-benzpyrene, and DDC show a regular geometric time course (91, 68, 30).

(g) DDC induction at various times of the day

No significant differences in ALV-synthetase activity were apparent when DDC induction was allowed to proceed for 18 hours before sacrifice at either 10 a.m. or 3.30 p.m. (Table 3-3). After a 24 hour dosage period sacrifice at 4 p.m. showed higher levels of ALV-synthetase than sacrifice at 10 a.m. The lower level of enzyme in the rats sacrificed in the morning after 24 hour dosing may not be significant however since morning sacrifice of 12 hour dosed rats produced the highest activity measured.

After obtaining the results of Table 3-3 the next batch of rats received (20 animals) was DDC dosed at 9-10 p.m. and sacrificed at 9-10 a.m. after 2 days fasting. Pooled livers were measured to have 3.9 units ALV-synthetase/mg homogenate, much less than the value of 8.9 previously obtained, demonstrating that a high variation in DDC inducibility existed between batches of rats.

A published study on Sprague Dawley rats (113) suggested that DDC was more effective if rats were kept under continuous lighting in short term inductions of ALV-synthetase, but a natural light cycle did not impair long term inductions relative to continuous light. Indirect evidence implying a circadian rhythm in haem production is provided by the light-dark variable activities of the

Dose period (h)	Dosage time	Sacrifice time	ALV-synthetase (liver homogenate) units/mg
12	9.30 p.m.	9.30 a.m.	8.9± 2.4
18	4.00 p.m.	10.00 a.m.	4.8± .4
18	9.30 p.m.	3.30 p.m.	5.0± 1.8
24	10.00 a.m.	10.00 p.m.	2.3± .8
24	4.00 p.m.	4.00 p.m.	5.4± .6

Table 3-3.

Diurnal variation as a factor in DDC induction of ALV-synthetase

Rats were deprived of food the morning before dosing commenced. DDC was administered by stomach tube as described in the methods. Each determination was made using 3 rats and all rats were from the same batch.

detoxifying enzymes hexobarbital oxidase and p-nitroanisole-O-demethylase (113). No practical significance of such a rhythm was established here.

(h) Variation in DDC induction within a single batch of rats

It was common to find that a limited variation in DDC induction occurred between cage mates received from the animal supply house. Exploitation of this variation by selecting only the most highly induced liver as starting material for ALV-synthetase purification was considered, but deemed impractical as discussed below.

Livers from 12 rats dosed with DDC for 12 hours were homogenized individually and the mitochondria isolated. Twelve rats, although more had been dosed, proved to be the most that could be handled conveniently by a single person, since mitochondria had to be isolated to determine ALV-synthetase activity - the ALV-synthetase assay procedure is too time consuming to permit storage of liver homogenates before deciding which homogenates should be used to isolate mitochondria. A mean specific activity of 7.7 ± 2.1 units/mg was determined and the three most active mitochondrial preparations had a specific activity of 10.6 ± 1.2. This was a relatively poor induction following a 12 hour DDC dosage period and would correspond to an approximate mean homogenate activity of 2.6 units/mg.

Since the variation between batches seemed much greater than within a batch, individual processing of livers was not deemed worthwhile to acquire starting material for enzyme purification. The failure of 12 hour

dosing to consistently give high ALV-synthetase activity led to the use of 24 hour DDC dosing following 24 hour prior fasting as standard enzyme inducing procedure for obtaining enzyme purification starting material.

(i) Genetic variation as a factor in susceptibility to DDC induction of ALV-synthetase

Rats of the Wistar-hooded type were obtained from the Institute of Medical and Veterinary Science, Adelaide, and dosed with DDC for 24 hours, but isolated mitochondria pooled from 12 rats showed little ALV-synthetase activity (1 unit/mg). Such a low activity was never noted after DDC dosing of Wistar-albino rats, routinely used here, suggesting that a genetic difference in inducibility between the strains existed. The variation of inducibility previously noted within the albino strain may also be genetic if inducibility is a multi-allelic character.

A drift in genetic constitution may also have gradually occurred in the albino rats used since the very high activities of ALV-synthetase in homogenates of up to 10 units/mg were only observed in the first year of this study.

A decline in inducibility to a maximum of 3-6 units/mg of homogenate was observed during continued investigations of enzyme purification procedures to be discussed in the following chapters. Gross and Hutton (30) have established genetic variation in DDC inducibility of ALV-synthetase between inbred strains of mice.

CHAPTER 4

AFFINITY CHROMATOGRAPHY OF MITOCHONDRIAL ALV-SYNTHETASE: ATTEMPTED PURIFICATION USING PLP ANALOGUE SIDE CHAINS ON CELLULOSE AND SEPHAROSE BASES

4.1. THE ISOLATION OF CRUDE MITOCHONDRIAL SAMPLES FOR INVESTIGATION OF PURIFICATION PROCEDURES

Mitochondrial rat ALV-synthetase has proved difficult to purify because it is extracted into buffer from freeze dried mitochondria as an aggregate of high molecular weight. This problem was first overcome in this laboratory by Whiting (21) who observed true solublization of ALV-synthetase by treatment with 0.8M NaCl with 1 mM DTE. This treatment, to obtain a solublized starting material, was used here and the details of this procedure are given in Chapter 2.

The solublized samples obtained by Whiting were then subjected to Sephadex G-150 filtration as an initial purification procedure. Initial experiments conducted here also used Sephadex G-150 columns, run with TDN (0.5 M NaCl) buffer, and confirmed that a purification of approximately 2 fold could be achieved as Whiting had reported. The Sephadex G-150 filtration of solublized mitochondrial extract has a number of disadvantages however. These are a long running time, limitation of sample volume and amount of protein that can be loaded and dilution of recovered sample. Therefore precipitation of solublized extract in 50% saturation ammonium sulphate solution, as described in Chapter 2, was used routinely to provide crude starting samples for investigations of purification techniques.

4.2. INTRODUCTION TO AFFINITY CHROMATOGRAPHY EXPERIMENTS

The isolation of a homogeneously pure mammalian preparation of ALV-synthetase remains an important goal in attempting to extend the knowledge of haem pathway regulation. In particular if a specific antibody to purified enzyme can be generated then direct experiments on the significance of enzyme induction and activation *in vivo* can be performed. A specific antibody should also facilitate an understanding of the relationship between cytosol and mitochondrial forms (see Chapter 1).

The most active fraction isolated here by a combination of procedures had an activity of 4,684 units/mg which implies that less than 0.2% of the protein in DDC-induced mitochondria is ALV-synthetase protein. The achievement of purification therefore requires techniques that can offer high selectivity for ALV-synthetase and produce good yields. As discussed in other chapters isoelectric focussing did not prove useful and electrophoresis gave variable yields and resolution of activity. The high activity sample mentioned above was purified by procedures including cation exchange, gel filtration and electrophoresis although the preparation was unstable and of insufficient amount to perform further experiments. Affinity chromatography was investigated at various times during this work and considered both as an initial and as a later step in the purification sequences used.

Affinity chromatography has been spectacularly successful in many applications where an immobilized

ligand has a high specific binding affinity for target molecules in solution (for reviews see 119, 120, 121). For example the PLP enzyme TAT has been purified 125 fold using a Sepharose 4B matrix, a 3,3'-diaminodipropylamine: succinate side chain and a PLP terminus (122). Elution in this case was achieved by specific ligand competition using 10 mM PLP after the column was washed with 0.5 M KCl to remove non-specifically bound contaminants. As a general principle free ligand competition rather than high salt should specifically elute bound apo-enzyme from an affinity column.

Whiting reported an 11.5 fold purification for rat mitochondrial ALV-synthetase (21), a 4.2 fold purification for chicken mitochondrial ALV-synthetase (23), and Woods and Murthy (19) achieved a 5.1 fold purification of foetal rat enzyme. These results were obtained using columns with PLP containing side chains. The elution conditions reported by Woods and Murthy (19) were unusual however, suggesting that the column they used was not acting as a true affinity substrate. The foetal enzyme was not released from a supposed PLP terminus column by successive washes with 10mM PLP, then 10 mM PLP with 0.5 M NaCl; release was achieved with 0.5 M NaCl, 50mM PLP.

Although Whiting reported an 11.5 fold purification of rat enzyme by affinity chromatography during work in this laboratory, he later found that he could not repeat this purification while working elsewhere. Difficulty in achieving affinity purification was simultaneously encountered in the work described here. Whiting in more recent work has established that the physical conditions described in the literature, and those which

he had previously used, to achieve PLP linkage to affinity column side chains were inefficient (unpublished result). The unsuitability of the chemical conditions to achieve linkage was not realized at the time of the work performed here because of the wide acceptance of the method, although it became apparent that side chain joining to PLP was incomplete and attempts were made to overcome this.

The experiments reported in this chapter were largely conducted using Sepharose bases because of the advantages of this substrate over cellulose claimed by Cuatrecasas (119) and because of the report by Miller *et al* (122), unavailable to Whiting at the time of his original investigations, that a Sepharose base was highly successful in TAT purification. The Sepharose base columns were largely investigated as a first step to achieve purification of crude mitochondrial samples, but it became apparent that the purifications that were achieved were probably by cation exchange from either unblocked carboxyl groups on the side chains or the phosphate group of the PLP moiety.

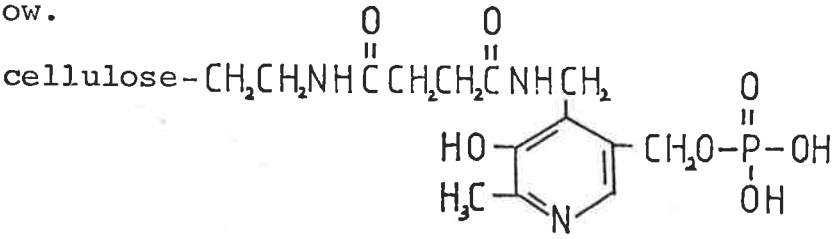
The investigations described in Chapter 5 led to the establishment of CM-Sephadex ion exchange as a useful step in the purification of ALV-synthetase so that samples purified by cation exchange were also used to investigate whether affinity chromatography could usefully extend the purification achieved by ion exchange. Such partially purified samples were mostly used to investigate the efficiency of the cellulose base column as used by Whiting and Elliott (21). The cellulose material

was investigated because it had formed the basis of a previously successful purification step.

4.3. RESULTS

(i) Cellulose base affinity chromatography using PLP as an affinity ligand

Amino ethyl cellulose was used as a matrix for the attachment of succinate-PLP side chains. The method used was that of Cuatrecasas (126) as adopted by Whiting (10) and the structure of the completed side chain is shown below.



Three separate syntheses were attempted, the latter syntheses with extended coupling times as described in Table 4-1. All results relevant to cellulose-base affinity chromatography have been summarized in Table 4-1.

The inefficient binding of ALV-synthetase by the first column synthesized, as indicated by the amount which eluted in the TD buffer alone, did not seem to justify further effort with this substrate. This was confirmed by applying a sample of PLP-depleted TAT to the column: no TAT activity bound. The sample of TAT used in this experiment was from haem induced rats, partially purified by heat treatment and centrifugation, and was kindly donated by Dr. A.M. Edwards.

Table 4-1. Cellulose base affinity chromatography

All ALV-synthetase samples applied, except for synthesis 2 experiment (i) were partially purified by previous cation exchange chromatography. The application of CM-Sephadex chromatography to crude samples of mitochondrial ALV-synthetase is described in Chapter 5. All applied samples were deprived of PLP by Sephadex G-25 filtration.

* Low concentrations of protein could not be reliably determined in solutions of high PLP concentration by the Lowry method: 0.5 M NaCl eluted fractions after 10mM PLP still contained a significant but uncertain amount of PLP in fractions where ALV-synthetase was eluted.

ıthesis No.	Synthesis Procedure		Sample Applied		Elution Buffer	% Eluted		Gross Purification
			units/mg	mg protein		protein	ALV-s	
1	Cuatrecasas (126) adopted by Whiting (10) Column: 3.5 x 1 cm.		85	6.5	TD	95	92	
					TD, 10mM PLP	*	8	
					TD, 10mM PLP, 0.5M NaCl	*	0	1.6
					Recovery		100	
2	As above but succinylation extended from 18 h. to 48 h. Pyridoxamine Pi concentration doubled during carbodiimide linkage and left 5 h. Column: 6.5 x 1.3 cm.	(i)	24	37	TDE	89	30	
					TDE, 10 mM PLP	*	34	
					TDE, 0.5M NaCl	*	36	6.4
					Recovery		100	
		(ii)	120	30	TDE	43	8	
					TDE, 10mM PLP	*	60	
					TDE, 0.5M NaCl	*	32	1.6
					Recovery		100	
3	As for 2 above Column: 10.5 x 1.3 cm.		41	30	TDE	48	0.5	
					TDE, 10mM NaCl	2.5	0	
					TDE, 10mM PLP	*	26	
					TDE, 0.5 M NaCl	*	24	Nil
					Recovery		50.5	
4	Synthesized by Whiting according to (126) and stored ≈ 18 months at -15°C Column: 2.5 x 1.3 cm.		280	9.5	TDE	93	56	
					TDE, 10mM PLP	*	13.5	
					TDE, 10mM PLP, 0.5M NaCl	*	14	3.9
					Recovery		83.5	

The first substrate was not subjected to any tests for the efficiency of chemical linkage procedures, but the second synthesis was performed with extended coupling times (see Table 4-1) and qualitative checks on the synthesis were performed. For the second synthesis TNBS staining indicated successful succinylation of amino side groups and, after coupling of the PLP side chain, the washed cellulose exhibited greater fluorescence under U.V. light than succinylated cellulose. This provided a qualitative indication that the affinity ligand was bound to be substrate. The efficiency of ALV-synthetase binding (70% and 92%) in two experiments with this column was satisfactory, but the failure of 10 mM PLP to completely elute bound activity is suggestive that non-affinity binding was also involved (e.g. to unsubstituted carboxyl termini of succinate side groups). The interpretation that ionic binding was involved on this column is encouraged by the further observation that a sample prepared only by ammonium sulphate precipitation was purified 6.4 fold, but a sample partly purified by prior CM-Sephadex cation exchange chromatography was purified only an additional 1.6 fold. The possibility that a relatively specific release of ALV-synthetase by 10mM PLP had occurred was investigated by electrophoresis of the PLP eluate from sample (ii) (Table 4-1). The result, presented as Fig. 7-1 in Chapter 7 was that the PLP eluate from the cellulose affinity column contained at least 5 bands of protein that were separated by electrophoresis at pH 8.8, ALV-synthetase apparently beinga minor component since

activity was not clearly associated with any of the well resolved bands.

It was hoped that a repetition of the modified synthesis used above would give a more satisfactory product. For the third synthesis, in addition to TNBS staining to demonstrate succinylation of amino ethyl groups, the succinate side chains were titrated against NaOH which indicated 0.25 m equivalents of carboxyl groups per gm of cellulose. After carbodiimide linkage of pyridoxamine phosphate the cellulose could be stained deep blue by the reagents of Leloir and Cardini (127) indicating the presence of bound phosphate. A sample of washed substrate was dried, digested in concentrated sulphuric acid, and total phosphate analysis conducted by the procedure of Leloir and Cardini (127). It was found that 0.034 m moles of phosphate were present per gm of cellulose, ie. 14% of the titratable carboxyl groups had been substituted with pyridoxamine phosphate. Table 4-1 indicates that this was an inefficient affinity chromatography column, although approximately half of the bound enzyme was released by 10 mM PLP.

A sample of cellulose-base affinity substrate synthesized by Whiting during work in this laboratory, and which had previously given useful affinity chromatographic purification, was investigated. The result (Table 4-1, 4) suggests that the side chains may not have been stable under the storage conditions. Whiting had previously achieved complete elution of bound ALV-synthetase by using 10 mM PLP elution.

(ii) Sepharose base affinity chromatography substrates

As a substrate for affinity chromatography Sepharose offers the advantage among others of accepting a high density of side chains (119). In addition Miller *et al.* (122) have described the synthesis of a *long* side chain PLP terminus matrix, using a Sepharose base, which they reported gave a 125 fold purification of the PLP cofactor enzyme TAT. Lower purifications were achieved using shorter side chains. The first substrate synthesis described here featured a longer side chain with an extra carbon unit but was otherwise the same as the side chain attached to Sepharose by Miller *et al.* A high degree of non specific binding to this material was observed and various other syntheses described here were undertaken to reduce the degree of non specific binding. It is likely however that unsubstituted carboxyl groups on side chains were responsible for continued observations of non specific (cation exchange) binding.

(a) Sepharose-spermidine-succinate-PLP substrate

This long side chain material was synthesized from Sepharose 4B (agarose) using the conditions described by Miller *et al.* (122). Sepharose was activated with 0.25 g CNB per ml of packed gel and 45 mg of spermidine added per ml of gel. TNBS staining indicated that spermidine had become attached to the Sepharose. Following treatment with succinic anhydride, TNBS staining confirmed efficient succinylation of the matrix. Pyridoxamine phosphate (0.2 g/ ml gel) was linked to succinyl termini using 0.1 g carbodiimide reagent/ ml gel. This amount of

pyridoxamine phosphate represents a 40 fold excess of the level used by Miller *et al.* in an attempt to ensure complete side chain completion.

This substrate gave a 4.3 fold purification of a crude mitochondrial preparation with 100% recovery of activity. This result, shown in Fig.4-1, shows that the substrate synthesized here is not acting as an affinity binding column by the criterion that competing ligand should release activity.

Prior to the preparation of this column, material was synthesized using the low level of pyridoxamine phosphate used by Miller *et al.* Three successive experiments with this material gave only 20-23% recovery of ALV-synthetase activity and showed elution behaviour similar to that shown in Fig.4-1. In one of these experiments the TAT activity present in a mitochondrial extract was also assayed: 10mM PLP released 70% of bound TAT activity but only 40% of bound and recovered ALV-synthetase activity which trailed after the TAT peak. This result suggests that ALV-synthetase may have more charged groups at its surface than TAT, leading to more stable ionic attachment to substrate side chains, and thereby complicating affinity chromatography procedures. Miller *et al.* (122) also found that a number of other PLP cofactor enzymes (not including ALV-synthetase) were much less effectively purified than TAT on their affinity columns.

To clarify the mechanism by which ALV-synthetase activity bound to the substrate described here, a sample of mitochondrial preparation was desalted and made to 0.1mM PLP before applying to a column run with PLP present at all

Fig. 4-1.

Sepharose - spermidine - succinate - pyridoxamine phosphate column.

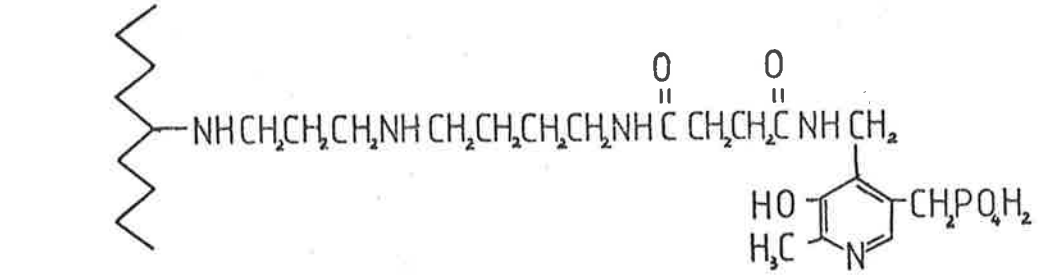
Column: 6 x 1 cm

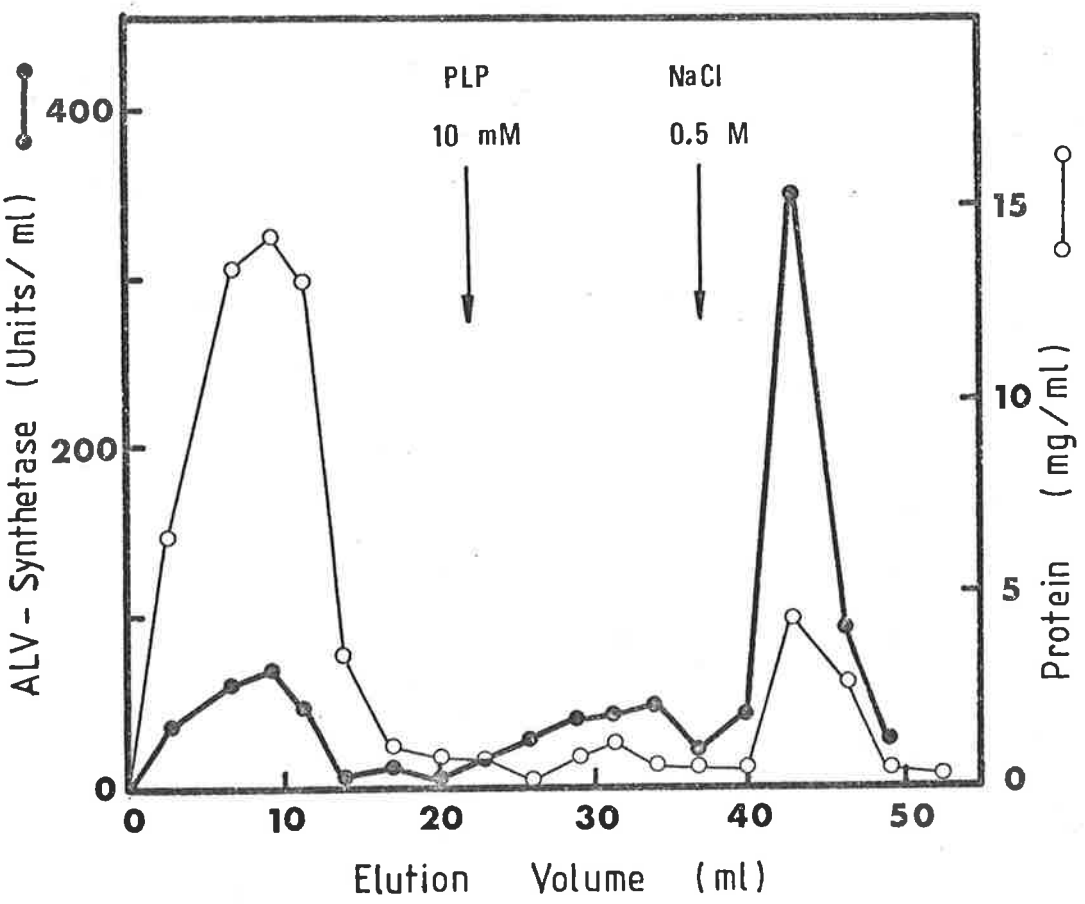
Buffer: TDE

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 180 mg desalted protein, specific activity 16 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	82	22
10 mM PLP released	4	17
0.5 M NaCl released	14	61
	100	100




PLP
10 mM
NaCl
0.5 M
400
200
0
ALV - Synthetase (Units/ml)
15
10
5
0
Protein (mg/ml)
0
10
20
30
40
50
Elution Volume (ml)

times. In this experiment shown in Fig.4-2 all ALV-synthetase activity bound, did not elute with 10mM PLP and was released by 0.5 M NaCl. A purification of 14 fold with recovery of 82% of applied activity was gratifying, despite the conclusion that affinity chromatography was unlikely to be the mechanism involved.

(b) Sepharose-diamino hexane-succinate-PLP substrate

It was considered that the intra-side chain amine groups present in spermidine based affinity side chains may have been sufficiently polar to cause non specific anion exchange binding of protein, and that this may have contributed to elution behaviour such as that shown in Fig.4-1. To determine whether elimination of an amine group would result in less non-specific protein binding, a side chain built on diamino hexane was synthesized: (the respective structures of the spermidine and diamino hexane side chain are shown with Fig.s4-1 & 4-3). Substrate was prepared using 37 mg of 1,6-diamino hexane per ml of CNBr activated gel, but otherwise by the method of Miller *et al.* (122).

The elution profile shown in Fig.4-3 demonstrates that this substrate also failed to provide an affinity binding matrix.

(c) Sepharose-γaminobutyric acid-PLP substrate

Efficient affinity chromatography purification requires that the immobilized affinity ligand should be sufficiently distant from the backbone of the matrix to minimize steric interference with binding, which

Fig. 4-2.

Sepharose - spermidine - succinate - pyridoxamine phosphate column.

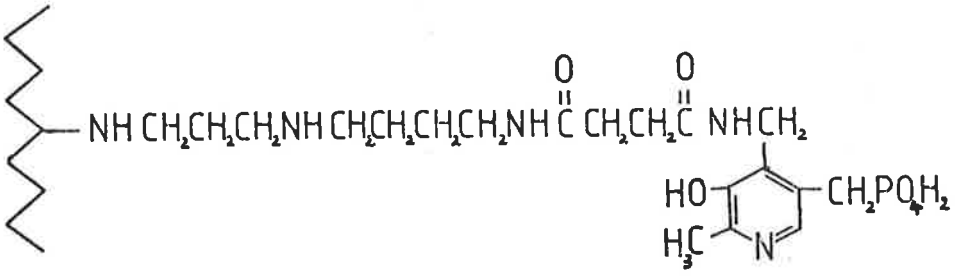
Column: 6 x 1 cm

Buffer: TDEP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 150 mg desalted protein, specific activity 18 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	90	3
10 mM PLP released	4	4
0.5 M NaCl released	6	82
	100	89



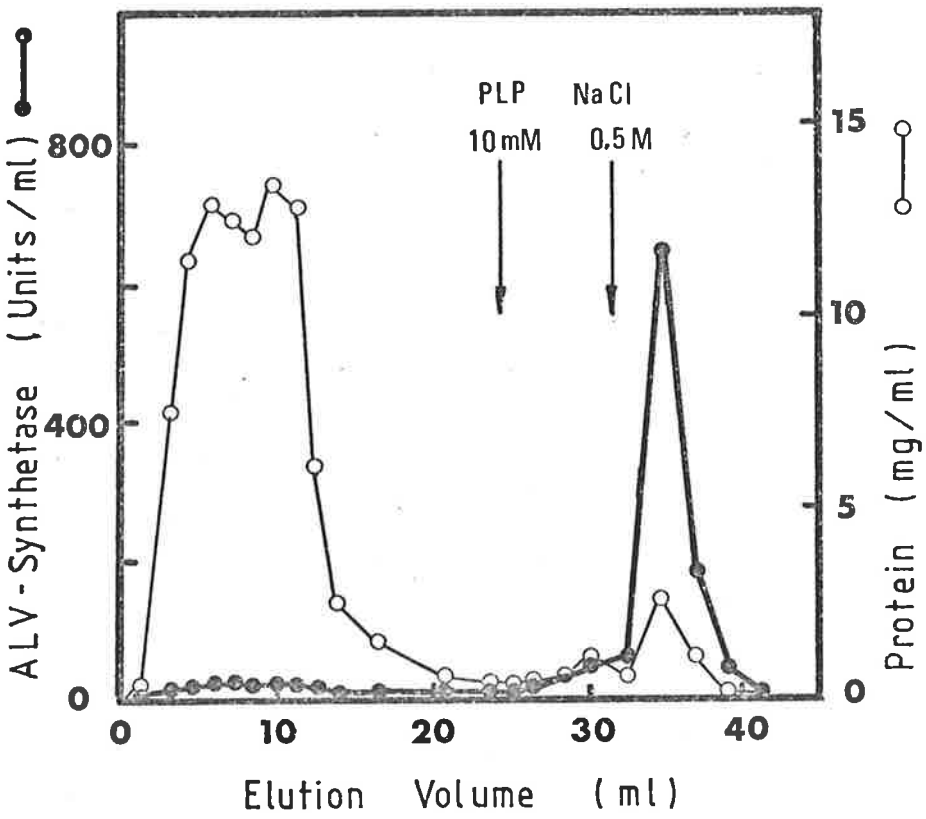

PLP
10 mM
NaCl
0.5 M
ALV - Synthetase (Units / ml)
Protein (mg / ml)
800
400
0
15
10
5
0
0
10
20
30
40
Elution Volume (ml)

Fig. 4-3.

Sepharose - diaminohexane - succinate - pyridoxamine phosphate column.

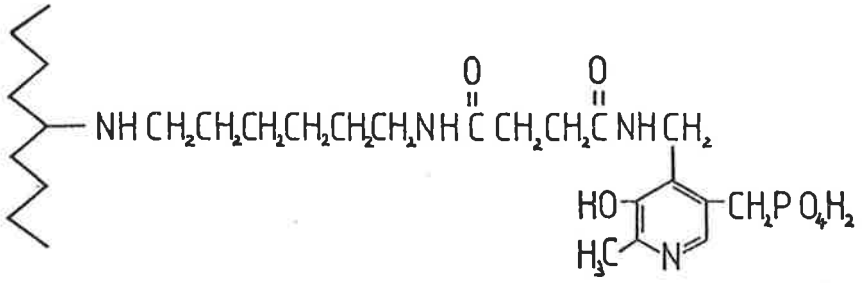
Column: 2 x 1 cm

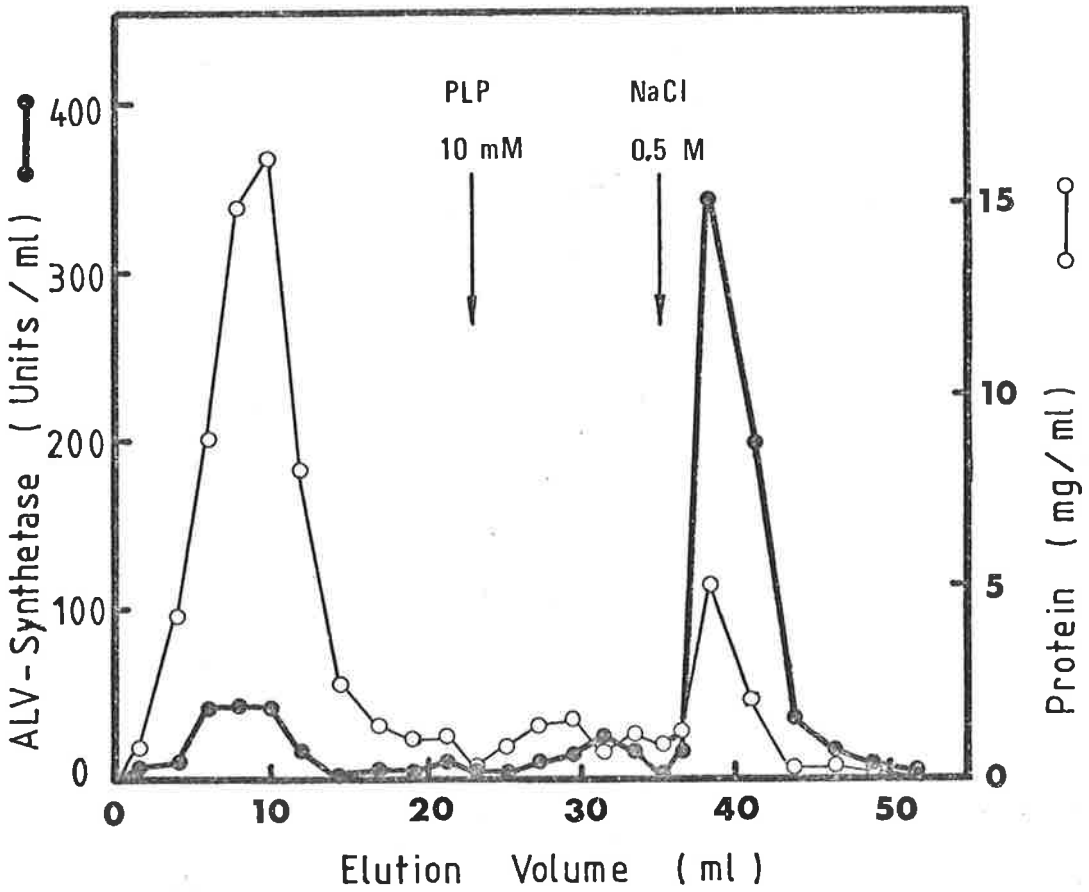
Buffer: TDE

Sample applied: solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 156 mg desalted protein, specific activity 15 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	77	18
10 mM PLP released	10	7
0.5 M NaCl released	13	74
	100	99


NH CH2CH2CH2CH2CH2CH2NH C CH2CH2C NH CH2
HO
H3C
N
CH2P O4H2


PLP
10 mM
NaCl
0.5 M
ALV-Synthetase (Units / ml)
Protein (mg / ml)
0
100
200
300
400
5
10
15
0
10
20
30
40
50
Elution Volume (ml)

therefore makes long side chains preferable (121). However an increased length of side chain might also increase non specific binding by analogy with the series of aliphatic-amine substrates studied by Shaltiel and ErEl (128, 132). These authors found greater attachment of protein samples to long side chain columns (attributed to hydrophobic interactions) and reported that high salt concentration could achieve elution from their polar side groups (cf. the failure of 10 mM PLP to elute ALV-synthetase from the long side chain columns reported previously). A short side chain affinity column was therefore synthesized to investigate whether a more selective substrate for ALV-synthetase would result.

The synthesis of a 4C side chain column was achieved using Sepharose activated with 0.15 g CNBr per ml of gel, treated with 0.1g γamino-butyric acid per ml of gel then 0.125 g of pyridoxamine phosphate with 0.06 g carbodiimide under the conditions described by Miller *et al.* (122).

This substrate also failed to act as an affinity binding substrate. In the experiment described in Fig.4-4 significant ALV-synthetase activity was released only by 0.5 M NaCl. Although only 27% of the applied ALV-synthetase activity was recovered in the salt-released fractions the co-release of only 1.4% of applied protein meant that a 19 fold purification was achieved. This short side chain substrate therefore did offer better discrimination against non specific binding than long side chain columns and an acceptable retention of ALV-synthetase activity, but it still did not behave as an affinity

Fig. 4-4.

Sepharose - GABA - pyridoxamine phosphate column.

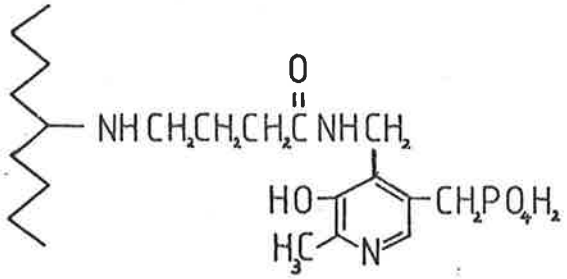
Column: 3 x 1 cm

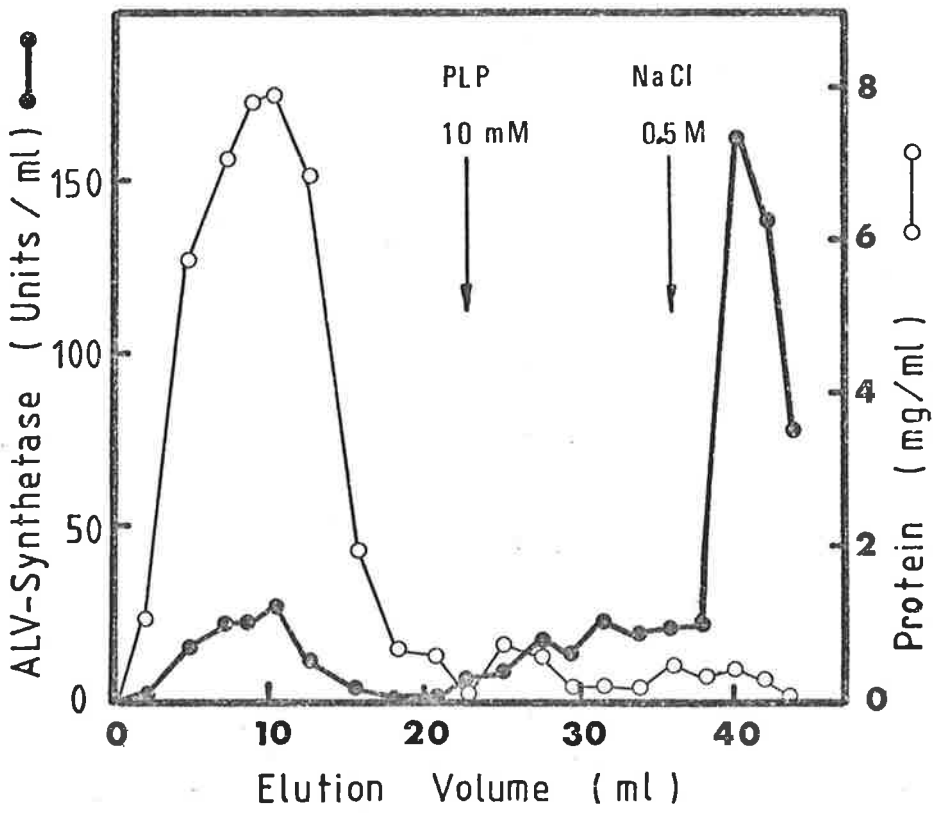
Buffer: TD

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 90 mg desalted protein, specific activity 28 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	92.2	8
10 mM PLP released	6.3	12
0.5 M NaCl released	1.4	27
	99.9	47


O
NH CH2CH2CH2C NH CH2
HO
CH2PO4H2
H3C
N


PLP
10 mM
NaCl
0.5 M
ALV-Synthetase (Units / ml)
Protein (mg/ml)
0
50
100
150
0
2
4
6
8
0
10
20
30
40
Elution Volume (ml)

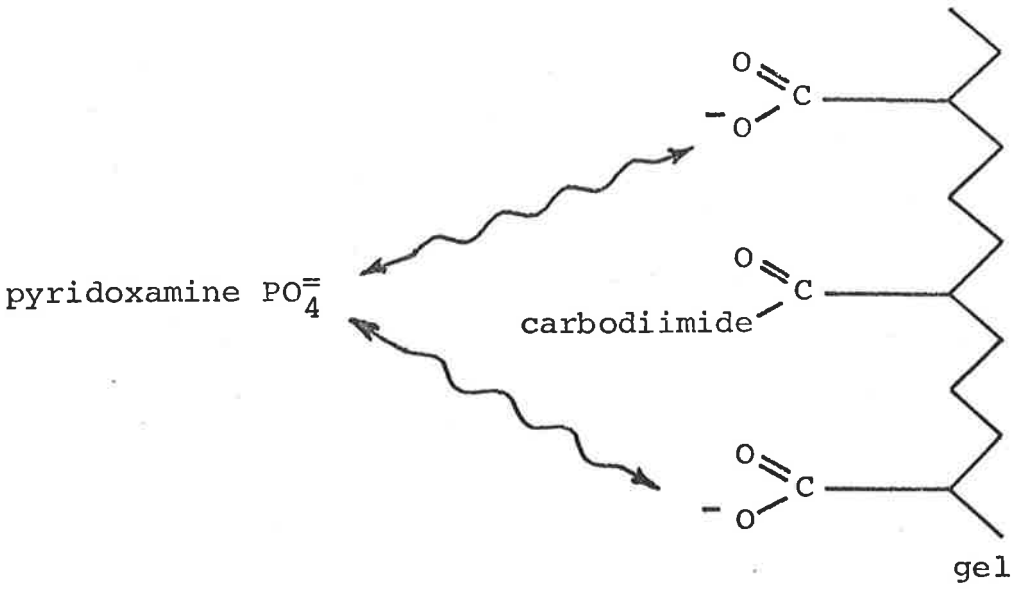
chromatography column. More results obtained using γ-aminobutyric acid side chain columns, and confirming the unacceptable affinity binding characteristics, are presented in the next section on low side-chain density material.

(d) Low side-chain density substrates

The results previously presented in this chapter suggested that the major cause of failure to observe acceptable affinity chromatography behaviour was the inefficiency of PLP linkage to the matrix side chains. The presence of unsubstituted carboxyl groups was an important concern since they could behave as ion exchange binding sites and give non-affinity binding.

Miller *et al.* (122) reported that, for a synthesis analogous to the spermidine containing matrix described here, approximately 14 μ moles of succinylated side chain was present per ml of gel and only 3 μ moles of pyridoxamine phosphate per ml of gel was bound after carbodiimide linkage at pH 4.7. This apparent completion efficiency of 21% is also likely to be a maximum estimate since Miller *et al.* estimated their binding by measuring the amount of free pyridoxamine phosphate recovered after carbodiimide linkage, not by analysis of the gel product.

A possible explanation for low blockage of carboxyl termini by pyridoxamine phosphate may have been electrostatic repulsion between the phosphate moiety and the negatively charged gel matrix as visualised below.



To circumvent this possibility Sepharose was activated with 3.85 mg (36 μ moles) of CNBr per ml of gel, a level of only 1.5% of that recommended for optimal activation by Cuatrecasas (119). The Aim of this was to obtain a lower density of substituted side chains on the matrix. γAmino butyric acid (0.04 g/ml gel) was linked to the activated Sepharose, the gel was then washed and titrated against NaOH. The titration indicated that 2 μ equivalents of carboxyl group were available per ml of gel. Carbodiimide and pyridoxamine phosphate, both 25 mg/ml gel, were added to this gel under the conditions of Miller *et al.*

Whole phosphate analysis of the product gel did not result in a significant detection of bound phosphate by the method of Leloir and Cardini (127). Because of the low sensitivity of this test however it was only possible to conclude that less than 50% of the available carboxyl groups were phosphate substituted. It was clear however that in this case the strategy of using a low density of side chains on the matrix had

failed to achieve the degree of chain completion by PLP that was aimed for.

The synthesized material was nevertheless tested for behaviour as an affinity chromatography substrate. The result presented in Table 4-2 confirms that binding to this material was both inefficient, presumably due to the low density of binding groups, and not affinity specific.

In further work a low side chain density substrate of the structure Sepharose-diaminohexane-succinate-PLP was synthesized on Sepharose activated by 6.8 mg CNBr per ml of gel. Quantative analysis was not attempted, but the completed substrate did retain a phosphate dependant stain indicating linkage of pyridoxamine phosphate. As indicated in Table 4-2 this material also failed to behave as an affinity chromatography substrate.

It is noteworthy that 87% of protein in the sample used to investigate the above substrates bound to a *long* side chain (diaminohexane - succinate) and only 34% to the *short* (γamino butyric acid) side chain column. Although a simple difference in the relative density of the side chains may explain this, the observation is also consistent with hydrophobic association with a long side chain. It was nevertheless clear that a low density of side chains was not a solution to the problem of synthesizing an adequately specific affinity-substrate for ALV-synthetase.

(i) Sepharose - γ amino butyric acid-PLP (Column: 8 x 1 cm)

Elution Buffer	% Eluted	
	Protein	ALV-s
TDE	66	53
TDE, 10mM PLP	*	10
TDE, 0.5 M NaCl	*	10
Recovery		73

(ii) Sepharose-diaminohexane-succinate-PLP (Column: 9 x 1 cm)

Elution Buffer	% Eluted	
	Protein	ALV-s
TDE	13	1.5
TDE, 10mM PLP	*	1.1
TDE, 0.5M NaCl	*	10.8
Recovery		13.4

* not determined, see Table 4-1

Table 4-2 Low side chain density substrates

The sample used to assess both substrates was previously purified by ion exchange on CM-Sephadex to a specific activity of 214 units/mg. PLP cofactor was removed by Sephadex G-25 filtration. The amount of protein applied to the columns was (i), 2 mg and (ii), 1.6 mg. Synthesis of column material is described in the text.

(e) Sepharose-γ aminobutyric acid-pyridoxamine substrate

The material described here was synthesized to investiage the potential for a non-charged side chain terminated by pryidoxal, rather than the 5'- phosphate derivative, to act as an affinity binding material for apo-ALV-synthetase (the structure is shown with Fig.4-5). The use of pyridoxamine during carbodiimide linkage eliminated the possibility that electrostatic repulsion would interfere with this step in synthesis as proposed in the previous section. If efficient linkage of pyridoxamine to free carboxyl termini was thereby achieved then a neutral matrix that should not behave as an ion exchange material would result. The charged 5'- phosphate group present on other columns synthesized here, as well as unsubstituted carboxyl groups, was expected to be simultaneously eliminated.

γ-Aminobutyric acid (0.1 g/ml gel) was reacted with activated Sepharose (0.15 g CNBr/ml gel) and then pyridoxamine dihydrochloride (0.125g/ml gel) was linked to the side chains using carbodiimide (0.04 g/ml gel) under the conditions used by Miller *et al.* (122).

A crude mitochondrial sample was purified by this substrate as shown in Fig. 4-5. The sequence of elutions here, in which 0.5M NaCl preceded 10 mM PLP, was chosen to avoid PLP interference with Lowry protein determinations. ALV-synthetase activity was released by 0.5M NaCl, a result expected if ionic or possibly hydrophobic (128) binding was involved, but this was not expected if true affinity binding was involved (122). The

Fig. 4-5.

Sepharose - GABA - pyridoxamine column.

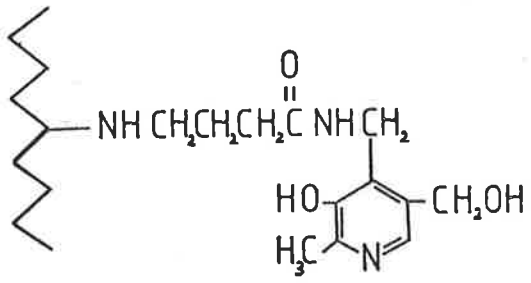
Column: 4 x 1 cm

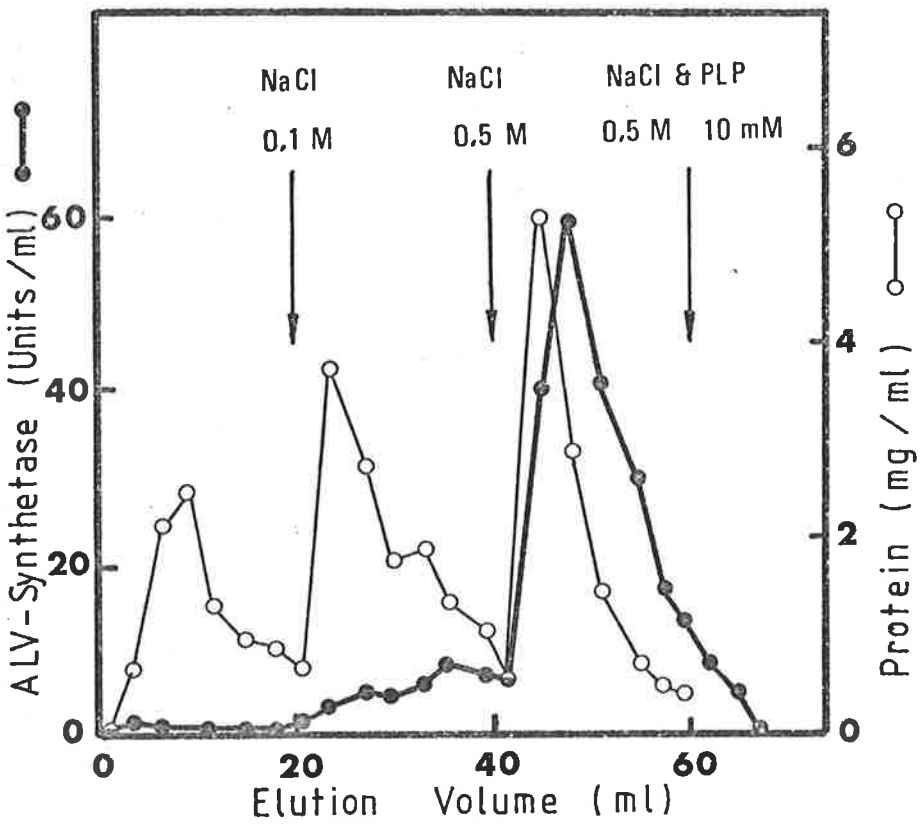
Buffer: TD

Sample applied: Solubilized mitochondrial extract, 0-50% ammonium sulphate precipitate. 95 mg of desalted protein specific activity 7 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	37	2
0.1 M NaCl released	28	9
0.5 M NaCl released	35	51
0.5 M NaCl 10 mM PLP released	not detectable	2
	100	64


O
NH CH2CH2CH2C NH CH2
HO
CH2OH
H3C
N


NaCl
0.1 M
NaCl
0.5 M
NaCl & PLP
0.5 M 10 mM
ALV-Synthetase (Units/ml)
Protein (mg/ml)
0
20
40
60
0
2
4
6
0
20
40
60
Elution Volume (ml)

observation that salt-released ALV-synthetase was retarded relative to total protein released by salt suggested that ALV-synthetase was in part being selectively absorbed to the substrate. The overal specificity for ALV-synthetase was poor however because 63% of total protein bound to the column in low salt concentration, presumably by a hydrophobic mechanism (cf.129), although ion-exchange properties can also be imparted by N-substituted isourea groupings generated during linkage to CNBr-activated Sepharose (121).

Application of a sample, partially purified by CM-Sephadex ion exchange chromatography, to this column resulted in 80% of the applied protein binding to the column. Enzyme activity was not eluted by 10mM PLP which suggested that the pyridoxal material was not a specific PLP site affinity column, and 0.5 M NaCl elution resulted in a recovery of only 1% of the applied activity. The unexpected observation that this substrate could be a *killer* column for ALV-synthetase was confirmed by investigations performed on a separate newly synthesized batch of substrate. No recovery of ALV-synthetase activity from the second batch of substrate was obtained in two experiments. This substrate was not investigated further.

Failure to recover activity from affinity columns may in part follow from the phenomenon which Wilchek and Hexter (121) describe as *the leakage problem*. A ligand that will inhibit an enzyme may lyse from the

matrix and bind to the enzyme; inhibitor-protein complex is then eluted and no recovery of activity results. No data is available on the effect of pyridoxal on the activity of ALV-synthetase however.

4.4. SUMMARY

Some of the substrates synthesized here gave useful purifications of relatively crude samples of mitochondrial ALV-synthetase. In no case was affinity chromatography demonstrated to be the exclusive mechanism by which purification was achieved.

The uncertain mechanism of action, and the inefficiency of achieving the desired chemical structure of substrates, meant that PLP affinity chromatography could not be used as the basis of a reproducible purification method.

CHAPTER 5

INVESTIGATION OF PROTEIN-BINDING CHROMATOGRAPHY MATERIALS FOR USE IN THE PURIFICATION OF ALV-SYNTHETASE

5.1. INTRODUCTION

The failure of Sepharose base affinity chromatography experiments to give acceptable purification of crude ALV-synthetase samples prompted an investigation of alternative chromatographic purification procedures. Kaplan (18) has reported that with the exception of calcium phosphate and hydroxyl apatite chromatography a wide range of conventional chromatographic materials were not useful in the purification of rat mitochondrial ALV-synthetase. The negative results obtained by Kaplan however derived from a study of crude enzyme known to be in a high molecular weight aggregate after extraction from mitochondria.

Using *solublized* rat mitochondrial enzyme, as described in Chapter 2, the investigations reported here showed that CM-Sephadex chromatography provided a reproducible purification of approximately 7 fold. Other materials investigated did not prove satisfactory.

5.2. RESULTS

(a) Sepharose-diaminohexane-succinate substrate

An investigation of a substrate with a succinate group at the terminus of the side chain was performed primarily to determine whether free carboxyl groups could produce the binding characteristics shown by certain affinity chromatography substrates described in the previous chapter. The substrate used here was synthesized as

described in section 4.3 (b) and represented the side chain, less PLP terminus, of the substrate which gave the result of Fig.4-3.

An additional objective was to investigate whether a succinate terminus could act as an analogue of the substrate succinyl-CoA and effect a specific binding of ALV-synthetase via the active site for this substrate. This was considered *ă priori* to be unlikely however due to the high Km of 20 mM for succinyl-CoA reported by Whiting and Elliott (21).

A crude sample of ALV-synthetase was purified 7.1 fold using the succinate terminated side chain as shown in Fig.5-1. 20mM succinate did not release significant ALV-synthetase activity showing that succinate analogue binding was unlikely to be important. A higher concentration of succinate was not used so as to avoid the complication of non-specific elution by succinate.

A protein sample previously purified 6 fold by salt elution from a Sepharose-diaminohexane-succinate-PLP column was applied to the succinate terminus column. The result of a 1.2 fold purification as shown in Fig.5-2 suggests that ion exchange binding was the predominant protein binding force on the preceding "affinity" terminus column.

(b) Hydrophobic chromatography

Shaltiel and ErEl (128) showed that aliphatic side chains bind proteins, presumably at hydrophobic sites, and that elution can be accomplished using high salt

Fig. 5-1.

Sepharose - diaminohexame - succinate column.

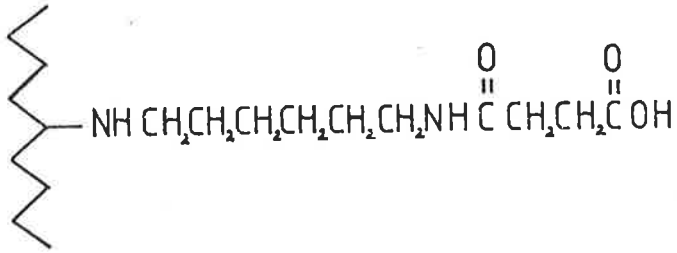
Column: 3.5 x 1 cm

Buffer: TDP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 178 mg desalted protein, specific activity 16 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	84	20
20 mM Na-succinate released	5	8
0.5 M NaCl released	10	72
	99	100


NH CH2CH2CH2CH2CH2CH2NH C CH2CH2C OH
O
O

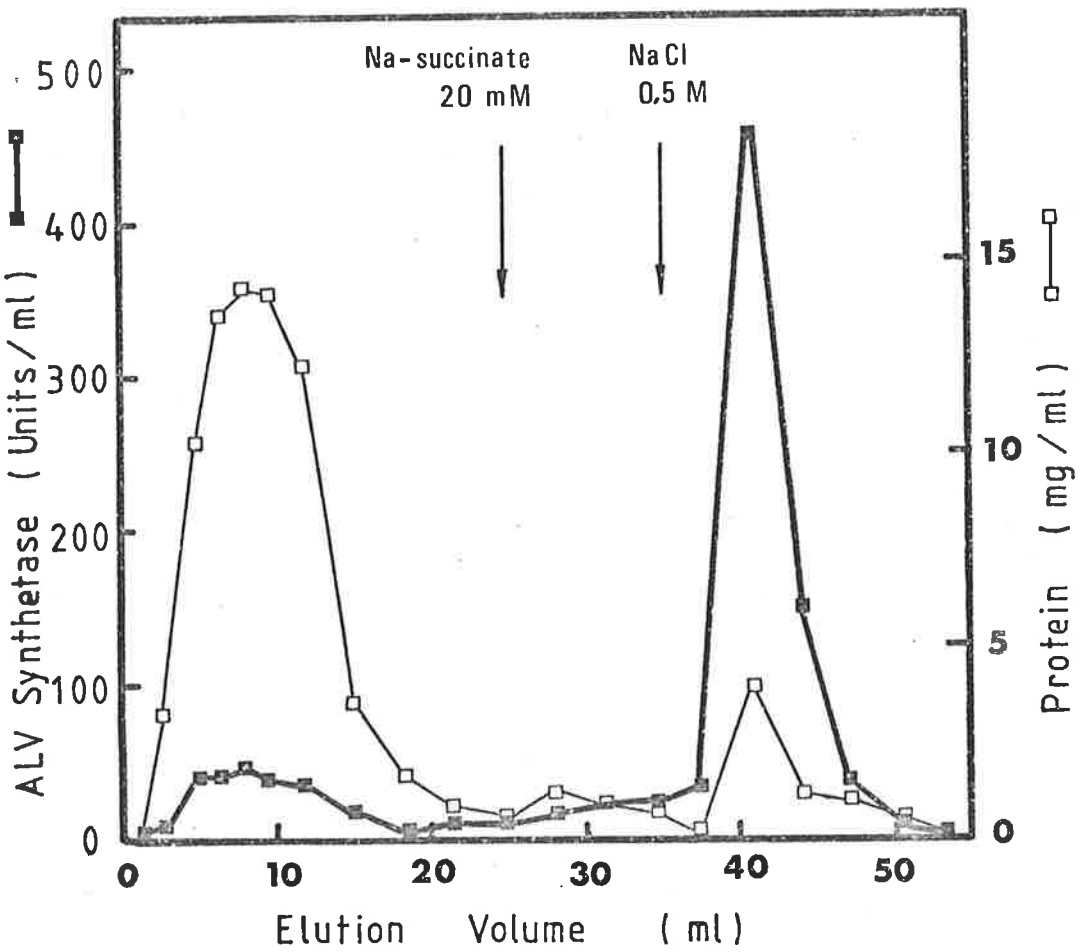

Na-succinate
20 mM
NaCl
0,5 M
ALV Synthetase (Units/ml)
Protein (mg/ml)
Elution Volume (ml)
0
100
200
300
400
500
5
10
15
0
10
20
30
40
50

Fig. 5-2.

Sepharose - diaminohexane - succinate column.

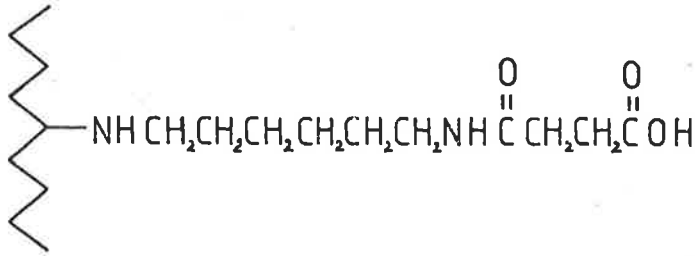
Column: 3.5 x 1 cm

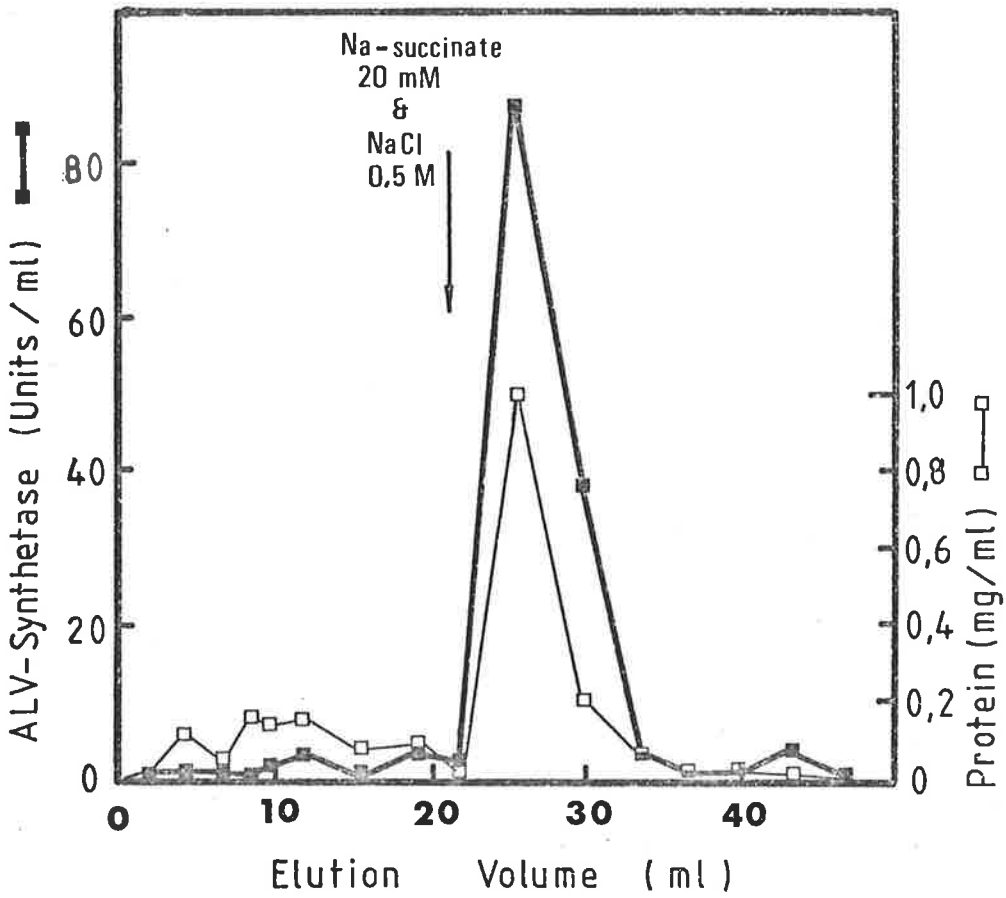
Buffer: TDP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate, salt eluted from a column of Sepharose - diaminohexane - succinate - pyridoxamine phosphate. 7.3 mg protein, specific activity 87 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	26	2
20 mM succinate, 0.5 M NaCl released	74	86
	100	88


NH CH2CH2CH2CH2CH2CH2NH C CH2CH2C OH
O
O


Na-succinate
20 mM
&
NaCl
0,5 M
ALV-Synthetase (Units/ml)
Protein (mg/ml)
0
20
40
60
80
0
0,2
0,4
0,6
0,8
1,0
0
10
20
30
40
Elution Volume (ml)

concentration. These authors used a series of amino terminated aliphatic groups and demonstrated that longer side groups resulted in greater binding of protein (128, 132).

Activated Sepharose was reacted with 1,6-diaminohexane under the conditions of Miller *et al.* (122) and TNBS staining indicated a high degree of substitution. As shown by Fig.5-3 essentially all protein and ALV-synthetase activity in a mitochondrial extract was bound by this ω-aminohexane side chain column. Elution with 0.5 M NaCl released 91% of applied ALV-synthetase and 74% of total protein. The 1.2 fold purification of ALV-synthetase thus achieved did not encourage further investigation of this material. A shorter side chain was then tested.

Activated Sepharose was reacted with 1,2-diaminoethane under the conditions described by Miller *et al* (122). A column of this 2C side chain substrate gave a 1.9 fold purification of crude enzyme sample using 0.5 M NaCl elution as shown in Fig.5-4.

(c) DEAE - Sephadex

Previous work with aminoalkyl-Sepharose columns suggested that ALV-synthetase might bind to a DEAE-Sephadex column. A preliminary experiment in which a crude sample of enzyme was applied to DEAE-Sephadex in TDP buffer and eluted with 0.5 M NaCl did result in satisfactory binding (83%), but a purification of 1.3 fold

Fig. 5-3.

Sepharose - diaminohexane column.

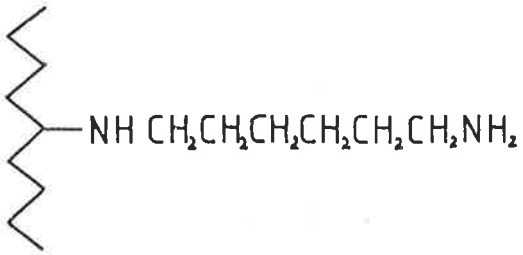
Column: 3.8 x 1 cm

Buffer: TDP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 80 mg of protein, specific activity 15 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	4	3
0.5 M NaCl released	74	91
	78	94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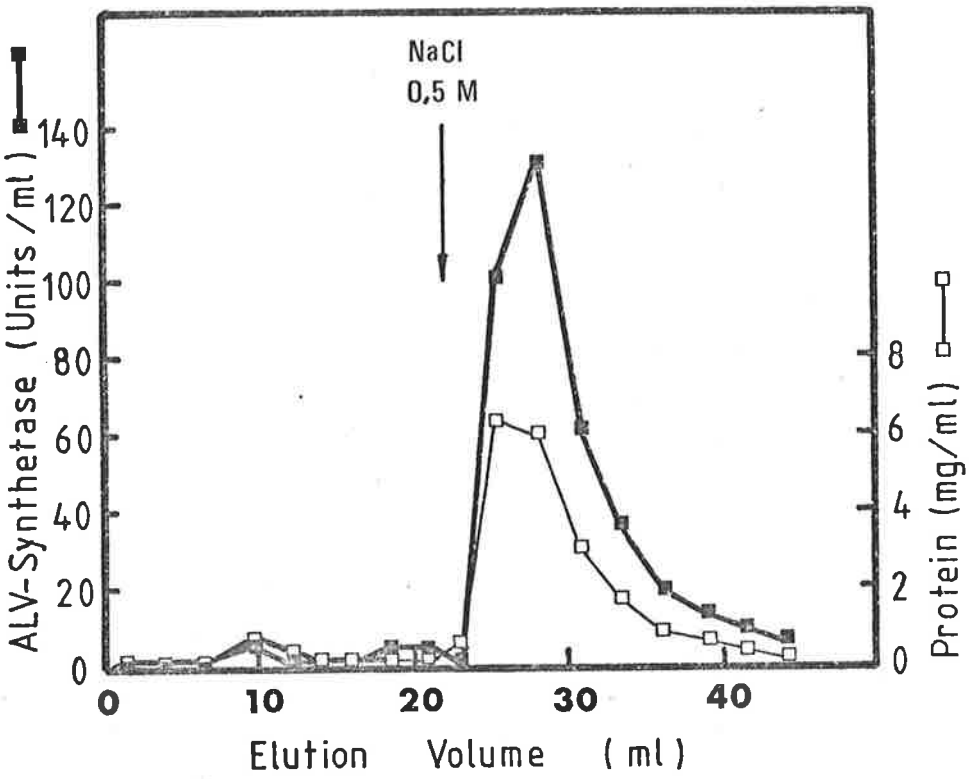

NaCl
0,5 M
ALV-Synthetase (Units/ml)
140
120
100
80
60
40
20
0
Protein (mg/ml)
8
6
4
2
0
0
10
20
30
40
Elution Volume (ml)

Fig. 5-4.

Sepharose - diaminoethane column.

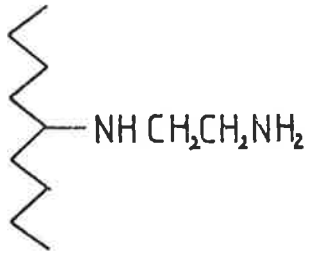
Column: 3.5 x 1 cm

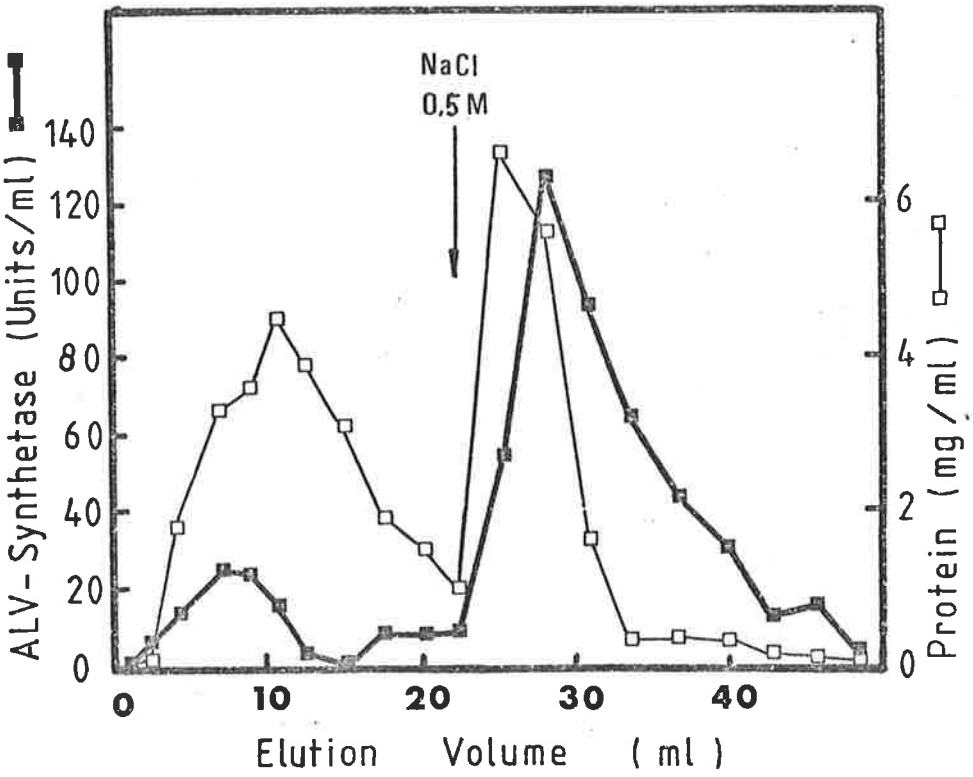
Buffer: TDP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 93 mg protein, specific activity 16 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	56	16
0.5 M NaCl released	44	84
	100	100


NH CH2CH2NH2


NaCl
0.5 M
ALV-Synthetase (Units/ml)
Protein (mg/ml)
0
20
40
60
80
100
120
140
0
2
4
6
0
10
20
30
40
Elution Volume (ml)

only was achieved (Fig.5-5).

During equilibration of the DEAE-Sephadex column used for the results of Fig.5-5 the material visibly bound yellow PLP from the equilibrating solution. Complications that may have derived from this binding include blockage of anion exchange sites by PLP and the possibility that the PLP immobilized on the DEAE-Sephadex might even act as an affinity ligand for ALV-synthetase.

Enzyme deprived of PLP by Sephadex G-25 filtration was therefore applied to a DEAE-Sephadex column equilibrated in TD buffer. Elution with a 0→0.5 M NaCl gradient gave no useful purification under these conditions (Fig.5-6).

Enzyme partially purified by prior CM-Sephadex chromatography was purified a further 2.2 fold using DEAE-Sephadex at pH 7.6, but higher pH failed to provide conditions allowing an improvement in the purification achieved (Table 5-1).

(d) CM-Sephadex

CM-Sephadex was expected to give useful purification of crude enzyme samples following the success of a free succinyl side chain column (Fig.5-1).

Fig.5-7 shows that further purification of ALV-synthetase bound to CM-Sephadex can be achieved using a NaCl concentration gradient. The gradient separation allowed a 5.7 fold purification for fractions containing 80% of the applied activity.

Fig. 5-5.

DEAE - Sephadex column.

Column: 5.7 x 1.3 cm (low salt)

Buffer: TDP

Sample applied: Freeze dried mitochondria disrupted in 0.8 M NaCl, 1 mM DTE; 0-50% ammonium sulphate precipitate. 64 mg protein, specific activity 9 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	36	17
0.5 M NaCl released	64	83
	100	100

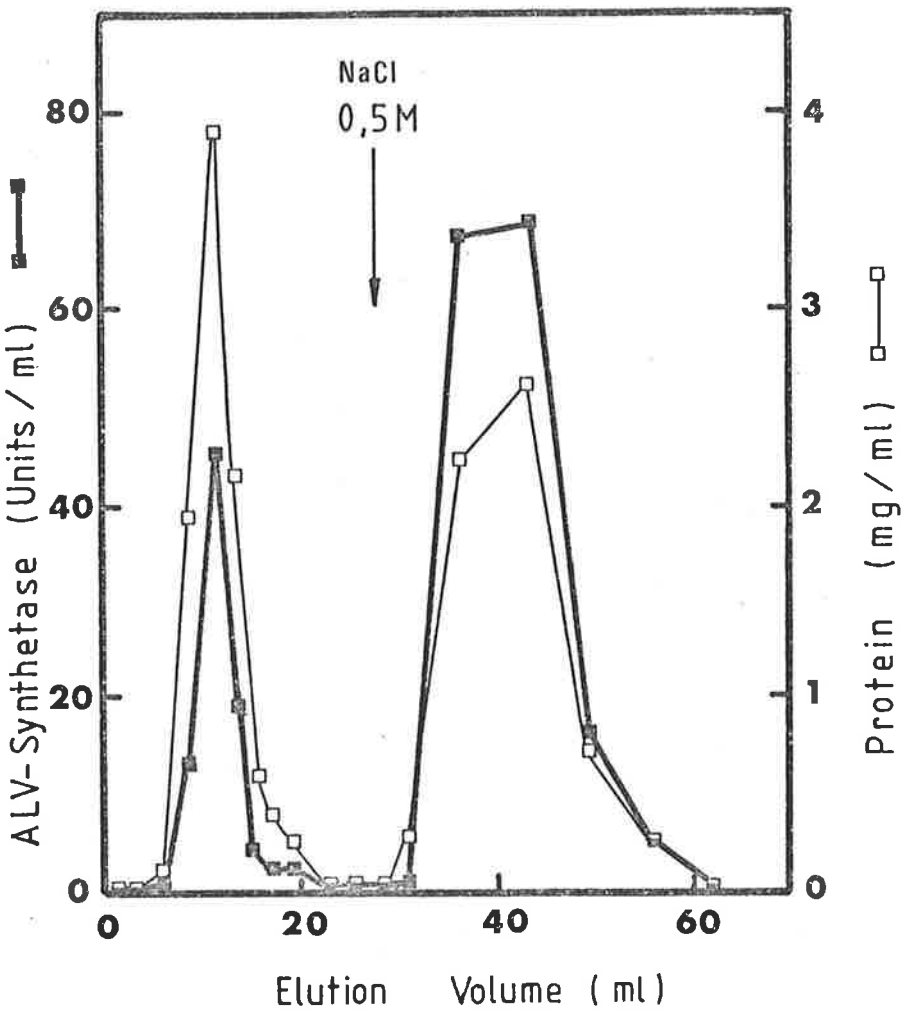

NaCl
0,5 M
80
60
40
20
0
4
3
2
1
0
0
20
40
60
ALV-Synthetase (Units / ml)
Protein (mg / ml)
Elution Volume (ml)

Fig. 5-6.

DEAE - Sephadex column.

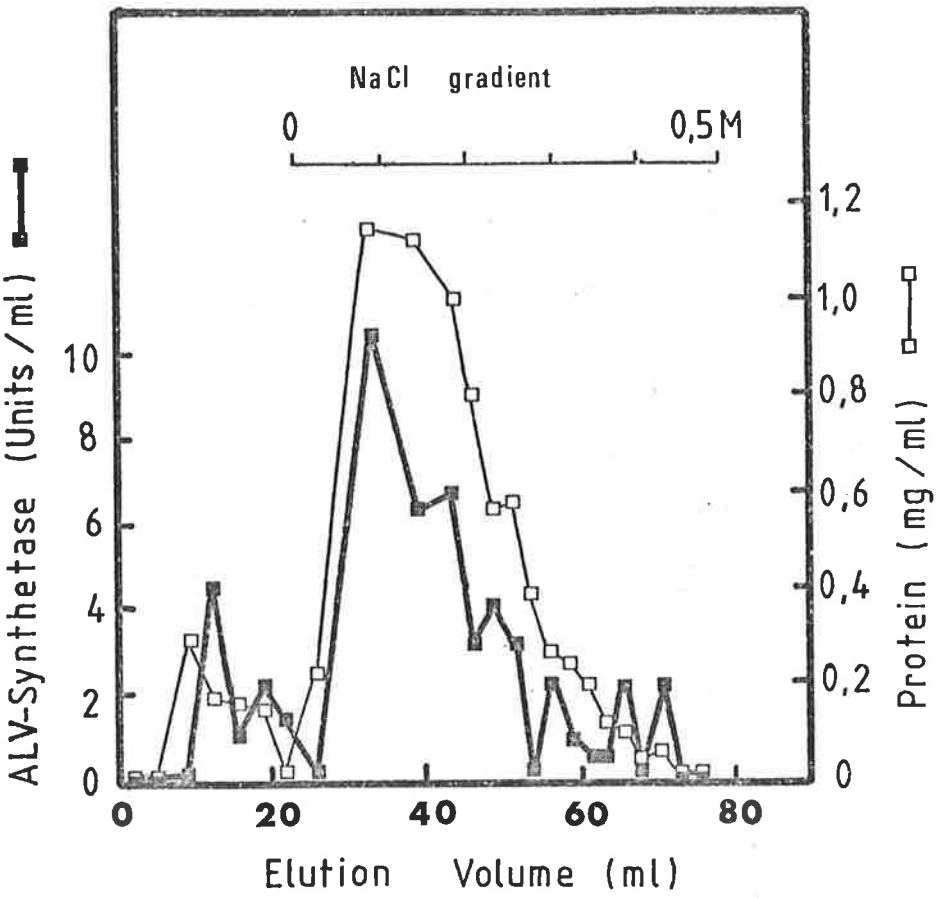
Column: 5.7 x 1.3 cm (low salt)

Buffer: TD

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 32 mg protein, specific activity 5 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	8	14
Salt eluted	92	86
	100	100


NaCl gradient
0
0,5M
ALV-Synthetase (Units/ml)
Protein (mg/ml)
0
2
4
6
8
10
0,2
0,4
0,6
0,8
1,0
1,2
0
20
40
60
80
Elution Volume (ml)

pH	% Bound Protein	% Bound ALV-s	Purification (fold)
7.6	32	70	2.2
8.0	41	69	1.7
8.5	56	64	1.1

Table 5-1.

DEAE-Sephadex chromatography of partially purified ALV-synthetase at high pH.

DEAE-Sephadex (0.2 g) was swollen in TDE buffer solutions (50 mM tris-HCl) at pH 7.6, 8.0 and 8.5. Samples of a CM-Sephadex purified ALV-synthetase preparation in pH adjusted TDE buffer were applied to columns of the DEAE-Sephadex (4 mg protein, 30 units ALV-s/mg). Elution with 0.5 M NaCl resulted in total recovery of applied ALV-synthetase and protein.

Fig. 5-7.

CM - Sephadex column.

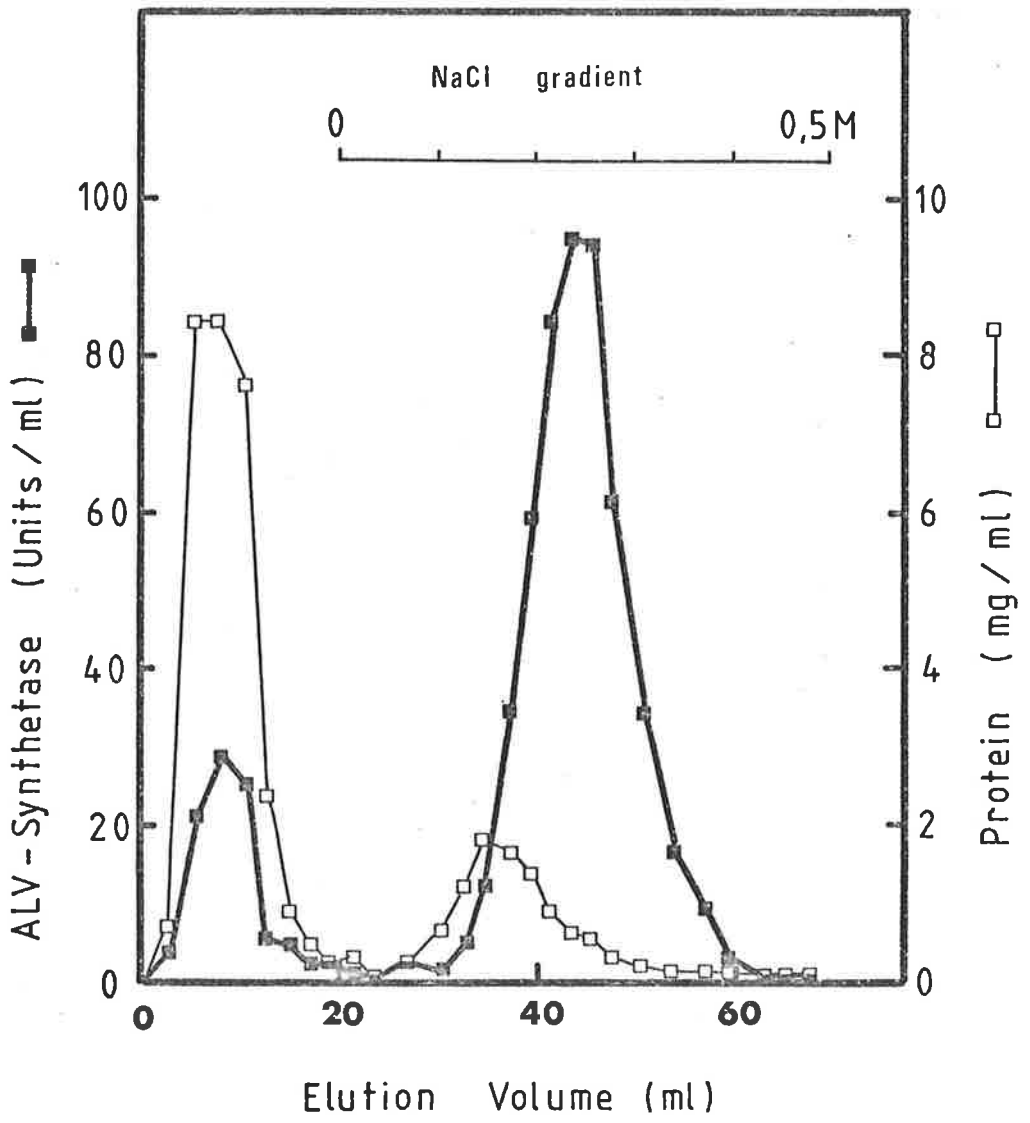
Column: 8 x 1.3 cm (low salt)

Buffer: TDP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 95 mg protein, specific activity 14 units ALV-s/mg.

Recovery (%):

Elution volume (ml)	Protein	ALV-s
0 -20	75	16
20-35	11	4
35-70	14	80
	100	100


NaCl gradient
0
0,5M
100
80
60
40
20
0
10
8
6
4
2
0
0
20
40
60
ALV - Synthetase (Units / ml)
Protein (mg / ml)
Elution Volume (ml)

Investigations using stepped increases in salt concentration as an elution procedure subsequently showed that 0.15 M NaCl elution was more effective than salt gradient elution to achieve separation of ALV-synthetase from other material bound to CM-Sephadex. Fig.5-8 shows an experiment in which 0.15 M NaCl elution gave a purification of 7.4 fold. In routine application the use of CM-Sephadex and 0.15 M NaCl reproducibly gave an approximately 7 fold purification of crude mitochondrial ALV-synthetase.

Attempts to achieve a more selective elution of ALV-synthetase by pH change (130) were not successful.

(e) Phosphocellulose

Phosphocellulose was tested to investigate its potential for purification after Cm-Sephadex chromatography, Fig.5-9 shows that phosphocellulose offers no further separation of proteins beyond that achieved by cation exchange.

(f) Hydroxyl-apatite

Whiting (10) found that guinea pig liver ALV-synthetase bound to hydroxyl-apatite and that a 4 fold purification resulted using 0.2 M Na phosphate as the eluant. Kaplan achieved a 1.3 fold purification of aggregated rat mitochondrial enzyme using hydroxyl-apatite (18).

Fig. 5-8.

CM - Sephadex column.

Column: 10 x 1.6 cm (low salt)

Buffer: TDEP

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitate. 1152 mg protein, specific activity 28 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	81.5	14
0.15 M NaCl released		
(i) 122-130 ml fractions	9.8	7
(ii) 130-151 ml fractions	7.2	53
0.5 M NaCl released	1.4	(0.2)
	99.9	74

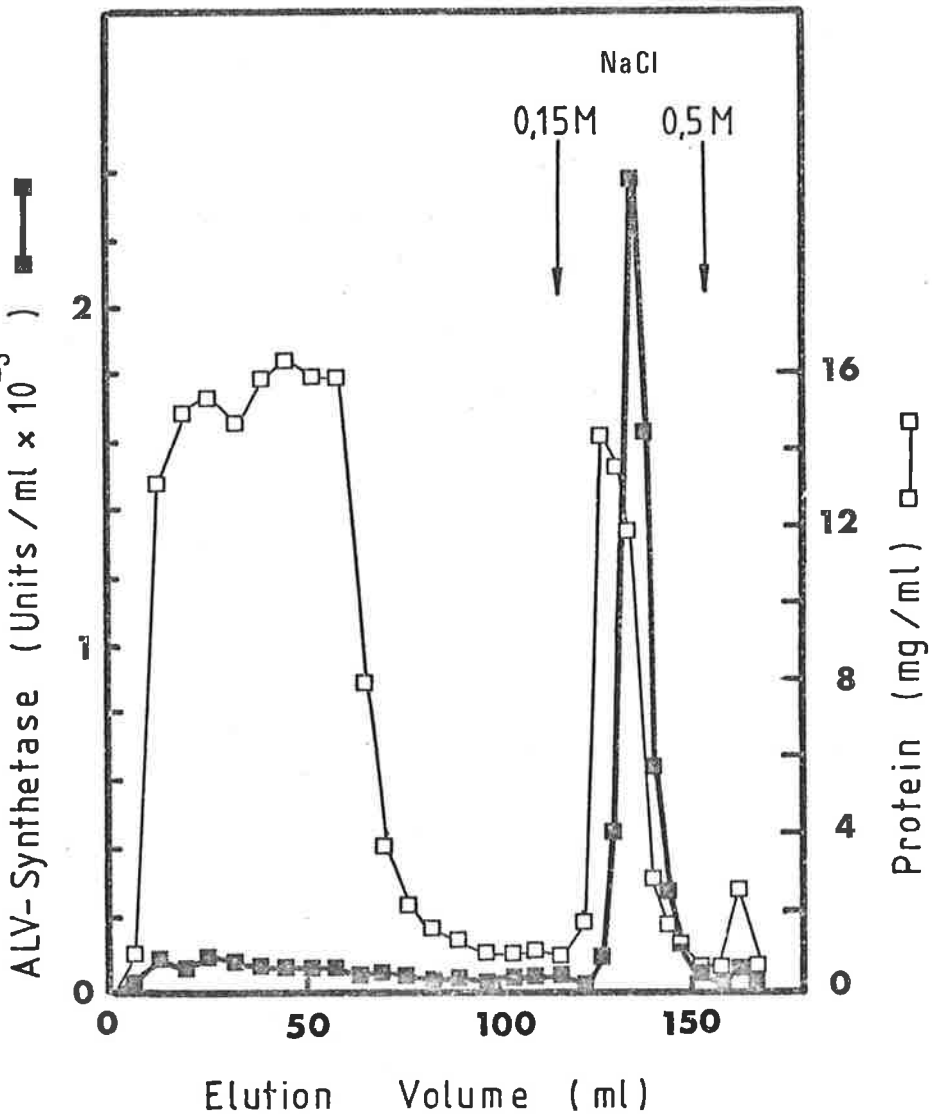

NaCl
0,15M
0,5M
ALV-Synthetase (Units/ml × 10^{-3})
Protein (mg/ml)
0
1
2
0
4
8
12
16
0
50
100
150
Elution Volume (ml)

Fig. 5-9.

Phosphocellulose column.

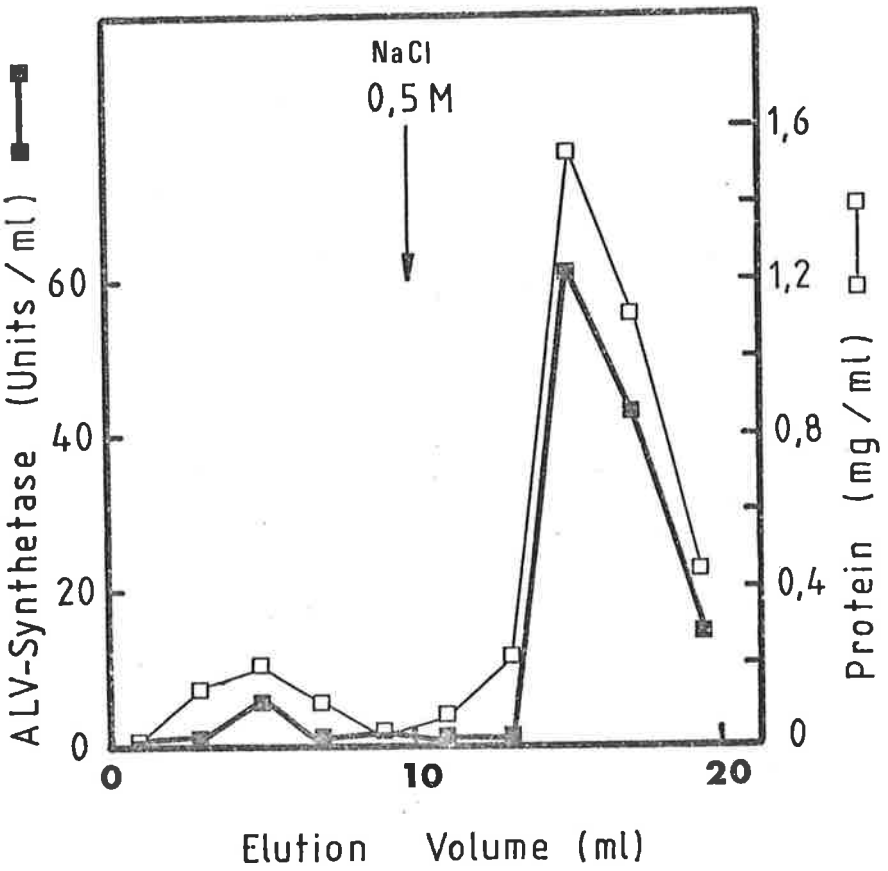
Column: 7 x 1 cm (low salt)

Buffer: TDEP

Sample applied: NaCl eluted from CM - Sephadex following solublization and 0-50% ammonium sulphate precipitation. 7.7 mg protein, specific activity 37 units ALV-s/mg.

Recovery (%):

	Protein	ALV-s
unbound fractions	7	4
0.5 M NaCl released	92	83
	99	87


NaCl
0,5 M
ALV-Synthetase (Units / ml)
Protein (mg / ml)
0
20
40
60
0
0,4
0,8
1,2
1,6
0
10
20
Elution Volume (ml)

Columns of mature hydroxyl-apatite were therefore used according to the recommendation of Levin (131). A small sample of ALV-synthetase that had undergone the preliminary purification sequence of Whiting and Elliott (sephadex G-150 filtration and 50% ammonium sulphate precipitation) was used in a preliminary experiment that gave quantitative recovery of enzyme activity and promised useful purification (Fig.5-10). However on a larger preparative scale hydroxylapatite columns became clogged when crude samples of ALV-synthetase were applied. Low recovery and low purification resulted using samples partially purified by *affinity* chromatography substrates. Hydroxyl-apatite columns were therefore abandoned as a preparative method.

5.3. REAGGREGATION OF ALV-SYNTHETASE AS A COMPLICATION OF CHROMATOGRAPHIC PURIFICATION

Turbidity was sometimes observed in solutions containing enzyme, particularly in the absence of high salt concentration. Reaggregation of the enzyme to high molecular weight following solublization and ammonium sulphate precipitation could be directly demonstrated using Sephadex G-150 chromatography run without NaCl present in the buffer. The Sephadex G-150 filtration of crude mitochondrial material gave estimates ranging to 40% reaggregation of enzyme activity which agreed with the variable observation of turbidity.

Fig. 5-10.

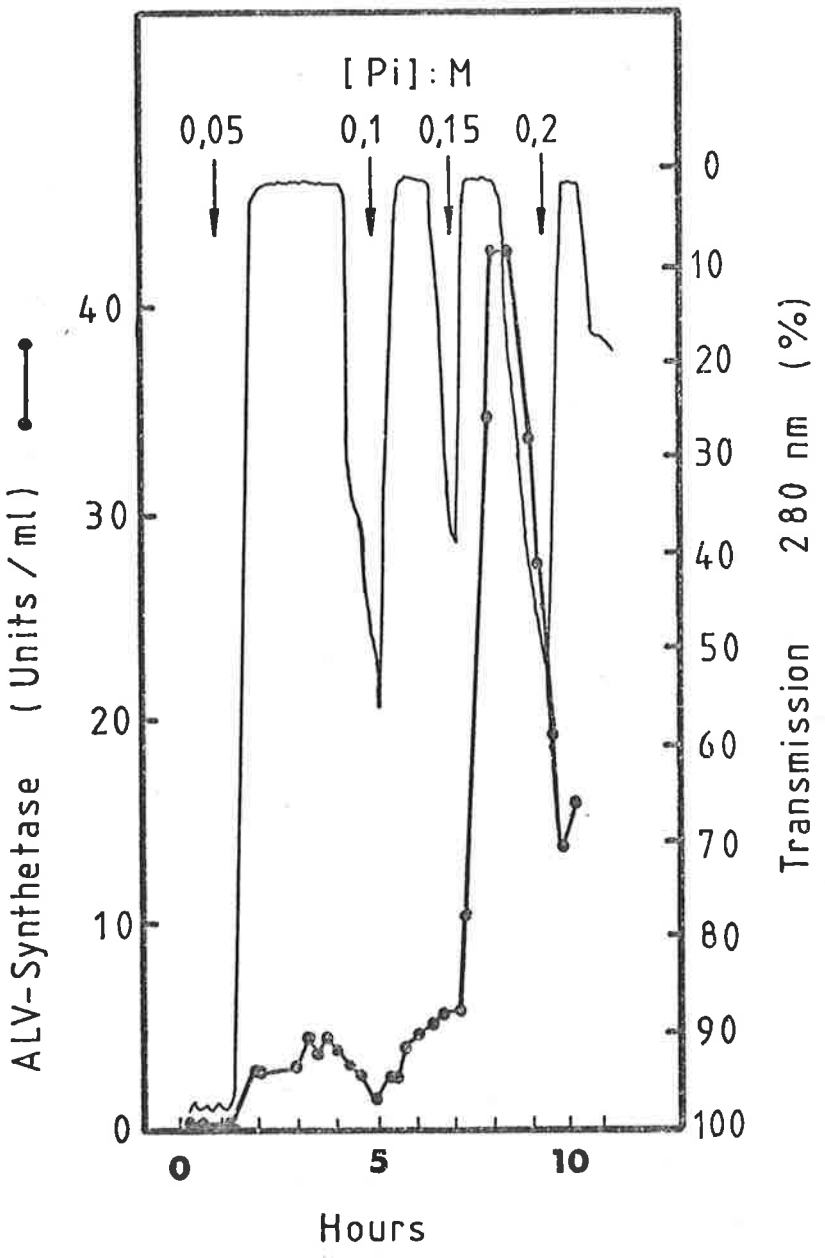
Hydroxylapatite column.

Column: 3.5 x 1 cm

Buffer: 0.05 M Na-phosphate, 0.1 mM PLP, pH 7.2.

Sample applied: Solublized mitochondrial extract, fractionated on Sephadex G-150 in 0.5 M NaCl, 0-50% ammonium sulphate precipitate equilibrated with phosphate buffer using Sephadex G-25. 3 mg protein, specific activity 39 units ALV-s/mg.

The small scale of this column meant that quantitative estimation of protein was impractical.


[Pi]:M
0,05
0,1
0,15
0,2
ALV-Synthetase (Units/ml)
Transmission 280 nm (%)
0
10
20
30
40
0
10
20
30
40
50
60
70
80
90
100
0
5
10
Hours

The ALV-synthetase solutions obtained by re-dissolving 50% ammonium sulphate precipitates in small volumes of buffer contained relatively high concentrations of this salt as a contaminant, but nevertheless turbidity indicating reaggregation was observed in these solutions. One such turbid sample was centrifuged and estimated to contain 19% of total protein and 8% of ALV-synthetase in an insoluble form (Table 5-2). Removal of contaminant ammonium sulphate by Sephadex G-25 filtration resulted in 44% of protein and 58% of ALV-synthetase becoming insoluble. A sample of this de-salted material was applied to a CM-Sephadex column and 60% of the enzyme activity failed to bind suggesting that reaggregated enzyme did not bind to the ion exchange column.

The question of whether enzyme sample that did not bind to CM-Sephadex would similarly be excluded from binding to DEAE-Sephadex was also investigated. Unbound ALV-synthetase from a CM-Sephadex column was rechromatographed on DEAE-Sephadex and only 16% of total protein and 15% of ALV-synthetase activity bound. The large proportion of sample excluded relative to crude mitochondrial material (Fig.s5-5, 5-6) indicates that non-binding to either DEAE- or CM-Sephadex is probably related to aggregation state, not to separate forms of the enzyme. Spontaneous disaggregation of the CM-Sephadex excluded material can explain the 15% of enzyme activity bound to the DEAE-Sephadex. In a further experiment a re-run of CM-Sephadex unbound material on a

$(NH_4)_2SO_4$ precipitate	Protein mg/ml	SA units/mg	Pelleted by 100,000 x g		
			Protein %	ALV-s %	SA units/mg
resuspended in TDEP buffer	71	25	19	8	11
desalted in TDEP buffer	23	17	44	58	22

Table 5-2. Aggregation to high molecular weight in a crude mitochondrial sample of ALV-synthetase

Mitochondrial extract was solublized, and precipitated by ammonium sulphate (50% saturation). The fraction resulting after resuspension of the ammonium sulphate precipitate in TDEP buffer (ammonium sulphate contaminated), and a sample of this material subjected to Sephadex G-25 filtration in TDEP buffer, were simultaneously centrifuged at 100,000 x g for 1 hour.

CM-Sephadex column produced a 25% binding of activity indicating spontaneous disaggregation. Column overload was not involved in exclusion of samples.

A purification of approximately 7 fold was generally achieved by CM-Sephadex chromatography, regardless of the binding efficiency of the sample. This results from the tendency of ALV-synthetase and total mitochondrial protein to reaggregate to a comparable degree in low salt solutions. For most experiments approximately 80% of ALV-synthetase activity bound to CM-Sephadex.

CM-Sephadex columns cannot be run under the high salt conditions established for solublizing ALV-synthetase. Therefore the non-ionic detergent Triton X-100 was tested as a means of increasing enzyme solubility and binding to CM-Sephadex, but proved to be ineffective.

5.4. SUMMARY

CM-Sephadex provided a useful purification of approximately 7 fold for crude mitochondrial ALV-synthetase. Other column procedures investigated were not useful as an initial step in purification and did not offer significant advantages as purification procedures after cation exchange purification.

Reaggregation of previously solublized ALV-synthetase under low salt conditions was shown to occur and was considered to explain the observed variation in binding of ALV-synthetase to chromatographic materials.

CHAPTER 6

ISOELECTRIC FOCUSING OF ALV-SYNTHETASE

6.1. INTRODUCTION

Isoelectric focusing is an electrophoretic method of separating amphoteric macromolecules according to their isoelectric points (pI) in stable pH gradients (for reviews see 133-136). Commercially available *ampholines* are mixtures of a large number of different aliphatic polyamino-polycarboxylic acids and they form a pH gradient when an electric potential is applied. Amphoteric molecules in the gradient, including proteins, migrate until they reach a position of electrical neutrality, and ideally remain stable at the position where pH equals pI. Molecules that differ by as little as 0.01 units in isoelectric point can be resolved by isoelectric focusing (136).

The technique results in concentration of protein as well as separation and has been investigated here both as a preparative and an analytical method. The pI of hepatic mammalian ALV-synthetase has not been reported, although a pI of 7.0 has been found for chicken enzyme (23), and a pI of 5.9 for rabbit reticulocyte enzyme (20).

6.2. RESULTS

(a) Isoelectric focusing on large scale sucrose density gradients

Preparative isoelectric focusing on sucrose density columns uses density gradients to stabilize the pH gradient and the focused protein zones. Experiments performed with a column volume of 440 ml, using commercially available equipment, requires 2 - 4 days to reach equilibrium. The 400 ml apparatus manufactured by the

LKB company also suffers from considerable diffusion during elution of the column, in the absence of the electrical field, and consequent loss of achieved resolution (136). Protein may also precipitate at its pI and fall through a sucrose gradient contaminating other fractions.

During initial investigations of ALV-synthetase purification procedures a preparative isoelectric focusing step using relatively crude enzyme was considered. After solublization and Sephadex G-150 fractionation by the procedure of Whiting and Elliott (21), samples were isoelectrically focused in an LKB 8100-20 (440 ml) column using pH 3-10 ampholine (1% w/v).

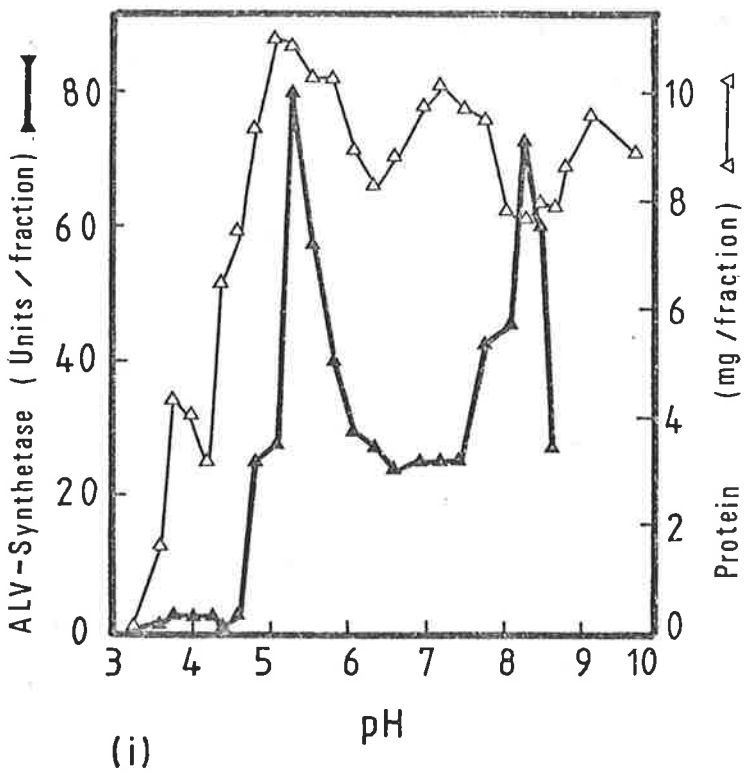
The first experiment (Fig.6-1 (i)) showed two peaks of ALV-synthetase activity and returned approximately 5% of the applied activity after a 48 hour focusing period. A repeat of this procedure (not shown) produced two peaks of ALV-synthetase at approximately the previously observed pI values and a recovery of 6% of applied ALV-synthetase activity. The low recoveries were probably due to the electrophoretic desalting of the ALV-synthetase and the resulting loss of PLP cofactor. Enzyme deprived of PLP, in TDE buffer, had a measured half life of 9.6 hours at $4^{o}C$, and over 48 hours only 1.5% of the initial activity would be expected to remain. Ampholines have a stabilizing effect on proteins however (133), so that greater recoveries than otherwise expected are possible. The losses of enzyme observed here clearly makes large scale preparative focusing impractical, although a compromise experiment was tried to avoid the unacceptable losses.

Fig. 6-1. Isoelectric focusing on LKB-430ml Apparatus

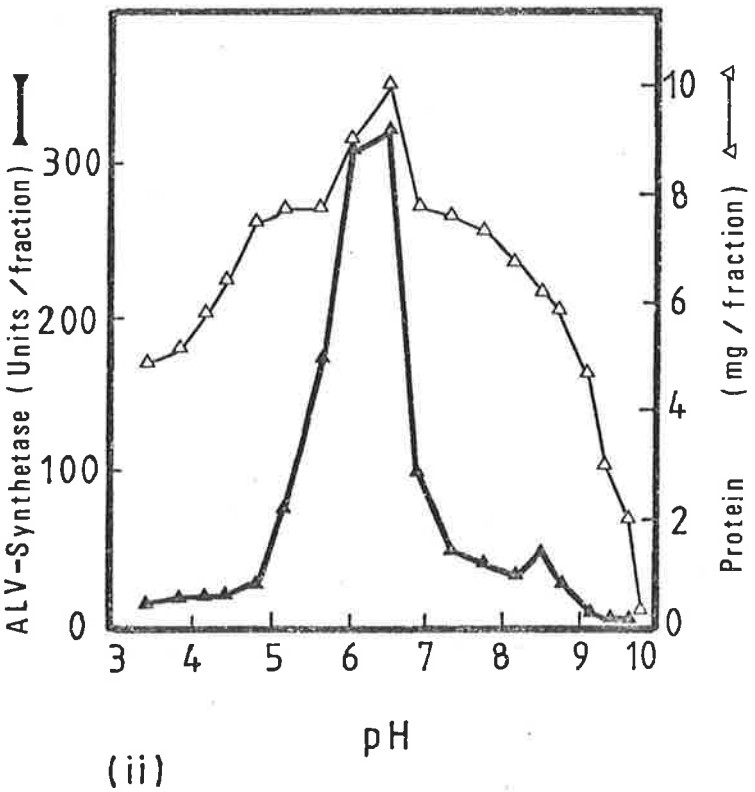
(i) Full scale experiment, run 48 hours.
Sample applied: solublized mitochondrial extract, Sephadex G-150 fractionated in TDEPN buffer, 0-50% ammonium sulphate precipitate: 200 mg protein, specific activity 25 units/mg.
Recovery: 5% of applied ALV-synthetase activity.

(ii) Half scale experiment, run 8 hours.
Sample applied: sequence as above (different preparation) : 115 mg protein, specific activity 48 units/mg.
Recovery: 24% of applied ALV-synthetase activity.

Protein determination: fraction samples were treated with 4% TCA and the precipitates analysed by the Lowry procedure. This precipitation is necessitated by carrier ampholine interference with direct determinations using the Lowry method.


ALV-Synthetase (Units / fraction)
Protein (mg / fraction)
0
20
40
60
80
0
2
4
6
8
10
3
4
5
6
7
8
9
10
pH

(i)


ALV-Synthetase (Units / fraction)
Protein (mg / fraction)
0
100
200
300
0
2
4
6
8
10
3
4
5
6
7
8
9
10
pH

(ii)

To reduce the focusing time and to obtain more economical use of ampholine a focusing gradient of only half the volume (and therefore length) was formed, and additional light electrolyte added to complete the column. After focusing for 8 hours a single peak of ALV-synthetase activity resulted (Fig.6-1 (ii)) and 24% of initial activity was recovered. The distribution of ALV-synthetase and protein in the pH gradient indicates that equilibrium was not achieved in the 8 hour period and therefore the half scale approach was neither preparative nor usefully analytical.

(b) Isoelectric focusing on small scale sucrose density gradients

The construction and use of the small scale isoelectric focusing apparatus is described in Chapter 2. Fig.6-2 shows two experiments using enzyme from the same CM-Sephadex purification batch and using wide range pH 3-10 ampholine. Well focused bands of protein were visible before fractionation so the diffuse and variable location of ALV-synthetase activity was disappointing, although good recovery was achieved. The wide range gradient did however imply that ALV-synthetase could be localized in the pH 5-9 zone of the gradient and narrow range ampholines were used for subsequent experiments.

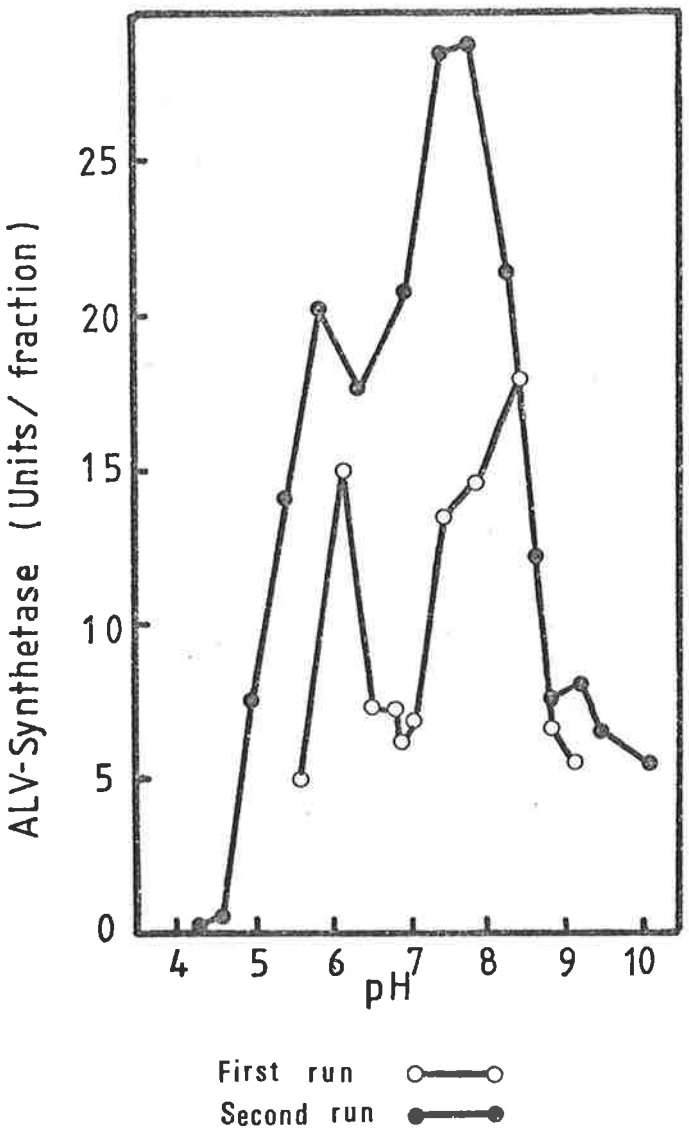
The use of Triton X-100 in isoelectric focusing has been recommended by Vesterberg (140) when conducting isoelectric focusing of aggregate form enzymes. It was therefore hoped that use of the detergent would give a reproducible peak of focused enzyme representing the free soluble form. A possible complication however is that

Fig. 6-2. Small scale isoelectric focusing using wide range pH 3-10 carrier ampholine

Sample applied was partially purified by CM-Sephadex chromatography to 159 units ALV-s/mg.

(i)
Sample: 1 mg protein
ALV-s recovery: 72%
Anode electrolyte: 0.2% (w/v) H_3PO_4
Focusing time: 4.5 hours

(ii)
Sample: 1.5 mg protein + 0.5 mg RBC lysate
ALV-s recovery: 92%
Anode electrolyte: 1% (w/v) H_2SO_4
Focusing time: 5 hours


ALV-Synthetase (Units/ fraction)
25
20
15
10
5
0
4
5
6
7
8
9
10
pH
First run
Second run

detergent molecules themselves may attach to ALV-synthetase molecules and cause focusing anomalies.

Fig.6-3 shows that 0.5% Triton X-100 present in the focusing gradient did not give any reproducible peak(s) of ALV-synthetase, even between experiments using samples of the same preparation, although a major peak always appeared in the range pH 7-7.8. Triton X-100 was not used in further sucrose gradient focusing experiments.

Enzyme samples of higher specific activity than used previously for focusing experiments gave the distribution of activity shown in Fig.6-4. The sample of activity 355 units/mg was purified by prior CM-Sephadex, Sephadex G-100 filtration and PEG precipitation (see Chapter 8) and was shown to give more discretely resolved protein bands by polyacrylamide gel electrophoresis than sample purifed only by CM-Sephadex fractionation. The broad distribution of enzyme throughout the gradient was therefore disappointing. The sample of 1660 units/mg was obtained by preparative electrophoresis (Cahpter 7).

(c) Isoelectric focusing in polyacrylamide gel

Polyacrylamide gels set in glass tubes were prepared by the method of Wrigley (141) and samples of enzyme applied at the gel head as recommended. Protein denatured at the top of the gel and no ALV-synthetase activity was recovered. To overcome this a half tube length of polyacrylamide gel was set according to Wrigley (141), sample applied in a small volume of 10% sucrose,

Fig. 6-3. Small scale isoelectric focusing using 0.5% Triton X-100 in the focusing gradient

All samples of ALV-synthetase were partially purified by CM-Sephadex chromatography.

(i) Sample applied: 1.9 mg protein, 164 units ALV-s/mg with 1 mg rat RBC lysate protein
Focusing time: 6.5 hours
Anode: 0.2% H_3PO_4
Ampholine: pH 5 - 8

(ii) Sample applied: same preparation as for (i), stored 5 days at 0°C: No RBC lysate added
Focusing time: 6.5 hours
Anode: 0.2% H_3PO_4
Ampholine: equal mixture of pH 5 - 8 and 7 - 9

(iii) Sample applied: 3 mg protein, 94 units ALV-s/mg with 0.5 mg RBC lysate
Focusing time: 7 hours
Anode: 1% H_2SO_4
Ampholine: equal mixture of pH 5 -8 and 7 - 9

(iv) Repeat of (iii) using sample stored 12 hours at 0°C between focusing experiments
Focusing time: 12 hours

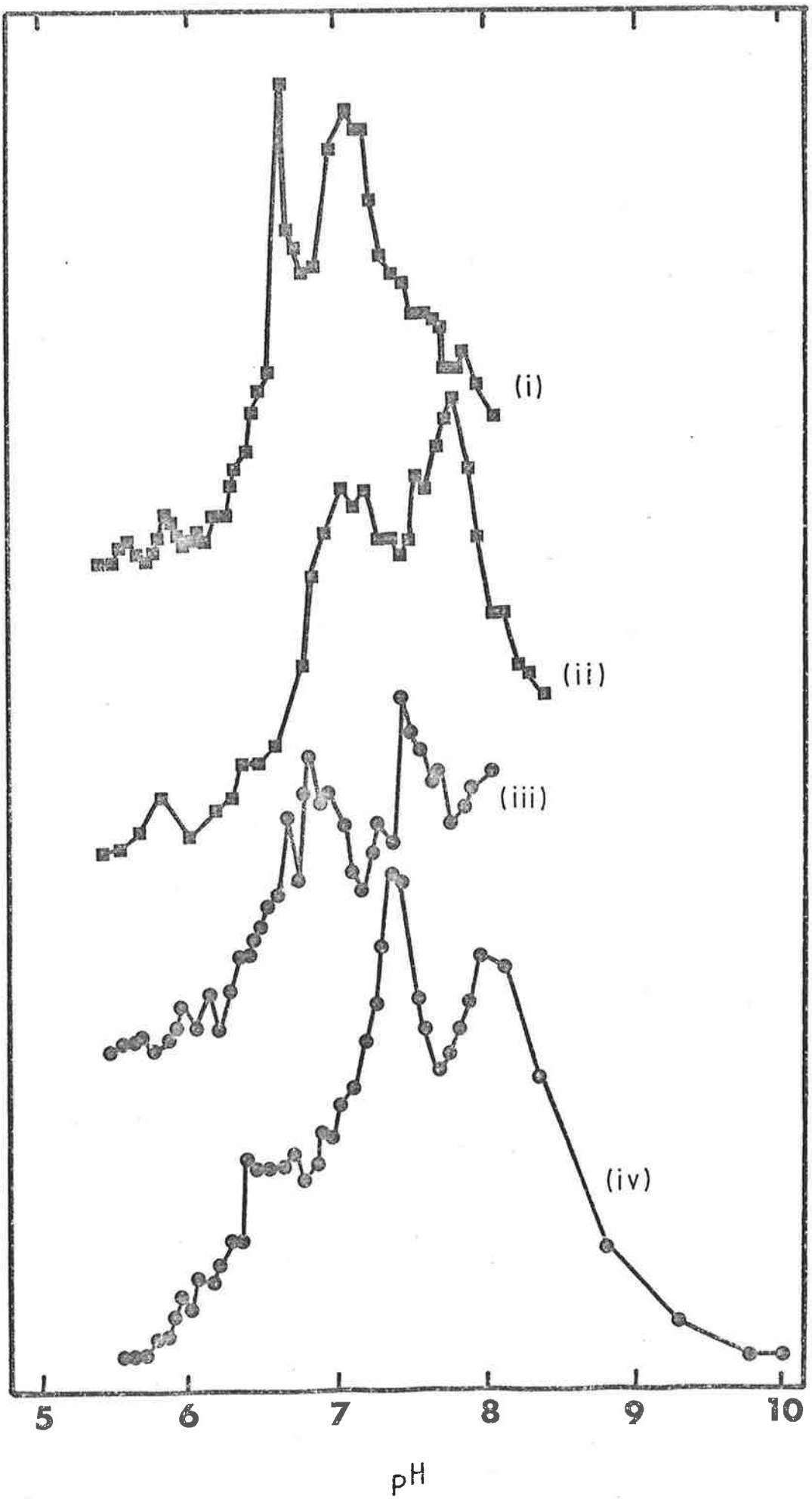
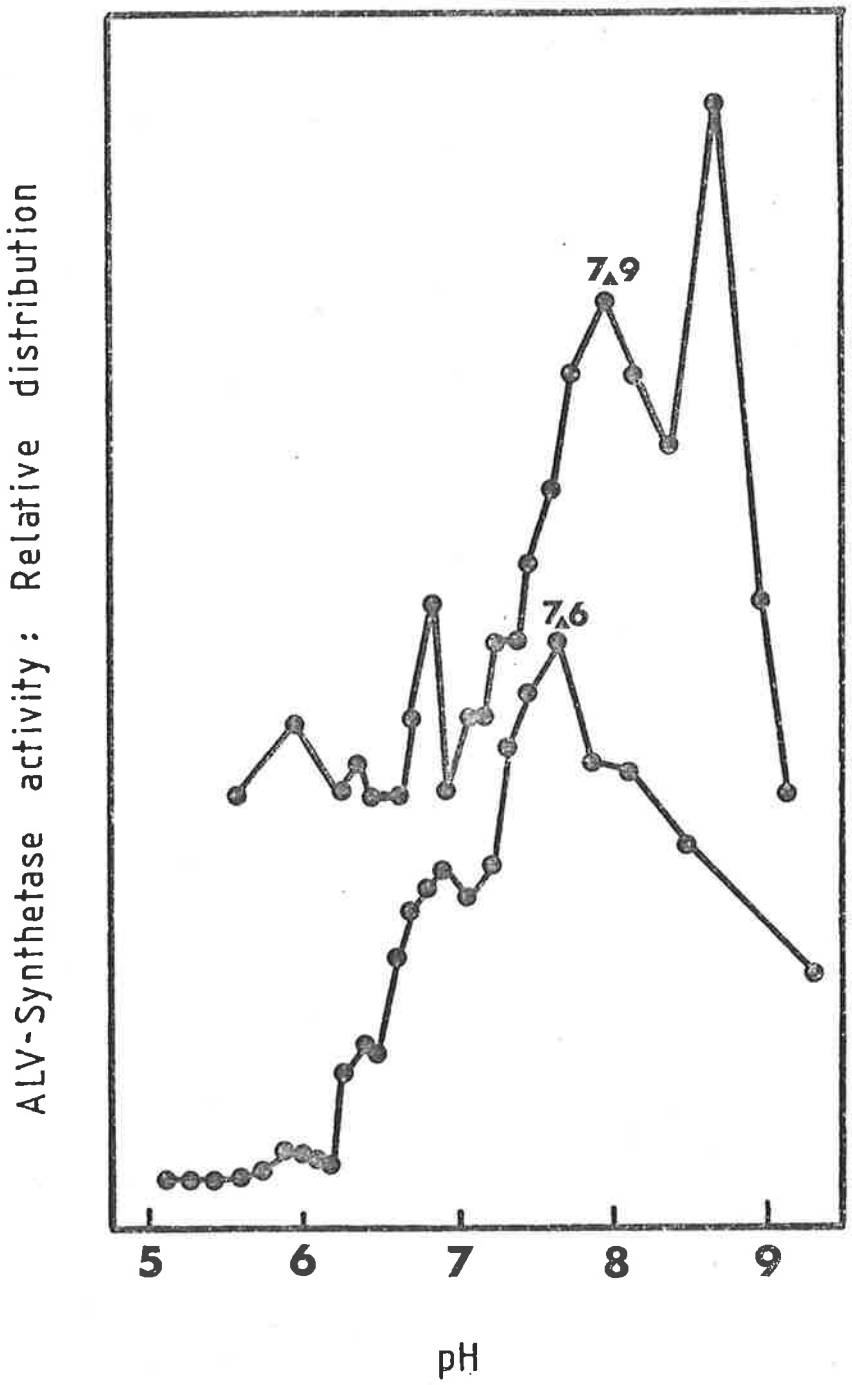

ALV-Synthetase activity: Relative distribution
(i)
(ii)
(iii)
(iv)
5
6
7
8
9
10
pH

Fig. 6-4. Small scale isoelectric focusing of high specific activity ALV-synthetase samples

Anode electrolyte was 1% (w/v) H_2SO_4

(i) Sample applied: 0.7 mg protein, 355 units ALV-s/mg
Focusing time: 6 hours
Ampholine: pH 5 -8

(ii) Sample applied: 23 µg protein, 1660 units ALV-s/mg
Focusing time: 5.75 hours
Ampholine: equal mixture of pH 6 - 8 and 7 - 9


ALV-Synthetase activity : Relative distribution
7.9
7.6
5
6
7
8
9
pH

then more gel set above the sample. Centre loaded gels gave very clear bands of focused haemoglobin (RBC lysate) and worked well for focusing the activity of a bacterial protease assayed for proteolysis. ALV-synthetase activity however was recovered as a broad zone between pH 6 - 8. The small amount of material resulting from sectioned tube gels was a severe disadvantage of this format, particularly for establishing the pH of sections.

Isoelectric focusing on slab gels however can handle amounts of protein comparable to gel electrophoresis, and the development of protein staining techniques that avoid interference from ampholine artifacts, makes focusing in slab gels an attractive technique (140, 142). The resolution can be better than obtained with sucrose density gradients since protein is held in a fixed position by the gel matrix during recovery procedures. Also the focusing of a high concentration of protein into a single band is not as undesirable as in sucrose density gradients since precipitated protein remains localized by the gel.

A slab gel based on Vesterberg (140), but containing 1 mM DTE and 0.5% Triton X-100, gave well resolved bands of stained protein both for an RBC lysate and a CM-Sephadex purified sample of mitochondrial ALV-synthetase. Coomasie-blue dye stained ten bands of protein from a CM-Sephadex purified sample resolved in a pH 5-8 gradient, and most protein was focused towards the high pH end. ALV-synthetase was not assayed in this preliminary exper-

iment and no time was left for further attempts using slab gel.

6.3. DISCUSSION

A double peak can be produced as an artifact due to arrival of protein from the near and far side of the pI if inadequate time is allowed for isoelectric focusing. After reaching optimal focus a peak may also be stable or undergo a degree of broadening depending on the protein itself, e.g. a focused band of β lactoglobulin A will destabilize, but β lactoglobulin B remains stable after focusing in a sucrose density gradient (137). Inadequate focusing time and destabilization of focused enzyme do not seem to be significant factors in the results obtained here using small scale sucrose density gradients, since samples of one preparation run for 7 hours or 12 hours at different times did not show important differences in the resolution quality of the peaks obtained (Fig.6-3 (iii, iv)).

The position a protein comes to occupy in a gradient remains constant relative to other markers so that extrapolation from marker proteins of known pI is a valid means of determining pI values (137, 138). In some experiments on small scale sucrose density gradients rat haemoglobin was included and was found to have a number of variants that reproducibly focused in the pH range 7-7.1. This addition therefore indicated that the pH measurement of fractions was being made reproducibly and provided a datum point from which to compare

the distribution of ALV-synthetase.

In the data presented here multiple peaks of ALV-synthetase were commonly observed but the pIs obtained were so variable that no definite pI values can be reported. Nor is it possible to conclude how many variants of ALV-synthetase may be present in rat liver mitochondria, although the appearance of 3 isoelectric variants in an experiment using enzyme of activity 1660 units/mg may be significant (Fig.6-4 (ii)). This high specific activity sample was purified by prior electrophoresis and it was established (Chapter 7) that no further bands of activity appear when electrophoretically separated samples were subjected to re-electrophoresis; this suggests a degree of homogeneity greater than other samples studied.

Aggregation of ALV-synthetase molecules would be favoured by the salt free conditions during isoelectric focusing and its occurrence might shift the observed pIs away from the true pIs). Aggregation might also cause the artifactual appearance of multiple forms of the enzyme and poor resolution of bands if the hypothesized aggregate forms had a widely variable composition. The resolution of ALV-synthetase was never comparable to the high resolution of visibly focused protein bands.

During focusing the gradient does not remain absolutely stable, but a small amount of *creep* (137) occurs. Although theoretically the ampholytes should remain trapped by the charge conditions at the electrodes, in practice the pH gradient slowly extends itself,

particularly towards the alkaline end of the gradient. In consequence when recoveries are plotted against pH, as they have been here for ease of comparison, a distorted distribution is displayed relative to the linear separation achieved. This is particularly noticeable at the high pH end when narrow range ampholine was used.

6.4. SUMMARY

Three isoelectric variants of ALV-synthetase may be present in rat liver mitochondria, but no firm conclusion is warranted and no reproducible value(s) of pI were obtained. The variable location of ALV-synthetase in focused pH gradients is presumed to result from aggregation of the enzyme with contaminant molecules.

CHAPTER 7

ATTEMPTS TO EXTEND THE PURIFICATION OF ALV-SYNTHETASE

7.1. ELECTROPHORESIS.

(a) Introduction

Kaplan (18) reported the failure of attempted electrophoretic purification of ALV-synthetase, and no other reports of electrophoresis under non-denaturing conditions have been published.

An electrophoretic system giving acceptable recovery of ALV-synthetase activity was established here although both the resolution of activity from total protein and recoveries of activity were variable.

(b) Electrophoresis on cellulose acetate strips

Electrophoresis on Sepraphore cellulose acetate strips (2.5 x 17 cm, Gelman Instrument Co.) was performed according to the directions of John and Feinberg (143) with 0.1 mM PLP included in electrolyte solutions (pH 8.0). Good recoveries of ALV-synthetase activity were obtained after electrophoresis of samples partially purified by CM-Sephadex ion exchange, but activity was broadly spread across the strips and was co-extensive with the stained pattern of total protein which showed no resolution into bands.

(c) Electrophoresis of ALV-synthetase in polyacrylamide gel

The method finally adopted for electrophoretic investigation of ALV-synthetase samples used 4% polyacrylamide gels set at pH 8.8 and has been described in Chapter 2.

No enzyme activity was recovered in prelimary experiments using the established electrophoretic methods of Ornstein & Davis (144, 145), but activity was recovered by using PLP in the anode electrolyte as described by Gelehrter *et al.* (146). The inclusion of PLP in the electrolyte gave a continuous supply of this cofactor to gels during electrophoresis and overcame the problem of electrophoretic removal of PLP from ALV-synthetase. The use of stacking gels (144, 145) did not improve the resolution of stained protein bands and was discontinued.

Gels of 4% polyacrylamide were found to be practical only when persulphate was used as a polymerization initiator. This low gel density was used to facilitate diffusion of protein from the gel matrix during recovery procedures.

DTE was essential for solublization of ALV-synthetase from the extracted mitochondrial aggregate (10) and was included at 4.5 mM in the gel mixture generally used here. The inclusion of DTE at 5 mM in a preliminary experiment using CM-Sephadex purified sample caused ALV-synthetase mobility to be reduced by 15% and altered the pattern of protein bands. Further experiments showed a less certain effect of DTE, nevertheless DTE was routinely included in gels since it was possible that DTE may have decreased the tendency for reaggregation to occur in some samples during electrophoresis.

Various methods of recovery of ALV-synthetase from tube gels showed that near quantitative recovery was possible

after freeze-thawing of 1 mm sections. This result formed the basis for the slab gel recovery procedure described in Chapter 2.

Electrophoresis was investigated here to provide a preparative step, but electrophoresis on slab gels did not become a reliable step because activity was oten lost and recovery ranged to only 60%. Investigations showed that denaturation at high pH, or loss of a physiological cofactor other than PLP, was unlikely to explain loss of activity during electrophoresis.

Variable recoveries of activity were obtained from tube gels using the pH 7.5 system of Williams and Riesfield (147) used with PLP in the electrolytes. This system runs at pH 8.0 during electrophoresis (147), and it was directly demonstrated that ALV-synthetase is stable over 3 hours, (the time allowed for tube gel electrophoresis) at pH values up to 8.6 in a 25 mM tris-HCl, 0.1 mM PLP buffer. It is therefore unlikely that pH is a major factor in the loss of activity during electrophoresis. The much lower mobility of ALV-synthetase and general protein in the pH 7.5 system was a disadvantage.

To replace any lost cofactors a mitochondrial extract from a non-porphyric rat was added to fractions of electrophoretically separated enzyme. The extract used did not improve the recovery of enzyme or demonstrate any otherwise inactive enzyme.

Although isoelectric focusing suggested that a component of ALV-synthetase samples may have acidic properties, no activity migrated into gels set at pH 7.5 or 8.8 when samples were loaded at the cathode (i.e. reverse

polarity).

The basis for the variability of recoveries was therefore not clear. Another problem was the variable tendency of ALV-synthetase activity to occupy a much larger zone of electrophoresed gels than expected from the profile of resolved total protein bands. This was a similar observation to that made for isoelectric focusing experiments and aggregation of proteins was considered to be the likely cause of poor resolution. Attempts to improve the resolution of ALV-synthetase by loading samples in Triton X-100 and inclusion of Triton X-100 in gels did not give any improvement.

It became apparent that CM-Sephadex purified material was not a suitable source of enzyme to achieve routinely acceptable resolution of ALV-synthetase by preparative electrophoresis. Electrophoresis of a sample previously cation exchanged then eluted from a cellulose affinity column by 10 mM PLP (Table 4-1, section 2(ii)) showed good resolution of protein and ALV-synthetase and encouraged the use of electrophoresis after more extensive purification procedures. Activity recovered from this experiment was not clearly linked to any band of protein and must have been a minor fraction of the protein present (Fig.7-1).

(d) Preparative electrophoresis

An early experiment with preparative electrophoresis using CM-Sephadex purified sample gave an 8 fold purification. The result was not reproducible and

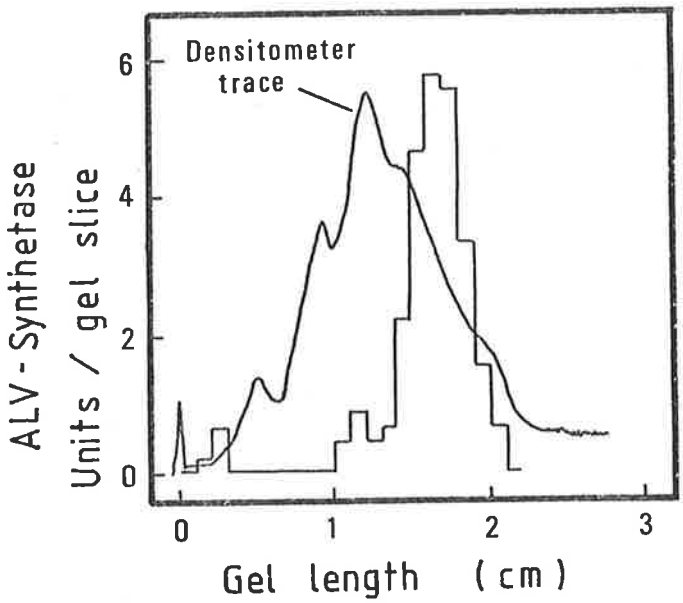


Fig. 7.1. Electrophoresis of ALV-synthetase purified by CM-Sephadex and affinity chromatography

Sample purification described in Table 4-1 (section 2 ii). Approximately 0.2 mg protein applied and run 2 hours on tube gels. One gel was stained and another sectioned for ALV-synthetase assay. All enzyme activity applied was recovered.

activity was often lost in subsequent experiments. The pattern of bands obtained also changed as observations continued, possibly due to a genetically determined drift in the constitution of the mitochondrial proteins (cf. discussion of genetic variability of ALV-synthetase inducibility, Chapter 3). The pattern commonly seen as experiments continued consisted of two leading bands free of ALV-synthetase activity, followed by a largely amorphous zone of protein which included ALV-synthetase.

Material partially purified by preparative electrophoresis was re-electrophoresed on tube gels. This established that ALV-synthetase could not be resolved from contaminant material by repeated electrophoresis either in the presence or absence of DTE although previous work had suggested that DTE selectively affected the mobility of proteins. Using higher gel density (7% compared to 4%) to increase the effect of molecular size sieving also failed to give improved resolution of ALV-synthetase from contaminant protein during re-electrophoresis.

By comparison with the electrophoretic profiles obtained from samples purified by CM-Sephadex alone, the additional step of either Sephadex G-50 or G-100 filtration in TDEPN buffer before electrophoresis gave an improved resolution of stained protein bands. The filtration procedure is discussed later in this chapter. Variable electrophoretic purification up to 8 fold was achieved using ion exchanged and Sephadex chromatographed samples, but ALV-synthetase continued to run as a broad

band similar in extent to a diffuse zone of stain between several resolved bands. This indicated that gel filtration to remove material of molecular weight different from soluble ALV-synthetase had not overcome the tendency for aggregation to occur during electrophoresis.

(e) Summary

Electrophoresis of ALV-synthetase gave purifications ranging to 8 fold using samples partially purified by CM-Sephadex chromatography.

The electrophoretic system established used 4% polyacrylamide gel set at pH 8.8 and utilized PLP in electrolyte solutions to provide this cofactor during electrophoresis.

7.2. CENTRIFUGAL REMOVAL OF AGGREGATED PROTEIN TO OBTAIN SOLUBLE ALV-SYNTHETASE.

Enzyme preparations were prone to develop turbidity at all stages of purification and this was attributed to aggregation of protein. Centrifugation was therefore used to reduce the amount of aggregated protein in samples.

During the course of the purification sequence described in Table 7-1 the CM-Sephadex purified material was stored 3 days at $0^{o}C$ and a precipitate appeared which was removed by centrifugation at 105,000 x g for 30 minutes. Table 7-2 (i) shows that 88% of the ALV-synthetase and 96% of total protein was recovered in the supernatant. A sample of this supernatant was stored at $-15^{o}C$ for 4 weeks and showed precipitation upon thawing; the sample was made 0.8 M with respect to NaCl to assist in dissociation of the protein aggregate, but after 16 hours at $0^{o}C$ tur-

FRACTION	Total activity Units x 10^{-3}	Protein mg	Sp. activity Units/mg	Yield %
homogenate	117	30,000	3.9	100
mitochondria	84	7,700	11.0	72
mitochondrial extract	58	2,860	20.2	50
0-50% ammonium sulphate ppt.	49	2,210	22.2	42
Sephadex G-25 desalted	53	2.250	23.4	45
CM-Sephadex	33	164	201	28
1st centrifugal clarification	10.8	115	94	*
2nd centrifugal clarification	2.4	24	99	*
Sephadex G-100	1.2	11.7	103	*

* not all material used for these procedures

Table 7-1.

ALV-synthetase purification sequence

Seventeen rats were made porphyric by the procedure given in Chapter 2. 156 g. wet weight of liver was isolated and 12.4 g. of freeze dried mitochondria recovered. Details of the procedures summarized here are given in the text.

(i) First centrifugal clarification (105,000 x g, 30 min.)

	Protein (mg)	ALV-synthetase (units)	Sp. Act. (units/mg)
Initial sample	120	12,260	102
Supernatant	115	10,800	94
Pellet	5.5	1,460	266

(ii) Second centrifugal clarification (3,000 x g, 10 min.)

	Protein (mg)	ALV-synthetase (units)	Sp. Act. (units/mg)
Initial sample	29.6	2,708	91
Supernatant	24.2	2,407	99
Pellet	5.4	301	56

Table 7-2.

Centrifugation of samples showing gross protein precipitation. Details are provided in the text.

bidity persisted. Table 7-2 (ii) shows that 89% of the total protein had remained soluble after centrifugation so that aggregation in this case had slightly increased the specific activity of the preparation.

Other observations similarly showed that aggregation was a persistent problem, regardless of the prior purification procedures carried out. A possible cause is that hydrophobic sites on the mitochondrial proteins (ALV-synthetase and contaminant proteins) gave rise to non-specific aggregation by Van der Waals association. This is supported by the observation that although the pellets of aggregated protein could be homogeneously resuspended after centrifugation, a true solution of protein was not obtained even in solutions containing 0.8 M NaCl.

7.3. POLYETHYLENE GLYCOL (PHASE PARTITION) PRECIPITATION

Polyethylene glycol (PEG) shows the interesting properties of selectively removing lipoproteins at low concentrations (152), and simultaneously desalting and concentrating precipitated protein. Initial results showed that little precipitation of ALV-synthetase occurred at 5% (w/v) PEG (20,000 molecular weight, ref. 153), the range in which lipoproteins were reported to precipitate, and 65% of activity precipitated in the range 5-15% (w/v) PEG. All activity was insoluble at 20% (w/v) PEG.

Table 7-3 shows a summary of a purification sequence in which 0-5% (w/v) PEG precipitation was performed after ammonium sulphate fractionation of mitochondrial extract. The subsequent 5-20% (w/v) PEG precipitate contained 80%

FRACTION	Total activity Units x 10^{-3}	Protein mg	Sp. activity Units/mg	Yield %
homogenate	165.6	36,000	4.6	100
mitochondria	105.7	8,800	12.0	64
mitochondrial extract	86.6	3,880	22.3	52
0-50% ammonium sulphate ppt.	79.9	2,700	29.6	48
0-5% w/v PEG precipitate	2.8	58	48.5	-
5-20% w/v PEG precipitate	63.6	1,545	41	38
CM-Sephadex	40.6	138	294	25
Sephadex G-100	18.9	50	378	11

Table 7-3.

ALV-synthetase purification sequence including phase partitioning during preliminary purification

Fifteen rats were made porphyric by the procedure given in Chapter 2. 155g wet weight of liver was isolated and 12.6g of freeze dried mitochondria recovered

of the starting ALV-synthetase activity with a purification of 1.4 fold. CM-Sephadex chromatography then gave a further 7.1 fold purification; a fold of purification equivalent to that expected without prior PEG treatment. Although the preliminary 0-5% (w/v) PEG fractionation did not enhance the purification achieved the value of this fractionation was supported by the observation that a resuspended sample of the 0.5% (w/v) PEG precipitate developed turbidity upon standing 3 days at $0^{o}C$, while a similarly stored sample of 5-20% (w/v) PEG precipitate remained clear. After the Sephadex purification an improvement in the electrophoretic separation of protein into stained bands resulted, (relative to samples not prepared with 5-20% (w/v) PEG precipitation), but ALV-synthetase activity remained poorly resolved and could not be identified with a particular band.

In summary 5-20% (w/v) PEG precipitation appeared to reduce the tendency of total protein in mitochondrial samples to aggregate.

7.4. GEL FILTRATION OF PARTIALLY PURIFIED ALV-SYNTHETASE

Material that had undergone cation exchange and two centrifucations to remove aggregated material (Table 7-2) was run through a Sephadex G-100 column in a buffer containing 0.5 M NaCl, 1 mM DTE to maintain conditions promoting the solubility of ALV-synthetase. The result shown in Fig. 7-2 indicated that the conditions established to initially solublize ALV-synthetase from mito-

Fig. 7-2.

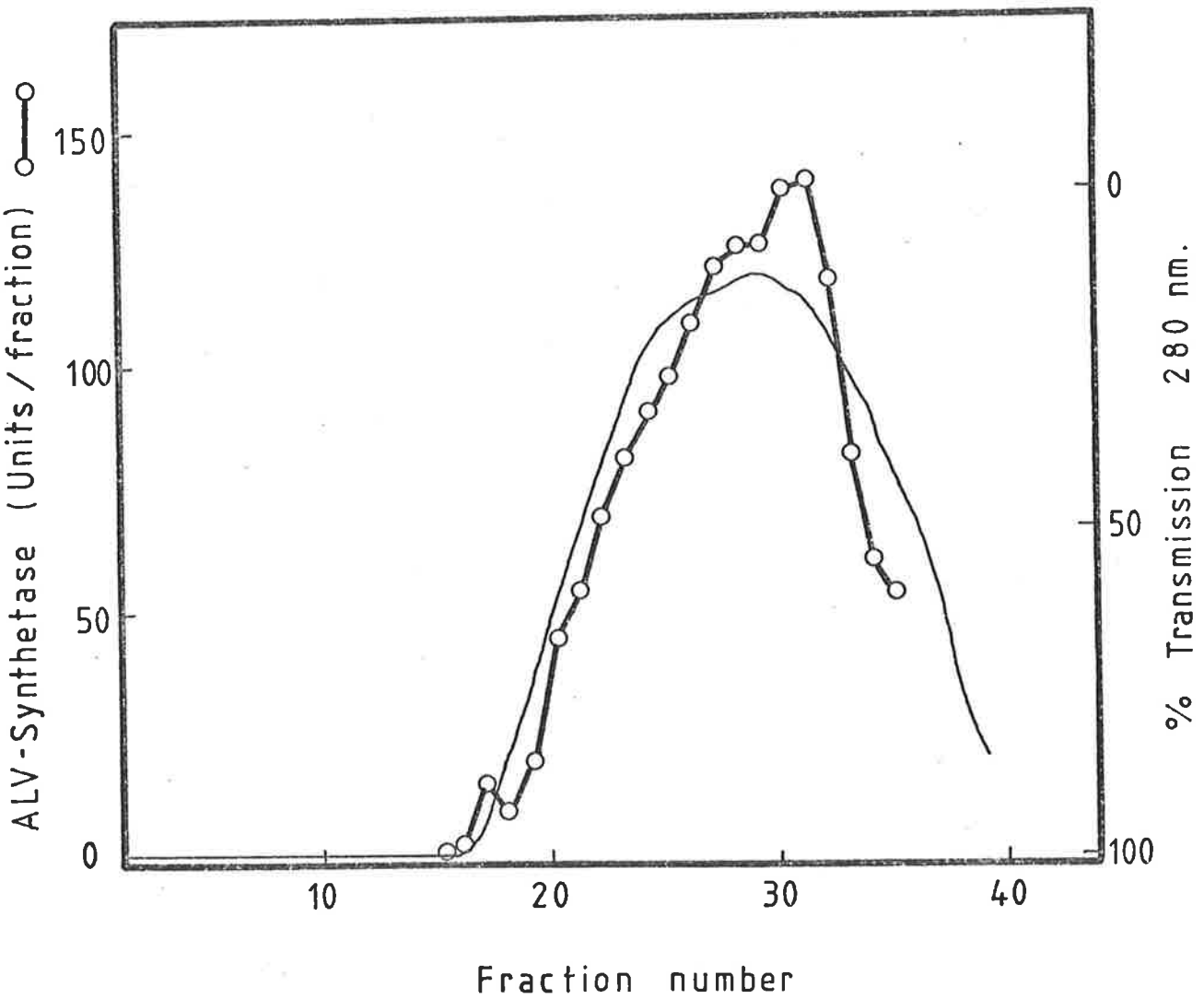
Sephadex G-100 chromatography after CM-Sephadex purification of ALV-synthetase.

Column: 52 x 1.8 cm

Buffer: TDEPN

Sample applied: Sample was at the stage of second centrifugal clarification as summarized in Table 7-1. 18 mg protein applied, specific activity 99 units/mg

Recovery: Fractions 24-35 contained 77% of the applied activity and representated a purification of 1.03 fold


ALV-Synthetase (Units / fraction)
150
100
50
0
0
50
100
% Transmission 280 nm.
10
20
30
40
Fraction number

chondrial extracts did not disrupt some bonds formed during reassociation of the enzyme with protein.

A higher proportion of soluble ALV-synthetase activity was observed when samples that had been 5-20% (w/v) PEG precipitated and CM-Sephadex purified, were fractionated on Sephadex G-100 in 0.5 M NaCl (see Fig. 7-3). Despite the apparent solubility of the fractions obtained from the experiment shown in Fig. 7-3, electrophoresis after G-25 desalting gave no better resolution of ALV-synthetase than was achieved with samples that had not been Sephadex G-100 fractionated.

Sephadex G-50 chromatography after CM-Sephadex purification was also investigated, but this gave no useful purification and no improvement of the electrophoretic separation properties of the enzyme.

7.5. ISOLATION OF A HIGH SPECIFIC-ACTIVITY SAMPLE OF ALV-SYNTHETASE

The most purified sample isolated during the investigations reported here had a specific activity of 4,684 units/mg although only 57 µg of this sample were obtained. This is the highest activity yet isolated from a mammalian source.

The sample, not otherwise described here, was purified by the following procedures. Solublized mitochondrial extract was 0-50% ammonium sulphate precipitated, 5-20% (w/v) PEG precipiated, CM-Sephadex fractionated, freeze dry concentrated, Sephadex G-100 fractionated then 0-20% (w/v) PEG precipitated. The

Fig. 7-3.

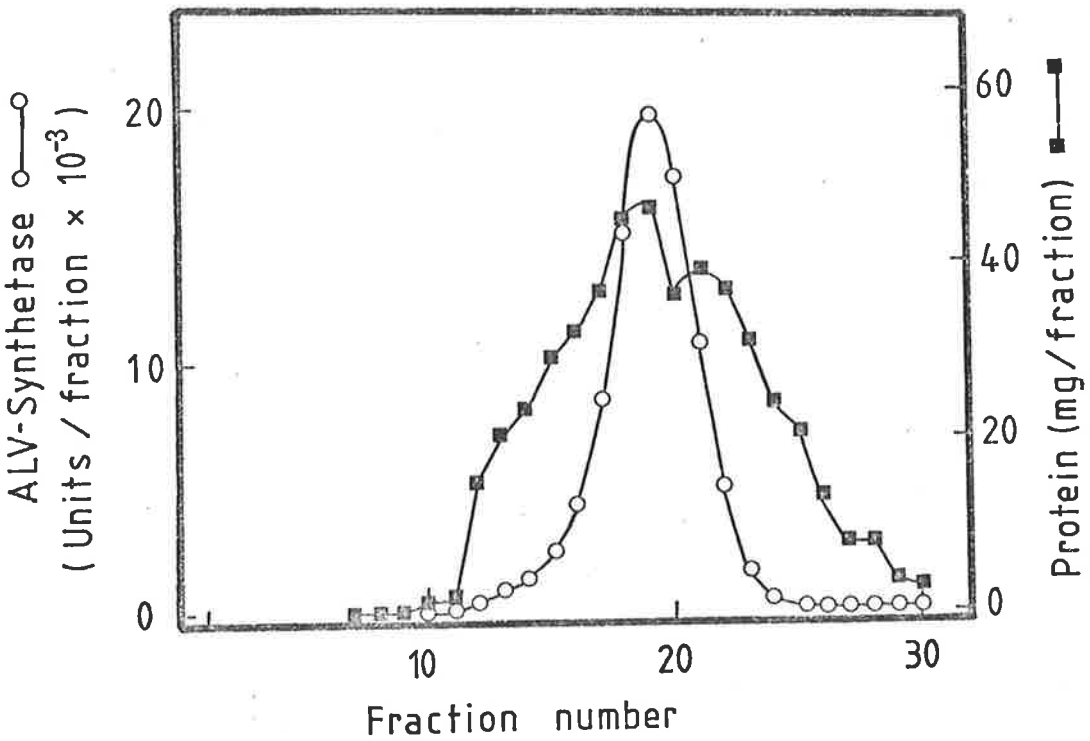
Sephadex G-100 chromatography after CM-Sephadex purification of ALV-synthetase.

Column: 42 x 3.8 cm

Buffer: TDEPN

Sample applied: Solublized mitochondrial extract, 0-50% ammonium sulphate precipitated, 5-20% (w/v) PEG precipitated, CM-Sephadex purified and freeze dry concentrated. 490 mg protein, specific activity 188 units/mg.

Recovery: Fractions 17-21 contained 80% of applied activity with average specific activity 357 units/mg giving a purification of 1.9 fold with respect to the applied sample.


ALV-Synthetase
(Units / fraction × 10^{-3})
Protein (mg/fraction)
0
10
20
0
20
40
60
10
20
30
Fraction number

resultant enzyme (1.36 mg, 728 units/mg) was preparatively electrophoresed and a further 6.4 fold purification was achieved. Final protein levels were estimated by the procedure of Schaffner and Weissman (108) using calibrated standard solutions for reference.

7.6. INSTABILITY OF ALV-SYNTHETASE IN DILUTE SOLUTIONS

Some of the enzyme described above (specific activity 4,684 units/mg) was stored 12 hours at $0^{o}C$ and the remainder frozen at $-15^{o}C$. When re-assayed for ALV-synthetase neither of the samples showed detectable enzyme activity. It was presumed that denaturation of the protein had occurred in the TDEP buffer due to the extreme dilution of 6.7 µg protein/ml. The possibility that protein had been lost by absorbtion to the glass storage vial was discounted after remeasuring the protein concentration.

A preliminary investigation of recovery from preparative electrophoresis, when high activity samples were obtained highly diluted during recovery, suggested that 20% sucrose gave better protection to the activity of recovered enzyme than carrier protein (5 mg BSA/ml). One sample stored in 20% sucrose however (23 µg/ml) lost all ALV-synthetase activity while a further sample of the same material undergoing isoelectric focusing for 5.75 h lost only 40% of its activity. This supports the previous suggestion (section 6.2 a) that carrier ampholytes may provide a protective environment for ALV-synthetase.

Although suitable storage conditions for dilute ALV-synthetase solutions may be found, the use of conditions that avoid very low levels of protein at all times may be necessary during any work to further the purification of mammalian ALV-synthetase.

7.7. SUMMARY AND SUGGESTIONS FOR THE EXTENSION OF THE PURIFICATION OF MITOCHONDRIAL ALV-SYNTHETASE.

A purification sequence that can provide approximately a 300 fold purification of ALV-synthetase from rat liver mitochondria has been described (section 7.5). The procedure has not been used extensively enough to establish the reproducibility of all steps and re-aggregation of proteins which has a variable incidence, depending on the batch, lowers the degree of purification achieved.

With regard to extending the purification of ALV-synthetase the use of affinity chromatography substrates synthesized under conditions that will ensure completion of the desired chemical structure seems worthy of further investigation (c.f. discussion in section 4.2). The prospect that affinity chromatography purification after cation exchange would give a sample more readily isolated by electrophoresis is encouraged by a preliminary result (Fig. 7-1).

The extraction procedure used to isolate starting material from mitochondria may also introduce a high proportion of proteins that are difficult to separate from ALV-synthetase. A preliminary observation that intact

mitochondria, suspended for 2 h at 37°C, lost 60% of the ALV-synthetase activity to the supernatant suggests that alternative extraction procedures may be possible and preferable.

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